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The World Journal of Biological Psychiatry

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The World Journal of Biological Psychiatry, 2010; 11: 683

EDITORIAL

Dear Colleagues,

It is my pleasure to welcome you to the fifth issue of the year 2010.

I am delighted to present to you a review on **Attention Deficit Hyperactivity Disorder (ADHD) in adults**. Michael Rösler and colleagues have undertaken an intensive literature review of peer-reviewed English language articles on the topic and compiled an up-to-date overview on the current discussions and treatment options. In line with this review, the Editors have selected several original articles and brief reports on the topic of ADHD in this issue. As a second topic for this issue, we have chosen schizophrenia.

Autism Spectrum Disorder (ASD) and **Attention Deficit Hyperactivity Disorder (ADHD)** are developmental disorders that overlap in a number of domains, sometimes complicating clinical distinction between both disorders. Although there is some evidence for a genetic overlap, there are no reports on genes that could differentiate between ASD and ADHD. Furthermore, it is not known whether this genetic overlap is influenced by co-morbid Substance Use Disorders (SUD). Bram Sizoo and colleagues present a study with 110 adult patients with ASD or ADHD with or without a lifetime history of SUD. The authors concluded that serotonergic genes could prove to play an important role in differentiating between ASD and ADHD and suggest that replication studies are conducted.

In an original article by Michael Rösler and German colleagues a 24-week, double-blind, placebo-controlled study in **adults with ADHD** is presented which investigates treatment with methylphenidate (MPH-ER) in adults with ADHD. The focus was to assess the medium to long-term effects of extended release methylphenidate on emotional symptoms and other psychopathology frequently seen in ADHD patients. The large-scale, multicenter treatment study included 363 patients who were randomized to MPH-ER or placebo at a ratio of 2:1. The duration of the titration period was 5 weeks followed by a maintenance phase of 19 weeks. The study shows that MPH-ER appears to be an effective treatment for emotional symptoms with ADHD, also obsessive-compulsive symptoms and problems with self-concept are affected positively.

Disturbed cholesterol and phospholipid metabolism has been associated with **schizophrenia**. Thus protein expression analysis in cerebrospinal fluid from patients may be a potential diagnostic tool for schizophrenia. Daniel Martins-de-Souza and colleagues investigated proteins from 17 first-episode schizophrenia patients and 10 healthy controls. Their findings suggest that the protein analysis of apolipoprotein E, apolipoprotein A-I, and prostaglandin-H2 D-isomerase in cerebrospinal fluid from patients might be a potential diagnostic tool for schizophrenia.

Rebecca Schennach-Wolff and colleagues examined **quality of life and subjective well-being** as predictors of symptomatic treatment outcome in **schizophrenia**. The study included biweekly PANSS ratings of 285 inpatients with schizophrenia spectrum disorders within a multicenter trial by the German Research Network on Schizophrenia. The Medical Outcomes Study-Short Form 36-Item Health Survey (SF-36), the Subjective Well-being Under Neuroleptic Treatment Scale (SWN-K) and the Adjective Mood Scale (AMS) were used. The findings highlight the importance of the patient's self-perception and especially of early improvement of quality of life and subjective well-being for symptomatic treatment outcome.

The influence of infectious agents on the pathogenesis of psychiatric disorders has been discussed intensively over the past few years. **Pre- and postnatal infections are risk factors for schizophrenia**. This may be explained by chronic infections or an altered immune status. However most of the studies have only focused on one single pathogen and not on the impact of different infectious agents. Daniela Krause and colleagues investigated the association between schizophrenia and various neurotrophic infectious agents. The study included 31 schizophrenic patients and 30 healthy matched individuals. The results show a higher prevalence of antibodies within schizophrenic patients. The findings emphasize the possible role of infectious agents in the pathogenesis of schizophrenia and indicate that it might not be one specific agent that is responsible for schizophrenic symptoms but the resulting immune response in the central nervous system instead.

Serge Brand and colleagues from Switzerland present a study with 2231 participants which aimed at investigating the **relation between burnout, depressive symptoms, satisfaction with life, and sleep complaints**. As a starting hypothesis the authors indicated that people with an optimistic attitude seem to be less vulnerable to stress and burnout. Interestingly, the findings suggest that among burnout symptoms emotional and physical exhaustion, but not depressive symptoms, are related to sleep complaints. Satisfaction with life is connected with low emotional and physical exhaustion and low pessimism and also contributes to favorable sleep.

In a brief report Yuval Bloch and colleagues from Israel represent their findings which emphasize positive effects of **repetitive Transcranial Magnetic Stimulation (rTMS)** on attention in **ADHD patients**. ADHD has been suggested to involve dopaminergic prefrontal abnormalities. A total of 13 ADHD patients were enrolled and there was a specific beneficial effect on attention ten minutes after the rTMS course. The authors wish to encourage future research on the possibility of amelioration of attention difficulties in patients suffering from ADHD by using high frequency rTMS directed to the right dorsolateral prefrontal cortex.

And lastly, Peter Höfer and colleagues present a letter to the Editors which focuses on **hyperprolactinaemia and acute psychosis**. The authors conclude the necessity of considering medication-induced enlargement of the pituitary gland and of avoiding surgery of a medication-induced adenoma of the hyophysis, especially in patients with a psychiatric case history.

Yours sincerely,

Siegfried Kasper, MD
Chief Editor

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REVIEW ARTICLE

Attention deficit hyperactivity disorder in adults

MICHAEL RÖSLER[1], MIGUEL CASAS[2], ERIC KONOFAL[3] & JAN BUITELAAR[4]

[1]*Neurozentrum, Universitätsklinikum des Saarlandes, Homburg/Saar, Germany,* [2]*Department of Psychiatry, Hospital Universitari Vall d'Hebron and Department of Psychiatry and Legal Medicine, Universitat Autònoma de Barcelona, Barcelona, Spain,* [3]*Hôpital Robert Debré, Centre Pédiatrique des Pathologies du Sommeil, Paris, France, and* [4]*Radboud University Nijmegen Medical Center, Department of Cognitive Neuroscience, Nijmegen, The Netherlands*

Abstract

Objective. To examine available literature regarding attention deficit hyperactivity disorder (ADHD) in adults. *Methods.* An electronic literature search of peer-reviewed English language articles using MEDLINE (without time limits) was undertaken. *Results.* Symptoms of ADHD in adults exert a substantial negative impact on daily life, including work, social life and relationships. Co-morbidities are common, further impairing quality of life. Diagnosis of adult ADHD can be difficult, as current criteria require evidence of symptom onset before the age of 7 years and impact on activities typically undertaken by children. Drug therapy is the first-line treatment for adult ADHD, particularly stimulant medication. However, methylphenidate (MPH) immediate-release tablets require three or more times daily dosing, which can impact on compliance, while demonstrating a loss of symptomatic benefit later in the day. Extended-release preparations of MPH, mixed amphetamine salts and dexamphetamine can provide symptom control for 6–12 h and the non-stimulant atomoxetine has demonstrated benefit in reducing ADHD symptoms. These therapies are generally well tolerated, but may be associated with adverse effects on the cardiovascular system, which need to be further assessed in controlled clinical trials. Psychological therapy may be beneficial in adults who continue to experience clinically significant symptoms while receiving pharmacotherapy. *Conclusion.* Further research in all areas of adult ADHD is urgently needed.

Key words: *ADHD, psychosocial intervention, pharmacotherapy, side effects, genetics*

Introduction

Attention deficit hyperactivity disorder (ADHD) is generally considered as a childhood disorder. ADHD is diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition, text revision (DSM-IV-TR criteria), when symptoms of inattention and impulsivity/hyperactivity, which significantly impair functioning at school and home, are present before 7 years of age (American Psychiatric Association 2000). A DSM-IV-TR diagnosis of adult ADHD requires fulfilment of the same criteria (American Psychiatric Association 2000). However, ADHD symptoms in adulthood can present in numerous ways, such as restlessness, difficulty in relaxing, and dysphoria, whereas attention deficits may manifest as a lack of concentration regarding details, forgetting appointments, and a failure to plan and organize activities and work. This may be compounded by disorganization, characterized by unfinished tasks and poor time management. Mood symptoms such as depression or irritability are common (Wender 1995; Asherson et al. 2007).

There is a growing interest in adult ADHD, due to its remarkable prevalence, symptomatic persistence, co-morbidity with several other disorders, and negative psychosocial impact. The objective of this review is therefore to examine the available literature regarding ADHD in adults.

Methods

Relevant articles were identified from an electronic literature search of peer-reviewed English language articles using MEDLINE (without time limits). The

Correspondence: Michael Rösler, Neurozentrum, Universitätsklinikum des Saarlandes, 66421 Homburg/Saar, Germany. Tel: +49 6841 1626350. Fax: +49 6841 1626335. E-mail: Michael.Roesler@uniklinikum-saarland.de

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primary research parameters were "ADHD", "adults", "amphetamine salts", "atomoxetine", "attention", "burden", "cognitive behavioural therapy", "driving", "effectiveness", "efficacy", "employment", "epidemiology", "dexmethylphenidate", "impact", "methylphenidate", "mixed amphetamine salts", "neurobiology", "parenting", "productivity", "psychosocial therapy", "quality of life", "relationships", "safety", "tolerability", and "treatment guidelines". The results presented here are based on original data from published clinical trials, review papers and meta-analyses. No assessment of the quality of the original trials was undertaken. The most important information from these sources was identified and is summarized here.

Epidemiology of ADHD in adults

In an international survey involving >11,000 adults, the mean prevalence of ADHD (DSM-IV-TR criteria) was estimated at 3.4%. Notably, ADHD was more common in higher-income countries, such as the United States and France (5.0–7.3%), compared with 1.8–1.9% for lower-income countries such as Colombia and Mexico (Fayyad et al. 2007a). An ADHD screen was also included in the US National Co-morbidity Survey Replication, which estimated an adult ADHD prevalence of 4.4% (Kessler et al. 2006). These surveys reported higher rates of ADHD in men than in women (Kessler et al. 2006; Fayyad et al. 2007a). It should be remembered that the actual prevalence of ADHD may be higher, as these estimates are dependent upon individual understanding and reporting of symptoms.

Diagnosis of ADHD in adults

There is evidence that ADHD symptoms can persist into adulthood and cause impairment, but there are no clear conclusions about the level of ADHD symptoms in adults that should be considered as grounds for intervention, or whether symptoms take a different form in adulthood (NICE guidelines, 2008). To confer a clinical diagnosis of ADHD in adults requires a thorough description of symptoms and everyday behaviours over a period of at least the preceding 6 months. However, a number of difficulties may be associated with arriving at such a diagnosis. In particular, the DSM-IV-TR criteria were developed for children and adolescents and cannot always be applied to adults. Symptoms of co-morbid conditions, such as bipolar disorder, may also appear similar to some within the ADHD spectrum, so differentiation from these is important (Asherson et al. 2007).

According to DSM-IV-TR criteria, ADHD in adults is essentially a retrospective diagnosis (McGough and Barkley 2004), in which impairment should be present before the age of 7 years, although the fifth edition of the DSM is considering an increase in the age of onset of symptoms to be present on or before the age of 12 years (American Psychiatric Assocation 2010). In order to confirm the presence of symptoms before the age of 7 years, evidence other than the subject's own personal recall is required, e.g., school records or parental commentary, although these may also provide insufficient unequivocal evidence (American Psychiatric Association 2000; McGough and Barkley 2004). Notably, failure to fulfil this age criterion may exclude a number of adults with significant ADHD symptom-related functional impairment. Indeed, in a study comparing individuals meeting the full DSM-IV-TR criteria for ADHD and those meeting the full symptom, but not the age criteria, the prevalence of divorce, learning disability, arrests and driving accidents were similar (Faraone et al. 2006a). In addition, patterns of co-morbidity, familial loading for ADHD and neuropsychological deficits are very similar for so-called late-onset ADHD and ADHD meeting the full DSM-IV-TR criteria (Faraone et al. 2006a,b).

The DSM-IV-TR ADHD symptom criteria may also be inappropriate in adults as they rely on observations relating to childhood activities (McGough and Barkley 2004). Barkley et al. (2007a) have recently proposed new criteria for ADHD in adults that are more age appropriate. However, validation of these criteria in independent samples is needed (Barkley et al. 2007a). Another complicating factor is the tendency for individuals under clinical assessment to under-report the severity of their symptoms (Kooij et al. 2008). Those individuals with subthreshold ADHD symptoms (i.e. fewer than the predefined six or of lesser severity) may exhibit less functional impairment than those meeting the full symptom criteria, but this group still demonstrates greater impairment than the general adult population (McGough and Barkley 2004; Faraone et al. 2006a,b). Furthermore, DSM-IV-TR states that symptoms should impair functioning at work/school and home (American Psychiatric Association 2000); however, in adults, functioning in numerous other settings may be affected by the disorder, such as social and leisure activities, parenting and intimate relationships (McGough and Barkley 2004).

Guidelines for the diagnosis and treatment of ADHD in adults have been developed by several organizations, including the National Institute of Mental Health, the American Academy of Child and Adolescent Psychiatry (AACAP), the Canadian ADHD Resource Alliance (CADDRA) and the British Association for Psychopharmacology (BAP) (Dulcan 1997; Jain et al. 2006; Nutt et al. 2007; NIMH 2009). All of these guidelines recommend

a comprehensive assessment including mental status, developmental history, rating scales completed by multiple informants, and a clinical interview to gain an insight into the patient's medical and psychiatric history, as well as their family's psychiatric history, background and mental status (Gibbins and Weiss 2007). A discussion of diagnostic instruments for the assessment of ADHD in adults is available in the online supplementary materials (available from: http://informahealthcare.com/doi 10.3109/15622975.2010.483249).

Neurobiology of ADHD

Neuroimaging has demonstrated several structural differences in the brains of adults with ADHD compared with unaffected individuals, including significant reductions in total cortical grey matter, prefrontal and anterior cingulate volumes and right putamen/globus pallidus grey matter (Seidman et al. 2006; Ellison-Wright et al. 2008). Adults with ADHD also exhibit selective thinning of the cerebral cortex in the networks that mediate attention and executive functioning, especially in the right hemisphere involving the inferior parietal lobule, the dorsolateral prefrontal and the anterior cingulate cortices (Makris et al. 2007).

Functional imaging techniques are also being used to investigate the neurochemistry of ADHD. Near-infrared spectroscopy has shown that patients with ADHD have reduced task-related increases in oxygenated haemoglobin in the ventrolateral prefrontal cortex, indicating reduced activation in this area. This was particularly marked for the task involving working memory (Ehlis et al. 2008). *N*-acetyl-aspartate/creatine ratios in the prefrontal corticosubcortical region and the left centrum semiovale were also higher in ADHD patients than controls (Fayed et al. 2007). The involvement of the dopamine transporter in ADHD has been supported by the results of a positron emission tomography (PET) study, which demonstrated its increased availability in adult ADHD patients versus matched controls (Spencer et al. 2007a). A separate PET study showed lower dopamine D2/D3 receptor activity in the caudate and potentially also in the hippocampus and amygdala (Volkow et al. 2007).

Several cognitive functions, such as response and behavioural inhibition, attention, working memory and planning, are impaired in subjects with ADHD (Hervey et al. 2004; Boonstra et al. 2005; Willcutt et al. 2005). Functional magnetic resonance imaging (MRI) studies using various types of inhibition tasks have consistently demonstrated reduced activation of the ventral prefrontal cortex and particularly the anterior cingulate cortex and the striatum in ADHD (Bush et al. 2005). Few functional MRI studies have been completed in adults with ADHD, but a single study, using the Counting Stroop task, reported evidence for reduced activation of the anterior cingulate (Bush et al. 1999).

A discussion of the genetic basis for ADHD, including candidate genes, is included in the online supplementary materials (available from: http://informahealthcare.com/doi 10.3109/15622975.2010.483249).

Impact of ADHD

ADHD has a significant negative impact on several aspects of daily life, particularly in the work and social domains. In a survey of US adults with ADHD and matched controls, the percentage completing high school and university was higher in the control population (Biederman and Faraone 2006). In a separate international survey, the prevalence of adults with ADHD was higher in those individuals whose education did not include attendance at university (5 versus 2% for those who did attend university) (Fayyad et al. 2007).

Professional and economic impact

The proportion of adults with ADHD in full-time employment is substantially lower than adults without ADHD (34 vs. 59% in one survey) (Biederman and Faraone 2006). This is reflected in the lower mean annual household income for ADHD adults versus controls. Adults with ADHD are also much more likely to change jobs frequently: over a 10-year period, they reported having a mean of 5.4 jobs, compared with 3.4 jobs for controls (Biederman et al. 2006a). The work productivity of adults with ADHD is also lower than that of other adults, due to concentration difficulties, disorganization, and a reduced ability to cope with a large workload (Biederman et al. 2006a; de Graaf et al. 2008). It has been estimated that an average of 35 days' work productivity are lost per year for each adult with ADHD (Kessler et al. 2005).

Social problems

Within the social realm, ADHD has been shown to lead to a number of relationship difficulties. The impact of ADHD on forming and maintaining personal relationships is evident in the higher prevalence of single (never-married) and divorced individuals (Biederman et al. 2006a; Fayyad et al. 2007). Adults with ADHD may find adjustment following marriage more difficult, and they are more likely to have negative perceptions regarding the state of their marriage than their non-ADHD partners. Spouses of adults with ADHD often have to cope with and compensate

for their partner's difficulties, which may place considerable strain on the relationship (Eakin et al. 2004). Problems in parenting have also been commonly described, with parents with ADHD more likely to have a lack of parental discipline and to engage in negative parent–child interactions (Harvey et al. 2003). In younger adults, ADHD may be associated with a lack of friendships and poor relationships with parents (Biederman and Faraone 2006; Biederman et al. 2006a; Asherson et al. 2007). In general, a high proportion of adults with ADHD may experience dissatisfaction with their family, social and professional lives. Notably, they perceive that ADHD has a lifelong detrimental impact (Biederman et al. 2006a).

Co-morbidities

A number of psychiatric co-morbidities affect many adults with ADHD. In particular, co-morbid mood disorders and anxiety disorders occur with a significantly greater frequency in adults with ADHD (by a factor of approximately four- to fivefold) (McGough et al. 2005; Kessler et al. 2006; Fayyad et al. 2007). In particular, the risk for co-morbid bipolar disorder is increased by as much as seven times in adults with ADHD versus the general population (Kessler et al. 2006). Furthermore, in adults with bipolar disorder, the prevalence of co-morbid ADHD is estimated at around 10% (Nierenberg et al. 2005). However, this association between adult ADHD and bipolar disorder has to be confirmed in studies outside the United States. Compared with adults in the general population, ADHD has also been calculated to present a three- or fourfold increased risk of a substance use disorder (Kessler et al. 2006; Fayyad et al. 2007). The proportions of individuals abusing alcohol, smoking and indulging in recreational drug use is around 1.6 times higher than in those without ADHD (Biederman et al. 2006a).

Sleep and activity disturbances

Problems with sleep are common in adults with ADHD. In general, longer latency to sleep onset and fragmented sleep with numerous nocturnal awakenings are associated with the condition, leading to poor quality sleep and daytime fatigue. Symptoms of inattention are thought to increase an individual's need for sleep, while symptoms of hyperactivity are suggested to reduce duration of sleep during the night (Boonstra et al. 2007; Gau et al. 2007).

ADHD in adulthood is also associated with higher than average incidences of traffic accidents and encounters with the law. Adults with ADHD are around twice as likely as adults in the general population to have been arrested (Biederman et al. 2006a), and the prevalence of ADHD in young adult prisoners has been shown to be significantly elevated when compared with controls (Rösler et al. 2004). Furthermore, adults with ADHD may be involved in more driving accidents than other adults. This phenomenon has been attributed to the impulsivity, inattention, loss of concentration and fatigue associated with the condition, resulting in overall poor driving performance and specific problems such as variable reaction times and risk taking (Fried et al. 2006; Richards et al. 2006; Fischer et al. 2007; Reimer et al. 2007).

Pharmacotherapy of adult ADHD: Efficacy

Current guidelines developed by AACAP, CADDRA and BAP all recommend combination therapy including psychoeducation, an initial trial of medication with titration to an individual effective dose, assessment of residual symptoms and long-term community follow-up (Gibbins and Weiss 2007). According to the National Institute for Health and Clinical excellence (NICE) guidelines, pharmacotherapy is the first-line treatment for adults with ADHD, with either moderate or severe levels of impairment (NICE guidelines, 2008). Psychological interventions without medication may be effective for some adults with moderate impairment, but there are insufficient data to support this recommendation. According to the NICE guidelines, methylphenidate (MPH) is the first-line drug. If MPH is ineffective or unacceptable, atomoxetine or dexamfetamine can be tried (NICE guidelines, 2008). The primary goal of drug treatment is to alleviate all ADHD symptoms, with an additional requirement of a long duration of action, in order to maintain effectiveness throughout the working day.

The most widely studied drug for the treatment of adult ADHD is the stimulant MPH, which is available as immediate-release (IR) tablets dosed three times daily (tds), extended-release (ER) tablets dosed twice daily (bd) and the OROS MPH formulation utilizing an osmotic release oral system that is administered once daily (qd). Other treatment options for adult ADHD include atomoxetine, mixed amphetamine salts (MAS) and dexmethylphenidate. Lisdexamfetamine dimesylate (LD) is also now approved for the treatment of ADHD in adults in the United States. A summary of the key randomized, double-blind clinical trials evaluating these drugs in adults with ADHD in presented in Table I.

Methylphenidate immediate-release

In a 6-week study, MPH-IR 1.0–1.3 mg/kg per day significantly improved ADHD-RS total score versus

Table I. Summary of key randomized, double-blind, clinical studies of drug therapies in adults with ADHD.

Reference	Study design/population	Treatment regimens	Key outcome measures
Methylphenidate immediate-release			
Kuperman et al., 2001	Randomized, double-blind, parallel-group study 30 patients aged 18–60 years	Bupropion 300 mg/day or MPH-IR 0.9 mg/kg per day versus placebo for 7 weeks	**Percentage of patients achieving CGI-I ≤ 2:** placebo: 27%; bupropion: 64%; MPH-IR: 50% (P = ns)
Kooij et al., 2004	Randomized, double-blind, crossover study 45 patients aged > 18 years	MPH-IR 1.0 mg/kg per day versus placebo for 3 weeks	**≥ 30% Reduction from baseline in ADHD-RS:** placebo: 13%; MPH-IR: 42% (P = 0.011 versus placebo) **Reduction from baseline of ≥ 2 points on CGI-S:** placebo: 18%; MPH-IR: 51% (P = 0.011 versus placebo) **Treatment response (≥ 30% reduction from baseline in ADHD-RS + reduction from baseline of ≥ 2 points on CGI-S):** placebo: 7%; MPH-IR: 38% (P = 0.003 versus placebo)
Spencer et al., 2005	Randomized, double-blind, placebo-controlled, parallel-group study 146 patients aged 19–60 years	MPH-IR 1.0-1.3 mg/kg per day versus placebo for 6 weeks	**Mean ADHD-RS score at baseline and Week 6:** placebo: 35.9 ± 9.2 versus 28.0 ± 11.2 MPH-IR: 33.8 ± 8.6 versus 13.1 ± 10.3 (P < 0.0001 versus placebo) **Treatment response (CGI-I ≤ 2 + ≥ 30% reduction in AISRS score from baseline):** placebo: 17%; MPH-IR: 68% (P < 0.0001 versus placebo)
Methylphenidate extended-release			
Rösler et al., 2009	Randomized, double-blind, placebo-controlled, parallel-design study 359 patients aged ≥ 18 years	MPH-ER 10−60mg/day versus placebo for 24 weeks	**MPH-ER resulted in significant reductions in ADHD symptoms as rated with the WRAADDS (data not provided)** **Treatment response (30% reduction of the WRAADDS score):** placebo: 42% MPH-ER 61% (P = 0.001 versus placebo) **Percentage of patients 'much improved' or 'very much improved' on the CGI** placebo: 37% MPH-ER: 55% (P = 0.0003 versus placebo)
OROS methylphenidate			
Biederman et al., 2006b	Randomized, placebo-controlled, parallel-design study 149 patients aged 19–60 years	OROS MPH 36 mg/day versus placebo for 6 weeks	**Treatment response (CGI-I ≤ 2 + ≥ 30% reduction in AISRS score from baseline):** placebo: 39%; MPH-IR: 66% (P = 0.002 versus placebo)
Reimherr et al., 2007	Double-blind, placebo-controlled, crossover study 47 patients aged 18–65 years	OROS MPH 90 mg/day versus placebo for 4 weeks	**Mean change from baseline in total ADHD-RS score** placebo: −14%; OROS MPH: −41% (P = 0.003 versus placebo) **Percentage of patients achieving CGI-I ≤ 2:** placebo: 22%; OROS MPH: 54% (P = 0.018)

Medori et al., 2008	Randomized, double-blind, placebo-controlled, parallel-group, dose-response study 402 patients aged 18–65 years	OROS MPH 18, 36, or 72 mg/day versus placebo for 5 weeks	**Mean change from baseline in CAARS-O:S total score:** placebo: –7.6 ± 9.93 OROS MPH 18 mg/day: –10.6 ± 10.34; 36 mg/day: –11.5 ± 9.97; 72 mg/day: –13.7 ± 11.11 (all $P < 0.05$ versus placebo) **Mean change from baseline in CAARS-S:S total score:** placebo: –5.8 OROS MPH 18 mg/day: –10.4; 36 mg/day: –11.3; 72 mg/day: –14.4 (all $P < 0.01$ versus placebo) **Treatment response (≥ 30% reduction from baseline in CAARS-O:S total score):** placebo: 27% OROS MPH 18 mg/day: 51%; 36 mg/day: 49%; 72 mg/day: 60% (all $P < 0.001$ versus placebo)
Dexmethylphenidate			
Spencer et al., 2007	Randomized, double-blind, placebo-controlled fixed-dose study 221 patients aged ≥ 18 years	Dexmethylphenidate ER 20, 30 or 40 mg/day versus placebo for 5 weeks	**Mean change from baseline in ADHD-RS total score:** placebo: –7.9 Dexmethylphenidate 20 mg/day: –13.7; 30 mg/day: –13.4; 40 mg/day: –16.9 (all $P < 0.05$ versus placebo) **Mean change from baseline in CAARS-O:S total score:** placebo: –3.1 Dexmethylphenidate 20 mg/day: –10.0; 30 mg/day: –12.8; 40 mg/day: –9.6 (all $P < 0.01$ versus placebo) **Mean change from baseline in CAARS-S:S total score:** placebo: –7.2 Dexmethylphenidate 20 mg/day: –16.0; 30 mg/day: –12.7; 40 mg/day: –15.6 (all $P < 0.05$ versus placebo) **Percentage of patients achieving CGI-I ≤2:** placebo: 26%; dexmethylphenidate 20 mg/day: 47% ($P < 0.05$ versus placebo); 30 mg/day: 37% (P = ns versus placebo); 40 mg/day: 56% ($P = 0.003$ versus placebo)
Adler et al., 2008	6-month OLE phase of randomized, double-blind, placebo-controlled, fixed-dose study 170 patients aged ≥ 18 years	Dexmethylphenidate ER 20–40 mg/day	**Most common AEs (> 15% of patients):** headache (27.6%) insomnia (20.0%) decreased appetite (17.6%) **Mean change from baseline in total ADHD-RS score:** –10.2 ± 10.19 (patients switched from placebo) –8.4 ± 10.14 (patients maintained on dexmethylphenidate ER) **Percentage of patients reporting improvements in CGI-I scores** 95.0% of patients switched to placebo 95.1% of patients maintained on dexmethylphenidate ER **Percentage of patients rated as 'normal' or 'mildly ill' on CGI-S** 90.0% of patients switched to placebo 92.7% of patients maintained on dexmethylphenidate ER

(Continued)

Table I. *(Continued)*

Reference	Study design/population	Treatment regimens	Key outcome measures
Mixed amphetamine salts			
Weisler et al., 2006	Randomized, double-blind, placebo-controlled, parallel-group, dose-escalation study 255 patients aged > 18 years	Mixed amphetamine salts ER 20, 40 or 60 mg/day versus placebo for 4 weeks	**Mean change from baseline in ADHD-RS total score:** placebo-adjusted difference: mixed amphetamine salts ER: 20 mg/day: –6.6; 40 mg/day: –7.2; 60 mg/day: –7.8 (all $P \leq 0.001$ versus placebo) **Percentage of patients achieving mean change from baseline in ADHD-RS total score ≥ 30%:** placebo: 61%; mixed amphetamine salts ER: 20 mg/day: 74%; 40 mg/day: 80%; 60 mg/day: 82% (all $P < 0.001$ versus placebo) **Percentage of patients achieving CGI-I ≤ 2:** placebo: 27%; mixed amphetamine salts ER: 20 mg/day: 50%; 40 mg/day: 56%; 60 mg/day: 58% (all $P < 0.001$ versus placebo)
Atomoxetine			
Michelson et al., 2003	Randomized, double-blind, placebo-controlled study 280 patients aged ≥ 18 years	Atomoxetine 60–120 mg/day versus placebo for 10 weeks	**Mean change from baseline in CAARS:O-S total score:** placebo: –6.0 ± 9.3; atomoxetine –9.5 ± 10.1 ($P = 0.005$ versus placebo) **Mean change from baseline in CAARS:S-S total score:** placebo: –9.3 ± 14.0; atomoxetine –16.0 ± 16.2 ($P = 0.002$ versus placebo)
Michelson et al., 2003	Randomized, double-blind, placebo-controlled study 257 patients aged ≥ 18 years	Atomoxetine 60–120 mg/day versus placebo for 10 weeks	**Mean change from baseline in CAARS:O-S total score:** placebo: –6.7 ± 9.3; atomoxetine –10.5 ± 10.9 ($P = 0.002$ versus placebo) **Mean change from baseline in CAARS:S-S total score:** placebo: –11.6 ± 16.1; atomoxetine –17.3 ± 17.6 ($P = 0.008$ versus placebo)
Adler et al., 2006	Randomized, double-blind study 218 patients aged 18–50 years	Atomoxetine 40 mg bd or 80 mg qd for 6 weeks	**Mean change from baseline in CAARS:O-S total score:** atomoxetine 40 mg bd: –17; 80 mg qd: −13 ($P<0.001$)
Lisdexamfetamine			
Adler et al., 2008	Double-blind, placebo-controlled study 420 patients aged 18–55 years	Lisdexamfetamine dimesylate 30, 50 or 70 mg/day versus placebo for 4 weeks	**Mean change from baseline in ADHD-RS total score:** placebo: –8.2 lisdexamfetamine 30 mg/day: –16.2; 50 mg/day: –17.4; 70 mg/day: –18.6 (all $P < 0.0001$ versus placebo) **Percentage of patients achieving CGI-I ≤2:** placebo: 29% lisdexamfetamine 30 mg/day: 57%; 50 mg/day: 62%; 70 mg/day: 61% (all $P < 0.01$ versus placebo)

ADHD-RS, Attention Deficit Hyperactivity Disorder Rating Scale; AEs, adverse events; AISRS, Adult ADHD Investigator System Report Scale; CAARS:O-S, Conners' Adult ADHD Rating Scale: investigator-rated-short form; CAARS:S-S, Conners' Adult ADHD Rating Scale: Self-rated – short form; CGI-I, Clinical Global Impression – Improvement; CGI-S, Clinical Global Impression – Severity; ER, extended release; IR, immediate-release; MPH, methylphenidate; ns, not significant; OLE, open-label extension; WRAADDS, Wender–Reimherr Adult Attention Deficit Disorder Scale.

placebo, with the difference apparent from Week 2 onwards. Similar improvements were noted for patients with predominant inattention and predominant hyperactivity. Treatment response, defined as "much improved" or "very much improved" (score of ≤ 2) on Clinical Global Impression–Improvement (CGI-I) plus ≥ 30% reduction in ADHD Investigator System Report Scale (AISRS) score from baseline was achieved by significantly more patients receiving MPH-IR, compared with placebo (Spencer et al. 2005). In a smaller study, the percentage of responders to treatment with MPH-IR over a 3-week period (defined by a number of different criteria) was also significantly greater than that for placebo (Kooij et al. 2004). However, an earlier study showed that the percentage of patients achieving CGI-I ≤ 2 with bupropion 300 mg/day or MPH-IR 0.9 mg/kg per day was not statistically superior to that achieved by patients receiving placebo (Kuperman et al. 2001). A meta-analysis of pooled data from six separate studies evaluating MPH-IR in adults has demonstrated a mean effect size of 0.9 versus placebo (for ADHD-RS total score or global improvement), which was statistically significant ($P < 0.001$). Further analysis revealed that use of higher doses of MPH-IR (≥ 0.9 mg/kg per day) was associated with greater effect sizes (Faraone et al. 2004).

Methylphenidate extended-release

MPH-ER has been evaluated in adults with ADHD in a single study (Rösler et al. 2009). Treatment with MPH-ER resulted in significant reductions in ADHD symptoms versus placebo, including those of inattention and hyperactivity/impulsivity, which were maintained for up to 24 weeks of treatment. Significantly more patients receiving MPH-ER achieved "much improved" or "very much improved" on the CGI than patients receiving placebo (Rösler et al. 2009).

OROS methylphenidate

OROS MPH has been evaluated in several clinical studies in adults with ADHD. Over a 6-week period, significant reductions in AISRS score were observed from Week 3 onwards with OROS MPH 36 mg/day versus placebo. In addition, statistically significant reductions from baseline to endpoint for DSM-IV-TR symptoms of inattention and impulsivity/hyperactivity were observed for OROS MPH versus placebo ($P < 0.05$ for both). The percentage of patients achieving treatment response was consistently higher in the MPH OROS group (Figure 1) (Biederman et al. 2006b).

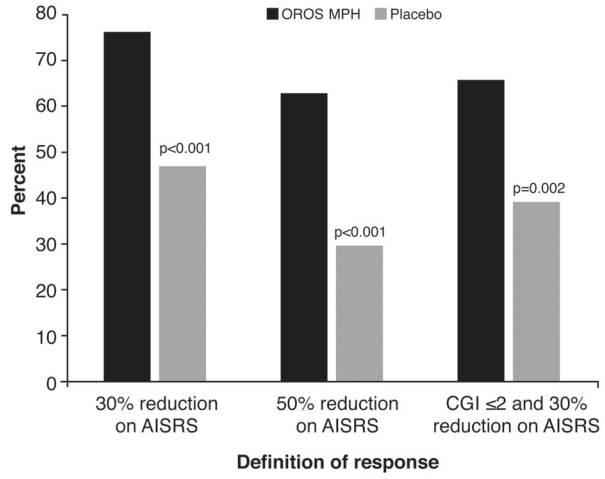

Figure 1. Treatment response with OROS MPH vs. placebo in a randomized, double-blind study (n = 149) (Biederman et al. 2006b). AISRS, Adult ADHD Investigator System Report Scale; CGI-I, Clinical Global Impression–Improvement; MPH, methylphenidate.

In a separate 6-week study evaluating OROS MPH 90 mg/day versus placebo, 38% of participants had co-morbid emotional dysregulation (ED) and 40% of participants had co-morbid ED plus oppositional defiant disorder. OROS MPH was associated with significant improvements in overall ADHD symptoms and also those of inattention and impulsivity/hyperactivity ($P < 0.01$ for all). Furthermore, symptoms of ED according to the Wender–Reimherr Adult Attention Deficit Disorder Scale (WRAADDS) were lower at study end for OROS MPH versus placebo (P = 0.002). Forty-nine percent of patients receiving OROS MPH achieved a ≥ 50% reduction from baseline in WRAADDS total score, compared with 15% receiving placebo (P = 0.007) (Reimherr et al. 2007).

In the Long acting MPH in Adult ADHD study, OROS MPH at doses of 18, 36 and 72 mg/day were compared with placebo over a 5-week, double-blind treatment period (Medori et al. 2008). Mean Conners' Adult ADHD Rating Scale: investigator-rated short version (CAARS:O-S) total score was reduced by a significantly greater amount from baseline to Week 5 in the OROS MPH groups than with placebo. There were no differences between the OROS MPH groups for this outcome measure. All doses of OROS MPH also significantly reduced the CAARS:O-S inattentive subscale score from baseline versus placebo ($P \leq 0.001$ for all comparisons). However, for the impulsive/hyperactive subscale score, mean change from baseline was significantly greater than placebo only for OROS MPH 72 mg/day (P = 0.003). Total CAARS self-report short version (CAARS:S-S) scores were significantly reduced from baseline for all doses of OROS MPH versus

placebo. In addition, severity of illness, as measured by CGI-S, was significantly reduced from baseline with all doses of OROS MPH versus placebo ($P <$ 0.05 for all comparisons) (Medori et al. 2008).

Dexmethylphenidate

Dexmethylphenidate ER 20, 30 and 40 mg/day was evaluated in adults with ADHD in a 5-week, fixed-dose, randomized, double-blind, placebo-controlled study. All doses of dexmethylphenidate demonstrated significant reductions from baseline in ADHD-RS total score, and inattentive and impulsive/hyperactive subscale scores versus placebo. The percentage of patients achieving CGI-I ≤ 2 was significantly greater than placebo for dexmethylphenidate 20 and 40 mg/day, but not for 30 mg/day. CAARS-S:S and CAARS-O:S total scores were also significantly reduced from baseline with all doses of dexmethylphenidate versus placebo (Spencer et al. 2007b). Following on from this study, 170 adults entered a 6-month, open-label extension phase to assess the long-term safety of dexmethylphenidate ER, with flexible dosing of 20–40 mg/day (Adler et al. 2008b). A total of 103 patients completed the open-label extension phase and effectiveness was evaluable in 102 patients. Dexmethylphenidate ER was well tolerated, with the most common adverse events ($>$ 15%) being headache, insomnia and decreased appetite. There were no clinically notable changes in vital signs of serious cardiac adverse events. Mean improvements in ADHD-RS score were –10.2 for patients switched from placebo to dexmethylphenidate ER ($n = 20$) and –8.4 for those maintained on dexmethylphenidate ER ($n = 82$). Respective CGI-I responder rates were 95.0 and 95.1% (Adler et al. 2008).

Mixed amphetamine salts

The effects of MAS extended release (MAS-ER) 20, 40 or 60 mg/day in adults with ADHD was compared with placebo over a 4-week study period (Weisler et al. 2006). All doses of MAS-ER demonstrated significant improvements from baseline in mean ADHD-RS total score versus placebo. Mean changes from baseline for ADHD-RS inattentive and impulsive/hyperactive subscale scores were also significantly superior to placebo, for all doses of MAS-ER. For the subgroup of patients with severe ADHD symptoms at baseline (ADHD-RS total score $>$ 32), MAS-ER 20 mg/day ($P = 0.001$) and 60 mg/day ($P = 0.0007$) achieved significant reductions in ADHD-RS total score. CGI-I ≤ 2 was achieved by significantly more patients receiving MAS-ER than those receiving placebo. In the same study, the duration of treatment effect was measured using the 4- and 12-h post-dose CAARS:S-S ADHD index. At both times, all doses of MAS-ER were associated with significant improvements versus placebo ($P < 0.05$ for all) (Weisler et al. 2006).

Patients completing this initial study were eligible to participate in a 24-month, open-label extension phase, where they received MAS-ER 20, 40 or 60 mg/day. Patients switching from placebo achieved a significant decrease from baseline in ADHD-RS total score, while those continuing on MAS-ER from the double-blind phase maintained their initial symptomatic improvement. For the ADHD-RS subscales, MAS-ER-naïve patients achieved significant reductions from baseline for inattentive and impulsive/hyperactive symptoms, respectively ($P < 0.001$ for both). Quality of life, as assessed by the Quality of Life Enjoyment and Satisfaction Questionnaire–short form (Q-LES-Q-SF), was significantly improved from baseline in the patients switching from placebo ($P = 0.042$), whereas those remaining on MAS-ER from the double-blind phase experienced little change (Biederman et al. 2005).

Atomoxetine

The noradrenaline reuptake inhibitor atomoxetine (60–120 mg/day; administered bd) has been evaluated in two identical trials of 10 weeks' duration. Both studies demonstrated significant improvements from baseline in investigator- and self-rated CAARS total scores for atomoxetine versus placebo. Inattentive and impulsive/hyperactive subscale scores were also significantly improved from baseline for atomoxetine compared with placebo (Michelson et al. 2003). A subgroup of patients from these studies ($n = 144$) who were considered to be experiencing emotional dysregulation (ED) (according to WRAADDS) was assessed for treatment response. For WRAADDS ED symptoms, mean scores decreased from baseline to Week 10 by a significantly greater amount with atomoxetine than with placebo ($P = 0.001$). In addition, these patients achieved significantly greater reductions in CAARS total score from baseline with atomoxetine ($P = 0.002$ versus placebo). This was a greater reduction than that reported for patients without ED (Reimherr et al. 2005).

Patients completing these studies were eligible to participate in a 3-year, open-label trial evaluating atomoxetine 50–160 mg/day, following a washout period of 4 weeks. After a mean of 40 weeks, there were significant mean changes from baseline in both investigator- and self-rated CAARS total scores (both $P < 0.001$). Inattentive and impulsive/hyperactive symptoms were also significantly improved from baseline with atomoxetine treatment ($P < 0.001$ for all) (Adler et al. 2005).

Once- and twice-daily dosing of atomoxetine (80 mg/day in both treatment arms) was compared in a 6-week study. Mean reductions from baseline in CAARS:O-S total score, and the inattentive and hyperactive/impulsive scores were significantly greater for atomoxetine 40 mg bd versus 80 mg qd (all $P < 0.001$) (Adler et al. 2006a). Quality of life was also assessed in this study, using pooled data for both dosing regimens. The mean mental component score of the 36-item Short Form health survey questionnaire (SF-36) was significantly improved from baseline, and all the individual domain scores also improved significantly over the 6-week study period (all $P < 0.001$ versus baseline). The physical component score slightly worsened during the study, but this was not statistically significant. However, individual scores for bodily pain and general health significantly improved from baseline (both $P<0.05$) (Adler et al. 2006b).

Lisdexamfetamine

Lisdexamfetamine is a prodrug, administered once-daily, which is converted to L-lysine and *d*-amphetamine following ingestion (Faraone 2008). Lisdexamfetamine was evaluated at doses of 30, 50 and 70 mg/day, over a 4-week period. At the end of the study, mean changes from baseline in clinician-determined ADHD-RS scores were significantly greater for all doses of LD versus placebo. In addition, significantly greater percentages of patients receiving LD achieved CGI-I scores ≤ 2. Improvement in both measures was evident from Week 1 onwards (Adler et al. 2008).

Pharmacotherapy of adult ADHD: Safety and tolerabilty

In general, stimulant therapy is well tolerated in the short-term, with the most commonly reported adverse events being tremor, anxiety, restlessness, diarrhoea or constipation, headache, dryness of mouth, dizziness, insomnia, loss of appetite (Faraone et al. 2004; Kooij et al. 2004; Spencer et al. 2005; Biederman et al. 2006b; Medori et al. 2008). However, despite this profile, a number of concerns remain regarding the efficacy of stimulants in the longer term (> 2 years) and the impact of these agents on the cardiovascular system.

MPH, MAS-ER and amphetamines are all associated with stimulatory effects on the cardiovascular system, leading to small but significant increases in blood pressure and heart rate, without effects on conduction (Goodman et al. 2005; Weisler et al. 2005; Biederman et al. 2006b; Reimherr et al. 2007; Medori et al. 2008). The Food and Drug Administration has also described cases of myocardial infarction, stroke and sudden death in adults and children whilst on stimulant therapy for the treatment of ADHD (Nissen 2006). However, an FDA review of sudden cardiac death among adults and children receiving stimulants (MPH and amphetamines) concluded that catastrophic cardiovascular events are extremely rare, with the odds of death due to injury from a variety of other catastrophic events (e.g., self-harm, motor vehicle accidences, fire, drowning) far exceeding those for sudden cardiac death in patients treated with stimulants. Furthermore, half the individuals who experienced sudden cardiac death were determined to have had pre-existing, although generally undetected, cardiovascular risks, or were using multiple drugs simultaneously (Newcorn et al. 2008).

In otherwise healthy adults, few have exhibited clinically significant abnormalities during clinical trials evaluating these agents. However, vital signs should be regularly monitored during treatment (Weisler et al. 2005; Wilens et al. 2005).

A review of clinical data from randomized, double-blind, placebo-controlled trials has investigated the potential cardiovascular effects of atomoxetine in children and adults with ADHD. Results showed that atomoxetine was associated with small but statistically significant increases in systolic blood pressure and heart rate in adults. In addition, the incidence of palpitations was higher with atomoxetine (3.7%) than with placebo (0.8%) (Wernicke et al. 2003). Of note, since the launch of atomoxetine three spontaneously reported cases of reversible drug-induced liver injury have been reported (Bangs et al. 2008). Atomoxetine should therefore be discontinued in patients with jaundice or laboratory evidence of liver injury and should not be restarted.

Stimulants are also associated with sleep disturbances. In studies evaluating MPH-IR in adults, difficulty sleeping was reported for 24% of patients versus 17% of patients receiving placebo (Spencer et al. 2005). Similarly, sleep problems (predominantly insomnia) have been observed for 12–18% of patients receiving OROS MPH in clinical studies, compared with 5–7% of patients receiving placebo (Biederman et al. 2006b; Medori et al. 2008). However, two studies have shown that although MPH may be associated with later sleep onset, and reduced sleep duration, the number and total duration of nocturnal awakenings is reduced, with a corresponding increase in the mean duration of within-night periods of uninterrupted sleep; i.e. improved sleep quality (Boonstra et al. 2007; Sobanski et al. 2008). In a 5-week clinical study, the incidence of insomnia was 13% for placebo and 21–30% for MAS-ER 20–60 mg/day (Weisler et al. 2006). In an open-label study of patients receiving MAS-ER 10–60 mg/day for 10 weeks, the incidence

of insomnia was 20% for patients receiving no prior ADHD therapy, 14% for patients receiving prior stimulant therapy, and 23% for patients receiving prior non-stimulant therapy (Goodman et al. 2005). In two identical 10-week studies, the incidence of insomnia with atomoxetine 60–120 mg/day was 21% compared with 9% for placebo (Michelson et al. 2003).

Effects of drug treatments on driving

The effects of stimulant treatment on driving performance in adults with ADHD have been investigated in two studies. MPH-IR (10–30 mg/day) significantly reduced weaving of the car during a 100-km driving test versus placebo, but variability in speed was comparable for these treatments (Verster et al. 2008). The effects of qd administration of OROS MPH and tds administration of MPH-IR on driving performance were compared in young men (aged 16–19 years) with ADHD. In this single-blind, crossover study, a driving simulator test was performed at 14:00, 17:00, 20:00 and 23:00. Mean Impaired Driving Score (IDS) was improved throughout all the tests following 1 week of treatment with OROS MPH (Cox et al. 2004). However, after 1 week of treatment with MPH-IR, IDS was significantly worse than with OROS MPH for the tests at 20:00 and 23:00 ($P = 0.01$). In 18 adults with ADHD, atomoxetine 1.2 mg/kg per day for 3 weeks demonstrated improvements in self-rated driving behaviour and driving simulator performance compared with tests performed prior to treatment initiation. However, no differences were observed by independent observers (Barkley et al. 2006).

Pharmacotherapy of adult ADHD: Considerations for treatment selection

When selecting drug therapy for ADHD, duration of daily symptom control is a key consideration. In general, the effects of MPH-IR usually last only about 4 h, although 12-h coverage may be provided with tds dosing (Wolraich and Doffing 2004). The development of MPH-ER preparations has allowed continuous effective management of the symptoms of ADHD over a longer time period (approximately 8 h) (Banaschewski et al. 2006), although their effect can be extended by the addition of an MPH-IR dose. Compared with multiple daily doses of MPH-IR, OROS MPH provides 12-h coverage of symptoms, with a similarly rapid onset of effect (Swanson et al. 2003). MAS-ER has also demonstrated significant reductions in ADHD symptoms when measured at 12 h post-dose.

Another important consideration when selecting a therapy for adults with ADHD is a convenient dosing regimen. Less frequent dosing of medication may be linked to greater persistence with treatment (Olfson et al. 2007), and overcoming the risk of forgetting mid-day doses due to inattention or disorganization (Biederman and Faraone 2005). A retrospective analysis has suggested that OROS MPH is associated with a greater adherence to and persistence with therapy than MPH-IR (Kemner and Lage 2006). In this analysis, significantly fewer individuals who initiated treatment with OROS MPH had a 15-day gap in therapy (85 vs. 97%; $P < 0.0001$), a 30-day gap in therapy (77 vs. 95%; $P < 0.0001$) or switched to another ADHD medication (27 vs. 68%, $P < 0.0001$). Moreover, patients who initiated therapy with OROS MPH stayed on therapy significantly longer (mean 199 vs. 108 days; $P < 0.0001$) (Kemner and Lage 2006). Similarly, results from a chart review of Spanish adults with ADHD reported that switching from MPH-IR to OROS MPH was associated with an improvement in compliance, as rated by the Simplified Medication Adherence Questionnaire (Ramos-Quiroga et al. 2008).

Longevity of clinical benefit is also important. Of the studies reviewed in this paper, the majority were of around 6 weeks' duration. One study, which evaluated 12 weeks of therapy with OROS MPH, demonstrated a sustained improvement in ADHD symptoms over this time (Medori et al. 2008), and 10-week studies evaluating atomoxetine reported similar outcomes (Michelson et al. 2003). A recent study reported that MPH-ER resulted in clinical and statistically significant reductions of ADHD symptoms over 24 weeks (Rösler et al. 2009). In addition, one study has indicated the prolonged benefit of MAS-ER over a 24-month period (Biederman et al. 2005).

Psychosocial treatment of adult ADHD

Although symptoms generally improve with medication, residual symptoms may still impact upon functioning in adults with ADHD. Adjunctive psychosocial interventions may, therefore, be beneficial (Safren et al. 2004).

A tailored form of cognitive behavioural therapy (CBT) was evaluated in 31 adults with ADHD who were continuing to experience clinically significant symptoms despite receiving medication. The CBT intervention consisted of 15 weeks of motivational interviewing and practice, repetition and review of skills such as organizing and planning, reducing distractibility, problem-solving, and adaptive thinking during times of stress. Those individuals in the control group received no psychological intervention. At study end, those patients receiving CBT plus drug therapy achieved a significantly greater improvement in ADHD symptoms, as measured by the ADHD-RS total score

and change in CGI-S ($P < 0.01$ for both measurements versus controls). In addition, the proportion of responders (those achieving a $\geq$ 2-point change in CGI-S) was higher in the CBT group versus controls (56 vs. 13%; $P < 0.02$) (Safren et al. 2004, 2005). CBT has also been applied to 43 adults with ADHD in a separate randomized, controlled trial. CBT was delivered as eight 2-h sessions, plus homework exercises. When compared with the control (no CBT) ADHD group, those receiving the intervention experienced significant improvements in their ADHD symptoms, organizational skills and a reduction in their levels of anger. Notably, the improvements in symptoms and organizational skills were maintained at 1 year following the intervention (Stevenson et al. 2002).

Dialectic behavioural therapy (DBT) has also been evaluated in adults with ADHD, in a pilot study involving seven patients. DBT sessions were attended by the patients on a weekly basis, for a 3-month period (Hesslinger et al. 2002). These sessions covered topics such as mindfulness, emotion regulation and impulse control. Participants were also required to undertake daily exercises by themselves outside of the DBT sessions and to read educational materials regarding ADHD. Following completion of the DBT course, ADHD symptoms were significantly improved from baseline (as measured using 16 relevant items from the Symptoms Check List; $P < 0.02$). In addition, depressive symptoms were also improved, according to measurements on the Beck Depression Inventory. In terms of neuropsychological outcomes, patients completing DBT demonstrated improvements in attention and psychomotor speed. However, no improvements in memory or executive function were observed.

Conclusions

Although generally considered a childhood disorder, there is now substantial evidence that ADHD symptoms can persist into adulthood, resulting in a significant negative impact on many aspects of daily life, particularly work and social domains. Epidemiological studies have demonstrated that ADHD in adults is a common phenomenon, affecting an estimated 3–4% of the population. Neuroimaging has demonstrated several structural differences to the brains of adults with ADHD compared with unaffected individuals, further supporting a diagnosis of ADHD in adults. Indeed, structural and functional brain imaging techniques converge in documenting changes in the prefrontal cortex and basal ganglia in adults with ADHD that are consistent with abnormalities in childhood ADHD and implicate persistent neurobiological alterations of brain circuits involved in attention regulation and executive functioning.

Diagnosis of ADHD in adults can be difficult, as the current DSM-IV-TR criteria require confirmation of symptom onset before the age of 7 years, and symptom expression in adults may differ from that in younger individuals. Several screening instruments and diagnostic interviews can be used to assess ADHD severity in adults, including the Wender Utah Rating Scale, The World Health Organization Adult Self Report Scale, the Conners' Adult ADHD Rating Scale and the WRAADDS. However, there is a need for further work in defining criteria and developing instruments for specific impairment in adult ADHD. Notably, the fifth edition of the DSM is considering an increase in the age of onset of symptoms to be present on or before the age of 12 years.

Drug therapy remains the first-line approach to treatment in adults with ADHD. A number of clinical studies have shown that treatment with stimulants (MPH-IR, MPH-ER, OROS MPH, MAS, dexmethylphenidate) and atomoxetine may reduce ADHD symptoms and improve quality of life. Selection of drug treatment must take into consideration duration of symptom benefit (on a daily and long-term basis), dosing regimen and safety aspects, in order to achieve the best outcomes for patients. Preliminary data suggest a benefit of adjunctive psychological interventions in individuals remaining symptomatic while receiving medication, but further investigation of such interventions is required.

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Supplementary material available online

Discussion of diagnostic instruments for the assessment of ADHD in adults.
Discussion of the genetic basis for ADHD.

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ORIGINAL INVESTIGATION

Do candidate genes discriminate patients with an autism spectrum disorder from those with attention deficit/hyperactivity disorder and is there an effect of lifetime substance use disorders?

BRAM SIZOO[1,2], WIM VAN DEN BRINK[3,4], BARBARA FRANKE[5,6], ALEJANDRO ARIAS VASQUEZ[5,6], PATRICIA VAN WIJNGAARDEN-CREMERS[1,2] & RUTGER JAN VAN DER GAAG[2]

[1]*Dimence Institute of Mental Health, Deventer, The Netherlands,* [2]*Karakter Department of Child and Adolescent Psychiatry, University Medical Center St. Radboud, Nijmegen, The Netherlands,* [3]*Department of Psychiatry, Academic Medical Center University of Amsterdam, Amsterdam, The Netherlands,* [4]*Amsterdam Institute for Addiction Research, Amsterdam, The Netherlands,* [5]*Department of Human Genetics and* [6]*Department of Psychiatry, Donders Institute for Brain, Cognition and Behaviour, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands*

Abstract

Objective. Autism spectrum disorder (ASD) and attention deficit/hyperactivity disorder (ADHD) are developmental disorders that overlap in a number of domains, sometimes complicating clinical distinction between both disorders. Although there is some evidence for a genetic overlap, there are no reports on genes that could differentiate between ASD and ADHD. Furthermore, it is not known whether this genetic overlap is influenced by co-morbid substance use disorders (SUD). *Methods.* A total of 110 adult patients with ASD (n=61) or ADHD (n=49) with or without a lifetime history of SUD participated in a study in which we genotyped polymorphisms in five known candidate genes for (one of) the disorders, i.e. the 5HTTLPR in *SLC6A4/5-HTT*, rs1800497 (TaqIA C>T) in *DRD2*, rs7794745 in *CNTNAP2*, rs1843809 in *TPH2*, and rs6565113 in *CDH13*. Genotyping was by Taqman-based analysis or by simple sequence length analysis, where appropriate. *Results.* ASD could be differentiated from ADHD with nominal statistical significance by the 5HTTLPR, and the polymorphisms in *TPH2* and *CNTNAP2*. The results were independent of lifetime SUD status. *Conclusions.* Serotonergic genes could prove to play an important role in differentiating between ASD and ADHD, but the results of this exploratory study need replication.

Key words: *Genetics, autism, ADHD, substance-related disorders*

Introduction

Genetics play a crucial role in the etiology of developmental disorders like autism spectrum disorder (ASD) and attention deficit/hyperactivity disorder (ADHD). Heritability of ADHD is estimated to be 76% (Faraone et al. 2005), and more than 90% in ASD (Steffenburg et al. 1989; Bailey et al. 1996). In the past decades many studies have attempted to identify the specific genes involved, often with conflicting results. Examples of studies documenting the multifactorial nature if both disorders have been presented for ADHD by Franke et al. (2009) and for ASD by Geschwind and Levitt (2007) and Happé and Ronald (2008). Complicating factors for gene identification are the polygenic nature of the disorders and the environmental influences on the phenotype. Furthermore, the phenotypes of both disorders are frequently complicated by the presence of comorbid psychiatric disorders. One of the most common comorbid conditions in ADHD is substance use disorder (SUD) (Biederman et al. 1998, 2008; Wilens et al. 2004; Kessler et al. 2006; Rommelse et al. 2010). SUDs are also reported to have a strong heritability ranging from 60 to 80%, dependent on the specific substance of abuse (Kendler et al. 2003). Interestingly, work by Ronald et al. (2008) indicated that there is evidence for moderate to substantial overlap in the genetic influences on ASD traits and

Correspondence: Bram Sizoo, Center of Developmental Disorders, Dimence Institute of Mental Health, Nico Bolkesteinlaan 1, Deventer, The Netherlands. Tel: +31 570 639600. Fax: +31 570 639629. E-mail: b.sizoo@dimence.nl

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ADHD behaviour. Also work by Reiersen et al. (2008) in young adult Australian twins suggests shared genetic influences on self-reported ADHD and autistic symptoms (Reiersen et al. 2008). Biederman et al. (2008) reported an overlap in familial risk between ADHD and drug dependence, but no overlap between ADHD and alcohol dependence (Biederman et al. 2008). Also, a twin study among girls by Knopik et al. (2009), showed no genetic overlap between ADHD and alcohol dependence (Knopik et al. 2009). To our knowledge, there are no studies reporting on a genetic overlap between ASD and SUD.

ADHD is a childhood onset condition that persists in about 50% of the cases into adulthood (Lara et al. 2009). ADHD is characterized by inattention, hyperactivity and impulsivity, but is rather heterogeneous with respect to the behavioural phenotype. Three main behavioural subtypes are recognized: an inattentive, a hyperactive-impulsive and a mixed subtype (American Psychiatric Association 2000). Dopaminergic and serotonergic neurotransmission genes have been implicated in ADHD aetiology (Sheehan et al. 2005; Nyman et al. 2007; Baehne et al. 2008). Dopaminergic genes have been most extensively studied in ADHD, especially focussing on dopamine transporter and receptor genes. For our purpose we selected the dopamine D2 receptor gene (*DRD2*), which is expressed in several brain regions like the basal ganglia and the prefrontal cortex, and is associated with the mesolimbic reward system and with inhibitory tasks. DRD2 has consistently been implicated in the pathophysiology of ADHD (e.g., Esposito-Smythers et al. 2009; Franke et al. 2009; Gizer et al. 2009). The TaqIA polymorphism (rs1800497) has been linked to *DRD2* expression levels (Laakso et al. 2005) and has also been implicated in the relationship between impulsiveness and the *DRD2* in alcohol-dependent men and women (Limosin et al. 2003). Sheehan et al. (2005) studied eight single nucleotide polymorphisms (SNP) in of the brain-expressed tryptophan hydroxylase (*TPH2*; a rate limiting enzyme in the synthesis of serotonin) and found a significant association between the T-allele of marker rs1843809 and ADHD (Sheehan et al. 2005). Recently, the first three genome-wide association studies (GWAS) for ADHD have been published. One of the most consistent findings was for the marker rs6565113 of *CDH13* (Franke et al. 2009). This gene codes for cadherin 13, a member of the cell–cell adhesion proteins, and also regulates neural cell growth. *CDH13* is expressed in brain regions showing volumetric reductions that have been implicated in ADHD. Interestingly, *CDH13* was also reported to be associated in GWAS with alcohol dependence (Johnson et al. 2006; Treutlein et al. 2009) and metamphetamine dependence (Uhl et al. 2008).

Autism spectrum disorders (ASD) include the autistic disorder (AD), Asperger's syndrome (AS), and pervasive developmental disorder not otherwise specified (PDD-NOS) (American Psychiatric Association 2000). ASD is a behaviourally defined disorder, with impairments in three domains: (1) reciprocal social interaction, (2) language, and (3) stereotyped responses and restricted interests. The very high heritability has prompted much genetic research. In searching for the genetic underpinnings of ASD, serotoninergic genes have received a lot of attention, e.g., the promoter variants (5HTTLPR) of the serotonin transporter gene (*SLC6A4*/*5-HTT*), which is associated with ASD (Muhle et al. 2004). The biological reason for assuming a more frequent presence of the long allele of the 5HTTLPR polymorphism in ASD, is that this polymorphism increases gene expression resulting in higher serotonin reuptake activity, as has been shown in autistic children (Huang and Santangelo 2008). The serotonin transporter gene has also been implicated in reward and addiction mechanisms (Oroszi and Goldman 2004), as well as in ADHD (Gizer et al. 2009). Another interesting candidate gene that is associated with ASD, is contactin-associated protein-like 2 (*CNTNAP2*) (Alarcon et al. 2008) encoding a neuronal cell adhesion molecule known to mediate cell–cell interactions and possibly involved in axon differentiation. It was suggested that *CNTNAP2* is associated with social and cognitive delay, and the polymorphism showed association with ASD (Bakkaloglu et al. 2008). The CNTNAP2 transcript has been found in frontotemporal-subcortical regions associated with executive function, and the development of "joint attention", which is considered to be at the core of the social deficit in ASD (Alarcon et al. 2008).

Substance use disorder (SUD) is defined as substance dependence or abuse (American Psychiatric Association 2000). The genetic component of SUD is primarily connected to the transition from unproblematic substance use to dependence. Kendler et al. (2003) have shown that SUD shares genetic factors with conduct disorder and with antisocial personality disorder, both of which are often co-morbid with ADHD.

In clinical practice, overlap between ASD and ADHD is assumed that can make the distinction at a diagnostic level confusing (Clark et al. 1999; Goldstein and Schwebach 2004; Banaschewski et al. 2005; Mulligan et al. 2008; Rommelse et al. 2010). The overlap between ASD and ADHD can be identified in different domains. With regard to brain structure, Brieber et al. (2007) studied similarities

and differences between ASD and ADHD patients. Both disorders shared structural deviations in the medial temporal lobe and in the inferior parietal lobe compared to normal controls. However, the ASD group differed from the ADHD group in grey matter abnormalities near the right temporo-parietal junction (Brieber et al. 2007). Saitoh et al. (1995) reported a smaller corpus callosum in ASD and ADHD patients compared to controls (Saitoh et al. 1995). In the personality domain, although ASD and ADHD groups show distinctive profiles, both disorders share a high temperament score of harm avoidance, and high character scores (self directedness, cooperativeness, and self-transcendence) on the Temperament and Character Inventory, compared to the norm population (Anckarsater et al. 2006; Sizoo et al. 2009a). The area of overlap that has received most attention is the neuropsychological domain. Most comparative studies have been conducted among children with ASD or ADHD reporting impairment of ADHD children in inhibition and working memory tasks and of ASD children in social tasks, planning and flexibility abilities (Luteijn et al. 2000; Sinzig et al. 2008a,b; Yerys et al. 2009). Planning impairments are reported in both groups although more pronounced in children with ASD (Booth et al. 2003). From other studies, it appears that executive functioning (EF), such as planning, working memory, impulse control, inhibition, and mental flexibility, does not clearly differentiate between ASD and ADHD in children (Ozonoff and Jensen 1999; Geurts et al. 2004; Banaschewski et al. 2005; Tsuchiya et al. 2005). Happé et al. have put this in a developmental perspective and suggested that EF deficits, are less severe in older children with ASD, compared to older children with ADHD (Happe et al. 2006). Likewise, Corbett and Constantine (2006) studied ADHD-like deficits in children with ASD with respect to attentional impairments. An important new finding is that although many neuropsychological measures do not discriminate between ASD and ADHD (mental flexibility, response inhibition and attention tasks), these measures do show a different pattern when stratified for SUD status. Then the ASD group with a lifetime history of SUD shows more impairment than the ASD group without SUD or any of the SUD subgroups in ADHD patients (Sizoo et al. 2010). There is evidence for substantial genetic overlap as well (Ronald et al. 2008). However, there are to our knowledge no reports on the role of candidate genes in differentiating between ASD and ADHD. Neither has the role of candidate genes in the occurrence of SUD comorbidity in ASD or ADHD been studied. Therefore, this study examines whether ASD and ADHD can be differentiated on the basis of five candidate genes tapping into two different neurobiological systems (potentially) involved in disease aetiology, i.e. (1) neurotransmission via dopamine or serotonin and (2) neural development/neuronal migration. Although single polymorphisms in SLC6A4/5-HTT and CNTNAP2 have been shown to be mainly associated with ASD and polymorphisms in DRD2, TPH2, and CDH13 with ADHD, there are other studies that did find these associations (e.g., DRD2 in ADHD; Todd and Lobos 2002). We hypothesize that these associations also allow for differentiating ASD from ADHD. In addition, the study explores whether these associations are modified by the presence of comorbid lifetime SUD. We discussed that three of the candidate genes that are associated with ASD or ADHD are also associated with SUD (*DRD2*, *SLC6A4/5-HTT*, and *CDH13)*. Elaborating on the first hypothesis, we propose that these three candidate genes will therefore not only differentiate between ASD and ADHD in the absence of SUD, but also in the presence of comorbid SUD.

Method

Subjects

Blood samples were obtained from a consecutive sample of 110 patients between January 2006 and June 2007, as part of a larger overall study on the co-morbidity of ASD and ADHD with SUD (Sizoo et al. 2009a,b). Subjects were recruited from two specialized diagnostic centers for adult patients with possible developmental disorders such as ASD and ADHD. After being diagnosed with ASD or ADHD, 191 patients ($n=100$ ASD and $n=91$ ADHD), were informed by their own clinicians about the study, and asked for permission to be approached by the research team. Exclusion criteria were: history of comorbid psychotic disorder, bipolar disorder, IQ less than 80, insufficient command of the Dutch language, and uncorrected visual or auditory impairment. Of the 167 patients that our team was allowed to contact, 138 agreed to participate and gave informed consent. Of those, 13 withdrew from participation for unaccounted reasons and two were excluded due to a total IQ below 80, leaving 123 participants in the total study, 110 of whom gave permission to draw blood for analysis. The 28 patients who gave informed consent for the other parts of the study, but did not participate in this genetic part of the study, did not differ from the 110 who did participate with respect to age, IQ, gender distribution and diagnosis. Approval for the study was obtained from the regional medical ethical committee. Privacy protection was guaranteed by

assigning a unique numeric code to patient data; the key of which was only known to the principal researcher. After complete description of the study to the subjects, written informed consent was obtained prior to their inclusion in the study.

The IQ was determined by means of the Dutch Wechsler Adult Intelligence Scale III in 85 patients (Wechsler 1997; Uterwijk 2000). For the remainder (n=38), the IQ had already been established elsewhere with valid methods.

DSM-IV diagnoses were based on current and retrospective assessment by experienced clinicians. For a diagnosis of ASD, semi-structured clinical interviews based on the Autism Diagnostic Interview Revised (ADI-R) were used (Lord et al. 1994) as were DSM-IV checklists and available information from schools and child psychiatric services concerning the development in childhood. The ADHD diagnoses were made according to a national protocol, including a semi-structured developmental history, and a DSM-IV criteria checklist for adult and childhood ADHD symptoms (Kooij et al. 2005).

As part of our study, a blind expert clinician (RvdG) reviewed 10 randomly selected charts and conducted 10 randomly selected clinical interviews, using the Autism Diagnostic Observation Schedule protocol for the diagnosis of ASD (Lord et al. 2000), and a DSM-IV checklist for adults for the diagnosis of ADHD (Kooij et al. 2005). His diagnoses were compared with the diagnoses that were routinely made at the centres. Inter-rater agreement was high with Cohen's values of 0.78 for the chart reviews and 0.81 for the clinical interviews. Seven subjects (5.7%) had been diagnosed with ADHD before being diagnosed with ASD in the centres. Because all ADHD associated symptoms reported by these subjects could – in retrospect – better be explained by ASD, we decided to use only the diagnosis ASD in these patients.

Procedure and assessments

To obtain representative results for ADHD, subjects with ADHD were required to cease stimulant medication two days prior to the assessments of the total study. Other medications, and coffee and nicotine consumption were not interrupted.

Substance use disorder (SUD) was diagnosed by the research clinician if the DSM-IV criteria for abuse or dependence were met. The substances that were investigated were alcohol, methadone, benzodiazepines, cocaine, heroin, amphetamines, cannabis, and combinations of these. Because behavioural addictions, like gambling, seem to share the same neurobiological underpinnings of craving and dependence, gambling was also included in our definition of SUD (Goudriaan et al. 2006; Potenza 2007). Three of the 110 subjects (2%) presented with comorbid gambling. Subjects with a lifetime history of substance abuse or dependence were designated as LTSUD. During the assessments none of the participants were noticed to be under influence of alcohol or illicit drugs.

Genetic analysis

DNA was isolated from blood using standard procedures. The genotyping was carried out in a CCKL quality-certified laboratory. Generally, 5% blanks as well as duplicates between plates were taken along as quality controls during genotyping.

Genotyping of the 5HTTLPR polymorphism in the promoter region of *SLC6A4/5-HTT* gene was performed by simple sequence length analysis. PCR was on 50 ng genomic DNA using 0.5 μM fluorescently labeled forward primer (FAM-5'-GGCGTT GCCGCTCTGAATGC-3') and reverse primer (5'-GAGGGACTGAGCTGGACAACCAC-3'), 0.25 mM dNTPs, 1× PCR optimization buffer A (30 mM Tris–HCl, pH 8.5, 7.5 mM $(NH_4)_2SO_4$, 0.75 mM $MgCl_2$), 10% DMSO and 0.4 U AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The cycling conditions for the polymerase chain reaction started with 12 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at the optimized annealing temperature (57.5°C), and 2 min at 72°C, then followed by an extra 10 min at 72°C. Subsequent determination of the length of the alleles was performed by direct analysis on an automated capillary sequencer (ABI3730, Applied Biosystems) using standard conditions.

The *DRD2* TaqIA C>T polymorphism (rs1800497), the *CNTNAP2* polymorphism (rs7794745), the *TPH2* polymorphism (rs1843809) and the *CDH13* polymorphism (rs6565113) were all genotyped using Taqman analysis (*DRD2*: assay ID: Taqman assay:C___7486676_10; reporter 1: VIC-A-allel, reverse assay; *CNTNAP2:* assay ID: Taqman assay:C__2661558_10; reporter 1: VIC-A-allel; assay ID: Taqman assay:C__11479729_10; *TPH2*: reporter 1: VIC-G-allel, reverse assay; *CDH13*: assay ID: Taqman assay:C__30498780_10; reporter 1: VIC-G-allel; all Applied Biosystems). Genotyping was carried out in a volume of 10 μl containing 10 ng of genomic DNA, 5 μl of Taqman Mastermix (2×; Applied Biosytems), 0.125 μl of the Taqman assay (40×) and 3.875 μl of water. Genotyping was performed on a 7500 Fast Real-Time PCR System and genotypes were scored using the algorithm and software supplied by the manufacturer (Applied Biosystems).

Statistical analysis

Genotype distributions were controlled using Hardy–Weinberg Equilibrium (HWE) testing. To explore differences between the diagnostic groups, we performed non-parametric and univariate ANOVA analyses. For each of the five candidate genes, we performed computations with both dominant and recessive models.

For each candidate gene we defined two genotype groups. The group suffix indicates the presence of an allele in the group (i.e. TaqIA-C denotes the presence of the C allele, incorporating both C/C and C/T subjects, while TaqIA-T is composed of both T/T and C/T subjects). To determine the effect of the presence or absence of a genotype group on the diagnosis (ASD or ADHD), we performed univariate logistic regression analysis with diagnosis as dependent variable and genotype group, LTSUD status, sex, and genotype group by LTSUD interaction as independent variables. The interaction term assesses whether the effect of the genotype group on diagnosis was modified by LTSUD (i.e. different between patients without and with LTSUD). In case LTSUD did not modify the relation between the genotype group and diagnosis, we removed the interaction term from the model and presented the odds ratio (OR), adjusted for LTSUD and sex.

Statistical analyses were carried out with SPSS 15.0 software, using two-tailed tests with $\alpha=0.05$. We maintained a significance level of $P=0.05$ for the logistic regression analyses.

Results

The 110 participants in this study consisted of 61 subjects with ASD, and 49 subjects with ADHD (Table I).

The ASD and ADHD group were very similar with respect to IQ and age, but there were significantly more males in the ASD group than in the ADHD group (80 vs. 63%, $X^2_{(1)}=3.99$, $P=0.046$). In addition, the prevalence of LTSUD was significantly higher in ADHD than in ASD patients (57 versus 30%, $X^2_{(1)}=8.53$, $P=0.003$). As a consequence, genetic comparisons between ASD and ADHD have to be adjusted for differences in sex and LTSUD.

The distribution of the three genotypes and the genotype groups for each of the candidate genes is shown in Table II. The analysis for the distribution of genotype groups in ASD and ADHD patients by means of logistic regression analyses is presented in Table III.

Genetic differences between ASD and ADHD patients

Carriership of the T-allele of the *CNTNAP2* rs7794745 polymorphism is significantly more often present in the ADHD group compared to the ASD group ($OR_{adj}=2.67$, 95% CI 1.13–6.29, $P=0.025$), whereas carriers of the G-allele of the *TPH2* rs1843809 polymorphism, and of the long (L) allele of the 5HTTLPR were significantly less frequent in ADHD compared to ASD patients ($OR_{adj}=3.00$, 95% CI 1.05–8.62, $P=0.041$ and $OR_{adj}=5.49$, 95% CI 1.74–17.32, $P=0.04$, respectively). In a post hoc logistic regression, the interaction between TPH2-G and HTTLPR-L was not statistically significant, indicating that these two serotonergic genotypes were independent. None of the interaction terms (genotype group by LTSUD) were significant, indicating that the associations between diagnosis and genetic factors were not statistically different for patients with or without LTSUD.

Discussion

This study aimed to examine whether known candidate genes could differentiate between ASD and ADHD, and whether the presence or absence of co-morbid lifetime SUD had any influence on this pattern.

Given the modest sample sizes it must be emphasized that the results are preliminary, calling for

Table I. Comparison of demographic characteristics and lifetime substance use disorder (SUD) characteristics in adults with autism spectrum disorder or ADHD.

	ASD ($N=61$)	ADHD ($N=49$)	Test statistic
Age – mean (SD)	35.7 (12.11)	32.5 (11.60)	$t_{(109)}=2.03$, $P=0.157$
Female – *N* (%)	12 (20)	18 (37)	$X^2_{(1)}=3.99$, $P=0.046$
Total IQ – mean (SD)	103.3 (13.96)	104.0 (10.72)	$t_{(73)}=0.06$, $P=0.807$
Lifetime SUD – *N* (%)	19 (30)	28 (57)	$X^2_{(1)}=8.53$, $P=0.003$
	Distribution of SUD types		$X^2_{(2)}=3.31$, $P=0.191$
Alcohol – *N* (%)	10 (53)	8 (29)	
Cannabis – *N* (%)	5 (26)	8 (29)	
Others – *N* (%)	4 (21)	12 (43)	

Table II. Distribution of genotypes and genotype groups for five candidate genes in adults with ASD or ADHD (n=110).

Candidate gene and marker	Genotype count (% of total sample)			Genotype group (% of total sample)	
DRD2 rs1800497 (Taq1A)	C/C	C/T	T/T	Taq1A-C	Taq1A-T
	76 (69)	32 (29)	2 (2)	108 (98)	34 (31)
CNTNAP2 rs7794745	A/A	A/T	T/T	CNTNAP2-A	CNTNAP2-T
	45 (41)	52 (47)	13 (12)	97 (88)	65 (59)
CDH13 rs6565113	T/T	T/G	G/G	CDH13-T	CDH13-G
	38 (35)	57 (52)	15 (13)	95 (86)	72 (66)
TPH2 rs1843809	T/T	T/G	G/G	TPH2-T	TPH2-G
	86 (78)	23 (21)	1 (1)	109 (99)	24 (22)
SLC6A4/5-HTT HTTLPR	L/L	S/L	S/S	5HTT-L	5HTT-S
	31 (28)	57 (52)	22 (20)	88 (80)	79 (72)

future replication. The results show that three candidate genes (5-HTT, TPH2, and CNTNAP2) differentiate with nominal statistical significance between the ASD and ADHD groups, regardless of the presence or absence of LTSUD. Whereas the presence of the serotonergic TPH2-G allele and HTTLPR-L predict ASD rather than ADHD, presence of CNTNAP2-T was significantly more prominent in ADHD compared to ASD. To test the post-hoc hypothesis that the effect of co-occurring polymorphisms is larger than the individual effects, we examined the distribution of a new composite variable over both diagnostic groups. This variable was set at value 0, but was given a value of 1 if the following conditions were fulfilled: (1) presence of TPH2-G, and (2) presence of HTTLPR-L and (3) absence of CNTNAP2-T. All three conditions were fulfilled in only five of the 61 ASD patients (8%), and only two of the 49 ADHD patients (4%). There was no statistically significant difference between the ASD and ADHD groups ($X^2_{(1)}$=0.772, P=0.38). Although this suggests that combinations of polymorphisms are not more powerful in differentiating between ASD and ADHD than single polymorphisms, this should be properly tested in future studies, as the numbers in our sample were too small to draw valid conclusions. Although this is, to our knowledge, the first time that candidate genes have been shown to differentiate between ASD and ADHD, there is, as yet, no use of candidate genes for clinical assessments, because single genes and even combinations of the investigated genes, are not able to effectively distinguish between the two disorders in individual patients. However, these findings are important for future research on the underlying differences in the developmental mechanism in ASD and ADHD. Importantly, the clinically complicating factor of comorbid SUD, does not seem to influence

Table III. Distribution of genotype groups in adults with autism spectrum disorder (ASD) or ADHD with or without lifetime substance use disorder (LTSUD).

	ASD		ADHD		Risk ADHD versus ASD
	No LTSUD	With LTSUD	No LTSUD	With LTSUD	Adjusted[b] OR (95%CI)
n=110	n=42	n=19	n=21	n=28	[P value]
TaqIA-C	43 (100)	17 (94)	21 (100)	27 (96)	n.s.
TaqIA-T	15 (35)	6 (33)	7 (33)	6 (21)	n.s.
CNTNAP2-A	38 (88)	17 (94)	15 (71)	27 (96)	n.s.
CNTNAP2-T	21 (49)	9 (50)	15 (71)	20 (71)	2.67[p] (1.13–6.29) [0.025]
CDH13-T	37 (86)	14 (78)	20 (95)	24 (86)	n.s.
CDH13-G	28 (65)	14 (78)	13 (62)	17 (61)	n.s.
TPH2-T	43 (100)	18 (100)	21 (100)	27 (96)	n.s.
TPH2-G	10 (23)	7 (39)	2 (10)	5 (18)	3.00[a] (1.05–8.62) [0.041]
5HTTLPR-S	31 (72)	11 (61)	17 (81)	20 (71)	n.s.
5HTTLPR-L	40 (93)	16 (89)	15 (71)	17 (61)	5.49[a] (1.74–17.32) [0.004]

[p]OR for ADHD versus ASD in the condition that the allele is present;
[a]OR for ADHD versus ASD in the condition that the allele is absent;
[b]OR adjusted for sex and LTSUD.

the differentiating ability of the polymorphisms in ASD and ADHD patients.

Moreover, given the small group size in our study, and the conflicting results in general in candidate genes studies, our findings require replication. It should be noted that personality profiles are different in ASD and ADHD (Soderstrom et al. 2002; Anckarsäter et al. 2006; Sizoo et al. 2009a), especially with respect to novelty seeking, which is associated with serotonergic pathways (Comings et al. 2000). Therefore, serotonin appears to play a role in the differentiation between ASD and ADHD in more than one domain. This requires further exploration in future research.

Our results (TPH2-G and HTTLPR-L predicting ASD and CNTNAP2-T predicting ADHD) seem to contradict the studies that were mentioned in the introduction comparing allelic distributions of candidate genes in clinical samples with those in normal controls. For example, Sheehan (2005) found the T-allele of the TPH2 variant rs1843809 overrepresented in ADHD patients of 179 nuclear families in Ireland. However, a recent meta-analysis of the findings in children refuted earlier findings for this SNP in ADHD, although heterogeneity between studies was noted (Gizer et al. 2008). Studies on the serotonin transporter gene give conflicting results on the allele involved in autism. For example, Raznahan et al. (2009) could not find a difference in S/L allele frequency (Raznahan et al. 2009). Huang and Santangelo (2009) found overrepresentation of the S-allele in US samples of autistic patients, but not in European and Asian samples (Huang and Santangelo 2008). Klauck et al. (1997) found an overrepresentation of the HTTLPR-L variant in ASD in contrast to other studies.

However, the current study differs from the studies mentioned in the introduction in that it compares ASD with ADHD patients, and not with healthy controls in the general population. Although in our study the ASD and ADHD samples are assumed to be independent, we know that there is some clinical overlap in symptoms. The results presented here show that ASD and ADHD patients in our samples differ from each other on three allelic subtypes (5HTTLPR-L, TPH2-G, and CNTNAP2-T), but the results also suggest that both diagnostic groups do not differ significantly on seven other allelic subtypes. The relative similarity in allelic distribution between ASD and ADHD patients could point to heterogeneity of these diagnostic constructs, as well as to significant overlap between ASD and ADHD. In that respect, only the TPH2-G allele (and not the TPH2-T allele) could differentiate between ASD to ADHD. Similar arguments apply to the other genotypes: only the L-allele of the 5HTTLPR could differentiate between the two groups of patients, and the CNTNAP2 rs7794745 T-allele was more prominent in the ADHD groups compared to the ASD group. Although Arkin (2008) found an overrepresentation of the CNTNAP2 rs7794745 T-allele in autism, this does not preclude a finding in ADHD. In fact, the gene now indeed also seems to be associated with other psychiatric disorders, in this case Tourette's syndrome (e.g., Bakkaloglu 2008).

This study not only underscores the polygenic nature of ASD. In conjunction with the fact that genetic liabilities are likely to be shared between ASD and ADHD (Rommelse et al. 2010), this could point to yet undiscovered endophenotypes shared by multiple disorders, and calls for cross-domain studies. It also raises the question as to how well the disorders are conceptually defined. Studying comorbidity patterns offer good opportunities for gaining a better understanding of the mechanisms involved (Angold et al. 1999). In addition, we need to look into the combined effects of multiple genes and into the associations between genes and phenotypes compared to genes and endophenotypes. In general endophenotypes are regarded to be more proximate to the genetic causes than phenotypes, although others have questioned this assumption (Flint and Munafo 2007). In future research we will also need to pay special attention to gene–environment (G × E) interactions to gain more insight in dynamics of disorders like ASD and ADHD that are characterized by their developmental nature. A number of strengths and limitations of this study should be mentioned. A clear strength of our study is that we were able to compose relatively large, and carefully diagnosed, groups of patients with ASD or ADHD. An additional advantage is that our hypotheses were based on previous research. However, for a candidate genes study even the group sizes in our study are still relatively small. We therefore maintained a significance level of $P=0.05$ for the logistic regression analyses in this exploratory study. Too stringent Bonferroni corrections would have introduced undesirable type II errors. However, had we applied corrections for multiple testing, the differentiating ability of HTTLPR-L would have remained statistically significant, with a nominal significance for the other two markers CNTNAP2-T and TPH2-G. It is an important and novel finding that candidate genes differentiate between two developmental disorders, but there is clearly a need for replication in future studies. Moreover, the genetic differences are not such that genotyping can be used already as a diagnostic tool. In addition, future research should also look at the genetic underpinnings of overlapping ASD and ADHD symptoms and at patients with a combined symptom profile in order to improve our

understanding of the neurobiological mechanisms involved in these symptoms and these disorders.

Conclusions

In our sample, the group of patients with ASD could be differentiated from patients with ADHD by polymorphisms in two serotonergic and one cell-adhesion gene: carriership of the long allele of the 5HTTLPR and of the *TPH2* rs1843809 T-allele, and carriership of the CNTNAP2 rs7794745 T-allele, respectively. This effect was not influenced by lifetime SUD, which is a common clinical comorbidity. However, the results require replication before clear conclusions about the strength of the effects can be drawn.

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Statement of interest

Dr Bram Sizoo reports no conflict of interests. Professor Wim van den Brink reports no conflict of interests related to the current manuscript. Dr Barbara Franke reports no conflict of interests related to the current manuscript. Dr Alejandro Arias Vasquez reports no conflict of interests. Dr Patricia van Wijngaarden-Cremers has received funding for RCT's from Eli Lilly and Jansen Cilag. She is an advisor for Eli Lilly and Jansen Cilag, but reports no conflict of interests related to the current manuscript. Professor Rutger Jan van der Gaag has received funding for RCTs from Eli Lilly and Jansen Cilag. He is an advisor for Eli Lilly and Jansen Cilag, but reports no conflict of interests related to the current manuscript.

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ORIGINAL INVESTIGATION

Twenty-four-week treatment with extended release methylphenidate improves emotional symptoms in adult ADHD

MICHAEL RÖSLER[1], WOLFGANG RETZ[1], ROLAND FISCHER[2], CLAUDIA OSE[3], BARBARA ALM[4], JÜRGEN DECKERT[5], ALEXANDRA PHILIPSEN[6], SABINE HERPERTZ[7] & RICHARD AMMER[2]

[1]*Neurocentre, University Hospital of the Saarland, Homburg/Saar,* [2]*Medice Arzneimittel Pütter GmbH & Co KG, Iserlohn,* [3]*Institute of medical Informatics, Biometry and Epidemiology, University of Essen,* [4]*Central Institute of Mental Health, Mannheim,* [5]*Department of Psychiatry and Psychotherapy, University of Würzburg,* [6]*Department of Psychiatry and Psychotherapy, University of Freiburg, and* [7]*Department of Psychiatry and Psychotherapy, University of Rostock, Germany*

Abstract

Objectives. Treatment investigations with methylphenidate in adults with ADHD focus preferentially on the classical psychopathology: inattention, hyperactivity and impulsivity. ADHD-associated emotional symptoms, which are frequently present at least in ADHD subpopulations, were studied rarely. The vast majority of the placebo-controlled trials had observation periods between 4 and 8 weeks. To assess the medium- to long-term effects of extended release methylphenidate (MPH-ER) on emotional symptoms and other psychopathology frequently seen in ADHD patients, we conducted a large-scale, multicenter treatment study. *Methods.* We performed a randomised, 24-week, double-blind, placebo-controlled study in adults with ADHD. The diagnosis was made on the basis of the DSM-IV criteria, which were confirmed by clinical history and a structured psychopathological interview and the use of rating instruments. 363 patients were randomized to MPH-ER or placebo at a ratio of 2:1. The duration of the titration period was 5 weeks followed by a maintainance phase of 19 weeks. The efficacy measures were the observer rated 10-item Emotional Dysregulation Scale (EDS) derived from the Wender-Reimherr Adult Attention Deficit Disorder Scale (WRAADDS) and a self-report, six-item Emotional Lability Scale (ELS) extracted from the long version of the Conners Adult ADHD Self Report Scale (CAARS:S:L). In addition we used the SCL-90-R for the assessment of ADHD associated and comorbid psychopathology. *Results.* MPH-ER was statistically superior to placebo in reducing emotional symptoms as assessed by the EDS and the ELS. Obsessive-compulsive symptoms and those of problems with self-concept declined until the end of the observation period. The decline was more pronounced in MPH-ER treated individuals. The effects remained robust during the entire maintenance period until week 24. Symptoms of anxiety, depression, anger and hostility, phobia, paranoid ideations and psychoticism were not improved. *Conclusions.* MPH-ER appears to be an efficacious treatment for emotional symptoms with ADHD. Also obsessive-compulsive symptoms and problems with self-concept were affected positively.

Key words: *Adult ADHD, methylphenidate, clinical trial, emotional symptoms, long-term effects*

Introduction

Attention deficit-hyperactivity disorder (ADHD) is a chronic disorder, which begins during early childhood and continues into adolescence. During adulthood the disorder can be found as full or partial clinical picture in 60% of patients (Weiss et al. 1985; Mannuzza et al. 1993). Data from recent research found a transnational prevalence of 3.4% for ADHD in adults (Fayyad et al. 2007).

The psychopathology of ADHD as defined by DSM-IV contains three major syndromes: attention disorder, hyperactivity and impulsivity. These major symptom domains are present at all life stages, although developmental factors may influence the psychopathology. An important finding in this respect is the robustness of attention symptoms during different life stages (Biederman et al. 2000). On one hand the core psychopathology of ADHD according to DSM-IV is

Correspondence: Prof. Dr. M. Rösler, Neurocentre, University Hospital of the Saarland, D-66421 Homburg/Saar, Germany. Tel: 06841 162 6350. Fax: 06841 162 6340. E-mail: michael.roesler@uks.eu

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widely accepted, on the other hand different concepts of ADHD comprise a broader spectrum of psychopathology (McGough and McCracken 2006). The concept of ADHD assessed by the Conners Adult ADHD Rating Scales (CAARS, Conners et al. 1999) refers to inattention/memory problems, impulsivity/emotional lability, hyperactivity/restlessness and problems with self concept. The psychopathological content of the Brown Adult Attention Disorder Scale (BAADS, Brown 1996) comprises organizing and activation to work, sustaining attention and concentration, sustaining energy and effort, managing affective interference and utilizing working memory and accessing recall. The Utah criteria of adult ADHD were designed by P. Wender (1995). The origin of this concept was created prior to DSM-III (1980). It is based on inattention, hyperactivity, poor temper control, affective lability, emotional overreactivity, impulsivity and disorganization. The comparison of the different conceptions of adult ADHD may lead to the hypothesis that beside the core psychopathology of inattention, hyperactivity and impulsivity phenomena of other domains like emotional symptoms can frequently be detected (Gibbins and Weiss 2007). The distinction between ADHD intrinsic emotional symptoms and those related to co-occuring affective and mood disorders is a major problem of current research. A core criterion to distinguish the two groups of psychopathology may refer to the course of the disorders. ADHD intrinsic emotional symptoms are phenomena in the frame of inattention, impulsivity and hyperactivity with a lifelong and chronic dimension, while emotional symptoms of depressive and anxiety disorders are part of the typical syndromes of these disorders, which may at least partly episodic phenomena. An unresolved problem is the considerable overlap between the psychopathological criteria of adult ADHD and Borderline personality disorder (Philipsen et al. 2008), which may lead to double diagnoses in many cases.

There is overwhelming evidence that inattention, hyperactivity and impulsivity can be improved by the administration of stimulant medications. More than 200 controlled studies in child and adolescent psychiatry, or even in adult psychiatry, have shown significant improvement of ADHD psychopathology with high effect sizes by far exceeding those of antidepressant or neuroleptic medications (Spencer et al. 1996, 2001, 2005, 2007, 2008; Faraone et al. 2004; Biederman et al. 2005, 2006; Jain et al. 2007; Reimherr et al. 2007). Meta-analytic investigations demonstrated that the effect sizes of MPH therapy in adults with ADHD may be lower as compared with children and adolescents (Kösters et al. 2008). The treatment with atomoxetine (ATX), a non-stimulant compound, can also improve inattention, hyperactivity and impulsivity in children, adolescents and in adults with ADHD (Michaelson et al. 2003; Adler et al. 2005). According to the treatment guidelines of national psychiatric associations or international committees in the most countries of the world MPH is recommended as first line treatment at all life stages. An alternative stimulant medication is amphetamine, whereas ATX is recommended in cases of non-response to stimulant medication or in cases with high abuse potential (Banaschewski et al. 2006; NICE 2008; Nutt et al. 2008).

Our knowledge regarding the effects of MPH treatment on ADHD associated symptoms in adults is limited. As far as we could find in the literature the study by Reimherr et al. (2007) is the only placebo-controlled investigation, which included oppositional and emotional dimensions of ADHD psychopathology. The authors found a positive response to MPH treatment. Because this investigation used a short 2×4-week crossover design no findings could be achieved regarding the robustness over time of this treatment effect.

It was the aim of the present study to assess emotional symptoms and other psychopathological phenomena, which are not included in the current DSM-IV concept of ADHD with different observer and self rating scales and to investigate the course of these symptoms under treatment with extended release-MPH over a period of 24 weeks. The data presented here are a secondary analysis of a research project, which has been described and published elsewhere (Rösler et al. 2009).

Methods and materials

Subjects

Subjects were outpatients with ADHD aged $\geq$18 years. For study inclusion the subject had to fulfil the DSM-IV criteria for ADHD. The diagnosis was established by psychiatric expert assessment including a German version of the ADHD Rating Scale – IV (ADHD RS-IV, DuPaul et al. 1998; ADHD-DC, Rösler et al. 2004). This instrument is based on the 18 psychopathological DSM-IV criteria for ADHD and the additional DSM-IV criteria referring to the age of onset, pervasiveness, functional disabilities and burden.

The German version of the Structured Clinical Interviews for DSM-IV disorders (SKID-I and -II, Wittchen et al. 1997) was used to assess axis I and II comorbid psychiatric diagnoses. Individuals with low intelligence (IQ$<$85), schizophrenia, bipolar disorder, acute depressive episode, acute anxiety disorders and other unstable psychiatric conditions were excluded, as were subjects with any serious medical illness. Also subjects with evidence of drug or alcohol dependence

during the preceding 6 months, pregnant or nursing women, persons who had participated in a previous drug trial in the last 30 days and individuals treated with any psychopharmacological drug in addition to study medication were not included. Regarding the prevalence of depressive and anxiety disorders the following comorbidity rates were found. Lifetime affective disorders (DSM-IV: 296, 300.4, 311) verum group: 21.2%, placebo group: 24.6%. Current affective disorders (DSM-IV: 300.4, 311) verum group: 13.7%, placebo group: 14.4%. Lifetime anxiety disorders (DSM-IV 300) verum group: 18.3%, placebo group: 26.3%. Current anxiety disorders (DSM-IV 300) verum group: 9.5%, placebo group: 11.9%. A depressive personality disorder (DSM-IV 301.9) or borderline personality disorder (DSM-IV 301.83) were found in 12 and 15%, respectively, in the verum group and in 8 and 16%, respectively, in the placebo population. A wash-out period of at least 2 weeks was necessary for any psychopharmacological drug before study inclusion. Urine screening for drugs of abuse was performed at the screening visit, at weeks 8 and 24, and could be repeated at any time of the study at the investigator's discretion.

Design

A multi-center, double-blind, randomized, placebo-controlled, 24-week study with parallel-group design was conducted. The participants were randomized to MPH-ER or placebo at a ratio of 2:1. MPH-ER is a MPH preparation manufactured by Medice (Germany) with a proportion of 50% immediate release MPH and 50% of extended release MPH. The effective time of action is about 8 h. The drug was described and compared with other long-acting MPH medications by the European guideline group (Banaschewski et al. 2006).

The study was approved by the ethical committee of the State of the Saarland and the regulatory authorities in Germany. All patients provided written informed consent. The study was registered with the Federal Opium Agency at the Federal Institute for Drugs and Medical Devices. Moreover the trial was registered at ClinicalTrials.gov (NCT00619840).

Study intervention

Medication was titrated b.i.d. after breakfast and lunch during the first 5 weeks by use of a flexible dose schedule to a maximum dose of 60 mg/day, starting with 10 mg/day. Lower daily doses were administered in the case of intolerable adverse events and if higher daily doses did not lead to an increased improvement. The interval between the two doses was 6–8 h. The minimum maintenance dose after week 5 was 20 mg/day. A standardised disease management programme consisting of seven sessions was administered to all participants of the study. The programme was designed especially for the study to avoid ethical objections to keeping subjects on placebo therapy for at least 24 weeks. Disease management sessions were performed at baseline and weeks 1, 3, 5, 8, 12 and 18. During these sessions patients received information about ADHD aetiology and symptoms, support in perception of symptoms and specific problems, help with the management of self-regulation and emotional problems, time management and performing daily routines.

Assessments

For the assessment of the inclusion and exclusion criteria each subject underwent a comprehensive clinical assessment by a certified psychiatrist using standardized rating scales and interviews. The examination included medical history, physical examination, vital parameters, body weight, liver function tests, complete blood count EEG and ECG in the case of a history of cardiac problems.

As mentioned above, ADHD was diagnosed according to DSM-IV criteria. Whenever possible, a retrospective assessment of childhood ADHD symptoms was made by report of informants. In addition, the German short form of the Wender Utah Rating scale (WURS, Wender 1995) was administered to all subjects (Retz-Junginger et al. 2002, 2003) in order to make sure that childhood ADHD symptoms were present by retrospective self reports of the patients.

Emotional symptoms were assessed by three different rating instruments. According to the approach of Reimherr et al. (2007) we selected the subscales affective lability, temper control and stress intolerance of the Wender-Reimherr Adult Attention Deficit Disorder Scale (WRAADDS, Wender 1995; Reimherr et al. 2005, German version: Rösler et al. 2008a) for the construction of an Emotional Dysregulation Scale (EMS). The WRAADDS is a investigator-rated semistructured interview. The 10 items of the above-mentioned EMS were assessed by a clinical expert. They are presented in Table I. Each item can be rated on a 0–2 Likert scale. Thus the maximum EMS score is 20. As second assessment procedure we used the subscales emotional lability and problems with self concept of the Conners Adult ADHD Rating Scale Self Report Long Form (CAARS-S:L, Conners et al. 1999). The Emotional Lability Subscale (ELS) contains six items (list of items: see Table I). The patients respond on a four-point Likert scale (0–3). The maximum score of the Conners ELS is 18. The scale regarding problems with self concept has also six items. The scoring

Table I. Emotional Dysregulation Scale (EDS) derived from the WRAADDS and Emotional Lability Scale (ELS) derived from the Conners adult ADHD Rating scale – self report version – long form.

EDS		ELS	
Domain	Item	CAARS-S:L	Item
Hot temper	Irritability	8	Easily frustrated
	Temper outbursts	19	Short fuse – hot temper
	Lack of control	23	Throwing tantrums
Affective lability	Mood fluctuations	30	Things set off easily
	Dysphoric periods	47	Unpredictable moods
	Boredom	61	Irritability
	Overstimulation		
Emotional over-reactivity	Overwhelmed, emotional		
	Reactivity		
	Impairment under stress		

follows the rules of the ELS items. Our third assessment instrument was the Symptom Checklist 90-Revised (SCL-90-R, Derogatis et al. 1977). We incorporated the subscales somatisation, obsessive-compulsive, insecurity in social contact, depression, anxiety and phobic anxiety. The WRAADDS and the CAARS-S:L were administered at baseline and at each visit. The SCL-90-R was used at baseline, week 8 and week 24.

Statistical analysis

The complete WRAADDS was used as primary variable for the trial (Rösler et al. 2009). The sample size was calculated for the primary variable only. In this secondary analysis the Emotional Dysregulation Scale (EDS) derived from the Wender-Reimherr adult attention deficit disorder scale and a self-report, six-item emotional lability scale extracted from the long version of the Conners Adult ADHD Self Report Scale (CAARS:S:L) were used. Only the final value of the EDS-WRAADDS score (Week 24) was included in the test statistics. Missing data were imputed using the LOCF procedure. The confirmatory analysis was performed on the intend-to-treat (ITT) population and separately in a per protocol (PP) population.

Group differences in the final values of scores at week 24 were compared using the Wilcoxon *U*-test.

This analysis is exploratory. If any p-values are given, these are to be interpreted as descriptive only.

Results

A total of 363 patients were recruited and randomized. Four patients were excluded from the ITT population because the WRAADDS Baseline was not available, and because of bad data quality/non-compliance at the trial site; 241 patients were randomized to MPH ER and 118 individuals to placebo. The distribution of genders was approximately equal in both treatment groups. There were no statistically significant differences in terms of subjects' mean age between the MPH ER and the placebo population. No differences in age of ADHD onset, body weight, IQ, ADHD severity, ADHD symptom score by ADHD-DC, WURS-k scores or CGI severity ratings could be found at the beginning of the treatment phase (Table II). The incidence of comorbid conditions according to SKID-I interviews demonstrated no significant difference between the MPH- and the placebo-population.

A total of 110 subjects discontinued the study prematurely. The drop-out rate was lower in the MPH ER group compared to the placebo group (24 vs. 43%; Fisher's exact test, $P<0.001$). The most common reason for drop-outs from the study in subjects receiving placebo was lack of efficacy (25%), whereas adverse events (13%) were the most common reason for drop-outs in the MPH ER group.

The ITT population consisted of 359 (241/118) patients and the PP population had 249 (183/66) persons.

Efficacy

The mean daily MPH dose at week 24 was 41.2 ± 18.2 mg in the MPH ER group. In the placebo group the mean daily dose was 40.8 ± 19.6 mg (Wilcoxon *U*-test, $P=0.94$). These are equivalent to 0.55 ± 0.27 mg/kg body weight MPH ER and 0.54 ± 0.29 mg/kg body weight placebo, respectively (Wilcoxon *U*-test, $P=0.99$).

The analysis of the course showed a decrease of emotional symptoms as measured by WRAADDS-EDS in both groups until the end of the observation period (Figure 1). The difference between placebo and verum was statistically significant from week 5 onwards (Wilcoxon test). The stability of the therapeutic effect between weeks 5 and 24 was robust. The effect size among the ITT population was 0.37.

Table II. Demographic and clinical characteristics of the sample (ITT). Data are presented as *N* (%) or mean±SD.

	MPH ER (ITT) *N*=241	Placebo (ITT) *N*=118	*P* values
Age (y)	35.2±10.1	33.8±10.6	Wilcoxon *U*-test *P*=0.24
Sex*			
Male	120 (50%)	58 (50%)	Fisher's exact test
Female	119 (49%)	60 (51%)	*P*=0.9
Missing	2	–	
Body weight (kg)	78.0±17.2	77.3±16.7	Wilcoxon *U*-test *P*=0.76
IQ	110.4±14.4	109.7±14.4	Wilcoxon *U*-test *P*=0.72
Age at ADHD onset (years)	5.8±2.0	5.7±2.2	Wilcoxon *U*-test *P*=0.53
WURS-k (Screening)	44.2±11.9	43.1±10.8	Wilcoxon *U*-test *P*=0.42
ADHD-DC score** (Screening)			Wilcoxon *U*-test
Inattention	7.6±1.0	7.8±1.1	*P*=0.16
Hyperactivity/Impulsivity	7.1±1.1	7.1±1.1	*P*=0.31
WRAADDS Score (Baseline)	44.8±7.2	45.5±6.8	Wilcoxon *U*-test *P*=0.45
CAARS-S:L (Baseline)	119.2±29.6	117.9±26.2	Wilcoxon *U*-test *P*=0.70
CGI Severity of illness (Baseline)	5.0±0.80	5.1±0.70	Wilcoxon *U*-test *P*=0.60
SCL-90 total score (Baseline)	82.5±56.8	82.3±51.3	Wilcoxon *U*-test *P*=0.80

The results of the PP population were pretty much the same. The effect size was 0.42. The Spearman correlation coefficient between the WRAADDS-EDS score and the WRAADDS total score at week 24 was 0.89 ($P<0.0001$) in the verum group and 0.92 ($P<0.0001$) in the placebo group.

The ELS score derived from the CAARS-S:L declined in the ITT verum group from baseline to week 24. The baseline score of the ITT verum patients was 10.7 and the endpoint score was 6.95. The ITT placebo patients had a baseline score of 10.9. They declined to 8.2. The difference between the two groups was statistically significant (Wilcoxon *U*-test: 0.006). The effect size was 0.3 (Table III). Regarding the PP population the results were similar. The effect sized was determined as 0.35. At the end of the observation period the Spearman correlation coefficient between the patient rated ELS and

Table III. SCL-90-R dimensions by treatment group, ITT population. The SCL-90-R subscales psychoticism and paranoid ideation are not considered.

SCL-90	MPH-ER			Placebo			
Dimension	Week	Mean	SD	Week	Mean	SD	Wilcoxon *U*-test *P*
Somatisation	0	0.58	0.63	0	0.60	0.61	0.76
	24	0.43	0.47	24	0.47	0.58	
Obsessive-	0	1.57	0.79	0	1.55	0.75	0.01
Compulsive	24	0.88	0.73	24	1.11	0.82	Cohen's *d*: 0.30
Interpersonal	0	1.13	0.84	0	1.14	0.80	0.22
Sensitivity	24	0.73	0.75	24	0.82	0.78	
Depression	0	1.18	0.86	0	1.19	0.87	0.26
	24	0.73	0.77	24	0.88	0.88	
Anxiety	0	0.86	0.69	0	0.81	0.65	0.10
	24	0.48	0.56	24	0.59	0.61	
Anger, hostility	0	1.03	0.89	0	0.97	0.75	0.40
	24	0.55	0.64	24	0.71	0.82	
Phobic anxiety	0	0.39	0.61	0	0.41	0.53	0.14
	24	0.20	0.40	24	0.30	0.49	

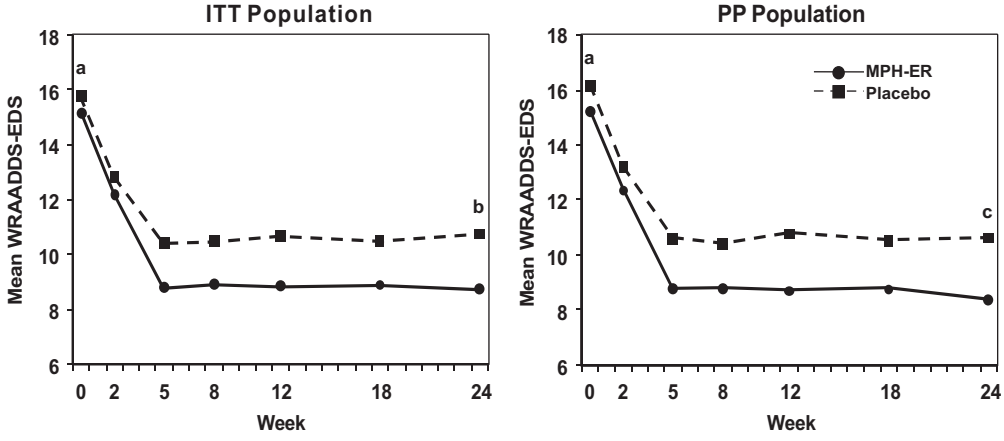

Figure 1. Mean score of the WRAADDS Emotional Dysregulation Scale (WRAADDS-EDS) during the 24-week observation period. The difference between MPH-treated persons and placebo group is significant from week 5 onwards. a=Not significant, bP=0.0009, cP=0.0013. ITT population: N=241 (MPH), N=118 (placebo). PP population: N=179 (MPH), N=85 (placebo).

the investigator rated WRI total score was 0.67 ($P<0.0001$) in the verum group and 0.75 ($P<0.0001$) in the placebo group. The correlation between the patient rated ELS and the investigator rated EDS at week 24 was 0.81 ($P<0.0001$) in the placebo group and 0.67 ($P<0.0001$) in the verum group.

The self concept score derived from the CAARS-S:L of the ITT patients treated with MPH-ER declined from 11.2 to 7.7 as compared with 11.6 to 9.1 in the ITT placebo group. The statistical comparison was significant (Wilcoxon *U*-test 0.009). The effect size was 0.29. Among the PP population MPH-ER we revealed similar results. The effect size was calculated as 0.40.

The results with the SCL-90-R are summarized in Table IV. We found no significant differences between the ITT MPH-ER population and the ITT placebo patients regarding symptoms of somatization, interpersonal sensitivity, depression, hostility, anxiety and phobic anxiety.

Obsessive-compulsive psychopathology declined in the placebo and the MPH-ER group. The improvement in the verum group was more pronounced as compared with the placebo patients (Wilcoxon *U*-test 0.01). The effect size was found to be 0.30. The results of the PP population were similar. The effect size concerning obsessive-compulsive symptoms was slightly higher (0.46)

Discussion

Numerous controlled studies and the two meta-analyses by Faraone et al. (2004) and Koesters et al. (2008) have demonstrated that short-term administration of MPH can reduce the classical psychopathology in adults suffering from ADHD. Thus it cannot be doubted that MPH is an effective treatment to improve inattention, hyperactivity and impulsivity. A medium-term controlled study has revealed, that these treatment effects are robust and do not decrease during 6 months of MPH-ER treatment (Rösler et al. 2009).

Individuals suffering from ADHD frequently present with additional psychopathology. They display emotional symptoms like affective lability, temper dyscontrol and emotional overreactivity (Wender 1995; Reimherr et al. 2005, 2007). The prevalence rate of this psychopathology is on a similar level like the classical symptoms of inattention, hyperactivity and impulsivity (Reimherr et al. 2005; Rösler et al. 2008b). Thus in adult psychiatry as well as in child and adolescent psychiatry there is an ongoing debate whether these symptoms are part of the ADHD spectrum psychopathology or should be better understood as independent comorbid conditions (Wender 1995; Walcott and Landau 2004; McGough and Barkley 2004; Brotman et al. 2006; McGough and McCracken 2006).

If the emotional symptoms improve by ADHD medication, one might have the hypothesis that emotional symptoms are rather part of the ADHD spectrum psychopathology and do not represent a comorbid condition. Indeed, there is growing amount of evidence that emotional symptoms improve when ADHD medication is administered. Reimherr et al. (2005) found that ATX ameliorates symptoms of affective lability, emotional overreactivity and temper control. In a recent study Reimherr et al. (2007) were able to demonstrate a positive effect of OROS-MPH on the psychopathology of emotional dysregulation. In our investigation we confirmed these findings. When MPH-ER was administered the reduction of emotional psychopathology was statistically

Table 4. CAARS-S:L Emotional Lability Scale (ELS) and problems with self-concept. ITT and PP population.

		Verum		Placebo			
CAARS-S:L	Week	Mean	Std Dev	Mean	Std Dev	Effect Size	Wilcoxon *U*-Test
ELSPP-Population	0	10.9	3.6	11.3	3.6	0.30	0.006
	24	6.7	3.8	8.2	4.7		
ELSITT Population	0	10.7	3.8	10.9	3.8	0.35	0.003
	24	6.9	4.0	8.2	4.7		
Self ConceptPP Pop.	0	11.3	4.3	12.0	4.0	0.40	0.003
	24	7.5	4.8	9.5	5.1		
Self ConceptITT Pop	0	11.2	4.3	11.6	4.1	0.28	0.009
	24	7.7	4.9	9.1	5.2		

significant better as compared with placebo. We found a small to medium effect size of 0.37 which was on the same level as we found regarding inattention, hyperactivity and impulsivity. The overall effect size of the WRAADDS was 0.39 (Rösler et al. 2009).

The results of the ITT and the PP populations were nearly identical. By comparison the effect size of the treatment study reported by Reimherr et al. (2007) was 0.70, which is medium to large. It should be noted that our mean daily MPH-ER dose was 0.55 mg/kg per day. This is relatively low. The patients of the Reimherr et al. (2007) study received higher MPH doses. They had 0.7 mg/kg per day on average. According to a meta-analysis by Faraone et al. (2004) high daily MPH doses are associated with growing effect sizes. Thus it seems evident, that the difference of the effect sizes is a consequence of our low dose regimen.

Our second procedure to assess emotional symptoms was the ELS derived from the CAARS Self Report Rating Scale (Conners et al. 1999). In both the ITT and the PP populations we found a significantly more pronounced decrease of the emotional psychopathology of the MPH-ER treated individuals in comparison with the placebo group. Our results are in contrast with an earlier study by Jain et al. (2007). These authors did not find significant differences between the placebo and MPH treatment effects regarding the impulsivity/emotional lability subscale of the CAARS. In this regard, it must be noted that the rating scales for the detection of emotional lability in our investigation differed from that used by Jain et al. (2007), who included symptoms of impulsivity. In our investigation only emotional symptoms were assessed.

Regarding the relation of the classical triade of inattention, hyperactivity and impulsivity with the psychopathology of emotional symptoms in adults with ADHD it is interesting to mention that the EDS and the ELS as well displayed medium to high correlations with the WRAADDS. This may support the notion that emotional symptoms contribute to the concept of ADHD substantially.

Not much was known so far regarding the robustness of MPH-ER related therapeutic improvement of emotional psychopathology. The OROS-MPH study by Reimherr et al. (2007) had an observation period of 4 weeks. The present study demonstrated that there was no decline of the therapeutic effect during 24 weeks as measured by clinical expert ratings and self reports of the patients as well. This is a new empirical finding.

Problems with the self-concept (Conners et al. 1999) and obsessive-compulsive symptoms can be interpreted as further and different types of psychopathology, which are frequently seen in patients suffering from ADHD (Moll et al. 2000; Gibbins and Weiss 2007). Problems with the self-concept are frequently classified as a consequence of the negative social impact of the disorder experienced by the patients over years. Obsessive-compulsive symptoms might be seen as an adaptive strategy or reaction to control for the disorganizational problems which occur in persons with ADHD (Weiss and Weiss 2004). Obsessive-compulsive symptoms as measured by the SCL-90-R may have such a character. The 10 obsessive-compulsive items of the SCL-90-R (Derogatis 1977) refer to concentration and working problems, procrastination, feelings of emptiness, stereotype behaviour, concerns regarding careless mistakes etc. It seems evident that there is a partial overlap between typical ADHD symptoms and phenomena of obsessive-compulsive conditions.

Both psychopathological domains – problems with self-concept and obsessive-compulsive symptoms – were affected positively by the administration of MPH-ER. We revealed a statistical significant treatment effect in favour of MPH-ER, which remained robust during the entire observation period of 24 weeks. The results allow for the speculation that symptoms which may develop as a reaction or as a coping strategy to the core ADHD psychopathology may decrease if an effective reduction of the classical ADHD psychopathology can be established. However, the study by Jain et al. (2007) had failed to demonstrate a specific effect on problems with

self-concept as measured by the CAARS-S:L. Based on the hypothesis that problems with self-concept may be a secondary psychopathological domain caused by deficits and complications during social life, there might be the possibility that the study by Jain et al. (2007) was not long enough to detect a decline of problems with self-concept.

Psychopathological symptoms of depression and anxiety were assessed in the majority of ADHD treatment investigations. We found no significant treatment effect on depression and anxiety as measured by the SCL-90-R. Our findings are in line with earlier research demonstrating no specific treatment effects on depression and anxiety by MPH (Spencer et al. 2005; Biederman et al. 2006; Jain et al. 2007). With regard to the discussion whether emotional symptoms are at least in part rather associated with comorbid anxiety or depressive disorder than with ADHD (Biederman 2004; McGough and Barkley 2004; Faraone 2005), it is interesting to note that emotional symptoms, as described above, respond to MPH-ER treatment, whereas typical signs of depression and anxiety do not show improvement. This may support the notion that emotional psychopathology is not associated with comorbid anxiety and depressive disorders but a distinctive and core domain of the adult ADHD spectrum psychopathology. Similar findings were already described by Reimherr et al. (2005). They found emotional symptoms to be a strong predictor of atomoxetine treatment response in adults with ADHD, while symptoms of depression and anxiety did not display any improvement.

There are several limitations that must be addressed. The first point is the relatively low MPH-ER dose in our study. Our patients received on average 0.55 mg/kg body weight. This is in the low range of the daily dose recommendations elaborated by treatment guidelines (Banaschewski et al. 2006; Gibbins and Weiss 2007; NICE 2008). The idea behind the low dose administration was that in treatments, which may last over months or years, high dose prescriptions of 1mg/kg/day or more may lead to concerns regarding the long term safety and tolerability of MPH. Thus we tried to find out whether low doses of MPH-ER may lead to significantly positive treatment responses. We expect that our low dose regimen had consequences in the way, that we did not exhaust the full therapeutic potential of the medication. Thus the magnitude of our effect sizes might have been limited.

A further point is the relatively high proportion of patients which terminated the study prematurely. A total of about 30% of our patients did not complete the entire observation period. This seems to be a general problem of medium- or long-term, placebo-controlled studies on adult ADHD. This new study type is three or four times longer than the short-term trials, which have been performed during the past three decades. Less than 50% of the randomised adult ADHD patients of a 26-week, placebo-controlled trial with atomoxetine reached the study endpoint (Adler et al. 2009). High rates of premature terminations may lead to concerns regarding the robustness of the treatment results. Thus it is important to note that the statistical analyses of the ITT and the PP patients revealed nearly identical results indicating a robust, small to medium treatment response in favour of low-dose MPH-ER with slightly higher effect sizes among the PP population. Apparently the study results were not decisively influenced by the number of patients dropping from the study.

We would like to sum up by saying that the treatment with low doses of MPH-ER in adult patients with ADHD over a period of nearly 6 months leads to a small to medium but robust improvement of emotional symptoms. This result was found with the methods of observer rating as well as with patients self report. A further finding was robust decline of problems with self-concept and of obsessive-compulsive symptoms.

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ORIGINAL INVESTIGATION

Different apolipoprotein E, apolipoprotein A1 and prostaglandin-H2 D-isomerase levels in cerebrospinal fluid of schizophrenia patients and healthy controls

DANIEL MARTINS-DE-SOUZA[*,1,4], THOMAS WOBROCK[*,2], INGA ZERR[3], ANDREA SCHMITT[2,4], JOANNA GAWINECKA[3], THOMAS SCHNEIDER-AXMANN[2], PETER FALKAI[2] & CHRISTOPH W. TURCK[1]

[1]*Max Planck Institute of Psychiatry, Munich, Germany,* [2]*Department of Psychiatry and Psychotherapy, University Medical School, Georg-August University Goettingen, Goettingen, Germany,* [3]*Department of Neurology, University Medical School, Georg-August University Goettingen, Goettingen, Germany, and* [4]*Laboratório de Neurociências (LIM-27), Inst. Psiquiatria, Fac. de Medicina da Universidade de Sao Paulo, Sao Paulo, Brazil*

Abstract

Objectives. To identify proteins differentially expressed in schizophrenia patients, we collected 50 µl cerebrospinal fluid from 17 first-episode schizophrenia patients and 10 healthy controls. *Methods.* Their proteins were separated by two-dimensional gel electrophoresis without using any depletion method and identified by mass spectrometry. *Results.* Approximately 550 spots were detected, six of which had significantly different intensities in schizophrenia compared to control specimens. We were able to validate in individual samples the upregulation of apolipoprotein E, apolipoprotein A1 and prostaglandin-H2 D-isomerase by Western blot analyses and detect the downregulation of transthyretin, TGF-β receptor type-1 and coiled-coil domain-containing protein 3 precursor. *Conclusions.* These findings may help to elucidate the disease mechanisms and confirm the hypothesis of disturbed cholesterol and phospholipid metabolism in schizophrenia, and thus reveal the final role players. Moreover, a grouped protein expression analysis of apolipoprotein E, apolipoprotein A-I, and prostaglandin-H2 D-isomerase in cerebrospinal fluid from patients might be a potential diagnostic tool for schizophrenia.

Key words: *Schizophrenia, CSF, apolipoprotein E & A1, prostaglandin-H2 D-isomerase, proteomics*

Introduction

Many efforts have been made in the field of global gene and protein analysis to identify schizophrenia (SCZ) biomarkers that could help diagnose the disease or give some indication about disease pathophysiology. Several transcriptome and proteome studies (Johnston-Wilson et al. 2000; Mirnics et al. 2000; Hakak et al. 2001; Vawter et al. 2001; Prabakaran et al. 2004; Martins-de-Souza et al. 2009a,b) on SCZ brain tissue have implicated a number of potential biomarkers. Despite transcriptome and proteome analyses have also furthered the understanding of SCZ pathogenesis on a molecular level, most of the potential biomarkers candidates (Martins-de-Souza 2010) have not been validated in peripheral fluids such as blood and cerebrospinal fluid (CSF).

Proteome analysis of CSF is a valuable approach for SCZ biomarker discovery as well as to reveal proteins that may be involved in disease pathogenesis. Despite the importance of such research and the publication of a number of reviews regarding the importance of CSF analysis from SCZ patients, until now only one research group has described the proteome analysis of CSF in SCZ (Huang et al. 2006, 2007, 2008). The proteome analysis of a different set of CSF samples from SCZ patients may enrich the knowledge in this field.

Correspondence: Daniel Martins-de-Souza, Max Planck Institute of Psychiatry, Kraepelinstraße 2–10, D-80804 Munich, Germany. Tel: +49 89 30622 630. E-mail: martins@mpipsykl.mpg.de

[*]Daniel Martins-de-Souza and Thomas Wobrock contributed equally to this article.

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Here, we present our initial findings from the proteome analysis of CSF from 17 first-episode SCZ patients and 10 healthy controls. We used a very simple method to extract the proteins without any protein depletion followed by two-dimensional gel electrophoresis (2DE) for protein separation, MALDI-TOF/TOF mass spectrometry (MS) for protein identification, and Western blot (WB) analysis for validation of the differentially expressed proteins. Our findings support the hypothesis that SCZ is a disorder of phospholipids breakdown (Gattaz et al. 1990; Yao et al. 2000, 2002; Schmitt et al. 2004) and propose potential biomarkers candidates to diagnostic ends.

Material and methods

Subjects and CSF samples

Seventeen first-episode SCZ inpatients and 10 healthy control subjects were enrolled in the study. The SCZ patients were taken from a sample of 61 first-episode SCZ patients (all of whom had the paranoid subtype according to ICD-10 and DSM-IV criteria) recruited at the Department of Psychiatry, Saarland University, Germany, in the years 2003–2006. After a complete description of the study, written informed consent was obtained from each patient and control. The protocol was in accordance with the Declaration of Helsinki and was approved by the local ethics committee. CSF samples were obtained by lumbar puncture and immediately stored at –20°C for 2 h; they were then stored at –80°C until further analysis.

Each patient underwent the following standardized procedures: biographical interview (Bassett et al. 1993); assessment of psychopathology (Positive and Negative Syndrome Scale [PANSS]) (Kay et al. 1987); assessment of disease severity (Clinical Global Impressions [CGI]) (Guy and Bonato 1976); and assessment of social functioning (Global Assessment of Functioning [GAF]) (Endicott et al. 1976). The diagnosis of SCZ was based on a consensus of two independent psychiatrists after they had conducted SCID I interviews (Wittchen et al. 1997). Additionally, we assessed the duration of illness (DUI), calculated from the beginning of the initial prodromal symptoms; the duration of untreated psychosis (DUP), calculated from the onset of diagnostic/characteristic positive symptoms; and familial risk factors (psychosis in first-degree relatives). All patients were treated with second generation antipsychotics, but no patient had been treated continuously for longer than 6 weeks. For each patient, the cumulative and daily antipsychotic dose was calculated in chlorpromazine equivalents (CPE), as suggested by reviews and studies focusing on second generation antipsychotics (Woods 2003) (see Table I).

The control subjects were healthy individuals recruited at the Department of Neurology of the University of Goettingen and they did not suffer from neurological, somatic or psychiatric diseases including substance abuse and had never been treated with antidepressant or antipsychotic medications. The CSF from controls were investigated in the CSF laboratory using routine diagnostics such as albumin content, albumin ratio, protein content, cell count, immunoglobulins, oligoclonal IgG and blood contamination. No alterations were revealed.

All patients and healthy controls were Caucasians of German nationality. None of the patients or controls had a history of alcohol or drug dependence, organic central nervous system (CNS) diseases, or severe physical illness. There were no significant differences considering controls and patients age (Mann–Whitney: $P=0.5303$) and gender (exact Fisher test: $P=0.5478$). SCZ patients and controls were comparable for mean age (31.2 vs. 30.8 years).

Further patient and control information is given in Table I.

Protein extraction and separation

Fifty μL of the CSF samples from each patient and control were precipitated with cold acetonitrile (−20°C) for 2 h at −20°C. Afterwards, samples were centrifuged at 12,000 rpm for 10 min. The pellets were collected for proteome analysis and the supernatant for peptidome analysis (not discussed here). Protein pellets were diluted in 50 μl of isoelectrofocusing (IEF) buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT). Five μL of each sample weer used for Bradford protein quantitation.

2DE was performed as described in Martins-de-Souza et al. (2007) by applying 100 μg of pooled proteins from SCZ or control CSF samples to IPG gel strips with a nonlinear separation range of pH 3–10. Proteins were detected by a silver nitrate staining protocol. The experiments were performed in duplicates (each protein has been tested using two sets of samples).

Determination of protein expression and identification

Determination of protein expression differences were made using the computational software PDQuest (BioRad, Hercules, CA). Briefly, all protein spots were detected and calibrated according their pI and MW based on known protein markers. Next, the spots volumes from SCZ and healthy controls 2-DE gels were determined and the correspondent spots were matched. Only proteins that appeared to be differentially expressed with a mean *n*-fold change

Table I. Clinical data of patients and healthy controls. All patients were first-episode schizophrenia, paranoid subtype.

Sample ID	Case	Age	Gender	DUI	DUP	PANSS total	CGI	GAF	Duration of treatment (days)	CPE daily dosage	CPE cumulative dosage	Previous cannabis abuse	Nicotine cig./day
200101	SCZ	37	M	294	8	89	5	29	7	200	1400	No	0.0
200201	SCZ	36	M	140	28	79	6	22	28	300	10500	Yes	18.0
200301	SCZ	21	M	56	26	91	5	40	5	100	400	Yes	15.0
200801	SCZ	44	M	5	4	93	6	35	11	450	4950	No	23.0
200901	SCZ	26	F	260	2	108	6	25	14	200	2800	Yes	10.0
201001	SCZ	27	F	8	6	100	6	30	9	1320	10560	Yes	7.0
201301	SCZ	27	F	112	32	89	5	48	0	0	0	Yes	12.5
202201	SCZ	36	M	33	9	103	6	25	5	400	1600	Yes	21.0
202401	SCZ	25	F	196	50	108	6	21	29	700	21000	Yes	20.0
203001	SCZ	38	M	138	46	87	6	23	15	400	5200	Yes	20.0
203101	SCZ	24	M	285	130	88	6	28	26	150	6750	No	10.0
203401	SCZ	36	F	98	70	87	6	28	23	300	6000	No	0.0
203601	SCZ	32	M	354	30	92	7	21	33	400	13200	Yes	20.0
204001	SCZ	21	F	22	4	70	5	45	4	600	1800	Yes	20.0
204401	SCZ	39	M	151	3	84	5	46	3	200	400	No	0.0
204901	SCZ	26	M	720	42	68	5	55	10	300	3900	Yes	20.0
205601	SCZ	31	M	294	8	74	6	35	7	200	2000	No	0.0
D712	CTRL	38	M	–	–	–	–	–	–	–	–	–	–
D508	CTRL	26	F	–	–	–	–	–	–	–	–	–	–
D825	CTRL	31	M	–	–	–	–	–	–	–	–	–	–
D830	CTRL	20	M	–	–	–	–	–	–	–	–	–	–
D843	CTRL	56	F	–	–	–	–	–	–	–	–	–	–
D1166	CTRL	25	M	–	–	–	–	–	–	–	–	–	–
D1167	CTRL	24	F	–	–	–	–	–	–	–	–	–	–
D1169	CTRL	22	F	–	–	–	–	–	–	–	–	–	–
D1044	CTRL	36	F	–	–	–	–	–	–	–	–	–	–
D1221	CTRL	30	M	–	–	–	–	–	–	–	–	–	–

Age, years at the collection; PANSS, Positive and Negative Syndrome Scale; CGI, Clinical Global Impressions; GAF, Global Assessment of Functioning; DUI, duration of untreated illness in weeks; DUP, duration of untreated psychosis in weeks; CPE, medication calculated in chlorpromazine equivalents (mg).

between SCZ and control CSF gels of at least ±1.8 in both experiments were excised for identification by MS, considering the sensitivity of the 2-DE gels silver-stained (Martins-de-Souza et al. 2008).

The protein identification by peptide mass fingerprinting was carried out as described in Martins-de-Souza et al. (2009c).

Western blot analyses

Twenty-five μg of protein from each sample were used for WB, within the linear detectable range for the used antibodies. The detailed WB procedure can be found in Martins-de-Souza et al. (2009c), but using IgG polyclonal anti-apolipoprotein E and anti-apolipoprotein A1 (Abcam, Cambridge, UK) or anti-prostaglandin-H2 D-isomerase antibodies (Cayman Chemical Company, Ann Arbor, MI) at a 1:1000 dilution. The used antibodies were general, not isoform-specific. The samples from the 17 patients and 10 healthy controls were analyzed by WB using the above mentioned antibodies. To replicate the experiment in a mixed set of samples, two rounds of WB were performed, each with 10 SCZ samples and seven control samples (some samples of the first round were randomly included in the second experiment) in order to validate the findings.

Statistical analysis

To rule out whether external interferences might have led to different protein expression that was not related to SCZ, we used SPSS 15 for Windows to analyze whether there was a correlation between the patients' and healthy controls' WB densitometry data and their respective sociodemographic data. All tests were two tailed and the significance level was $\alpha = 0.05$. The independent factor was diagnostic group (control subjects, SCZ patients). Distributions of all dependent variables were examined in the two groups by using histograms and the Kolmogorov–Smirnov test for normality (Lilliefors 1967). Though the power for the Kolmogorov–Smirnov test was not very high, because of the small sample size, the results nevertheless suggested a normal distribution of the data and thus parametric tests were used for

analysis. Stepwise linear regression analyses ($p_{in}=0.05$, $p_{out}=0.10$) with the independent variables gender and age, were performed for all dependent variables. Analyses of covariance (ANCOVA) were conducted to test for diagnostic group differences. The intervening covariates age, and storage time were added to our analyses if they showed a significant influence in the initial regression analysis. Correlations between variables were performed using Pearson product moment correlation coefficients, but Spearman correlation was used for the cumulative dose of CPE, DUI and DUP.

Results

On average, 540 protein spots (individual results of duplicate experiments: 523, 557, respectively) were detected in SCZ 2DE gels whereas 542 protein spots (536, 549, respectively) were detected in healthy control 2DE gels.

There were 468 matched spots between the groups, representing 86.5% of the average number of spots. When SCZ and healthy control 2DE profiles were compared, significant changes in relative abundance (> 1.8 fold difference) were found for six spots (~1.1%), corresponding to six distinct proteins with apparent altered regulation in the SCZ samples (three were downregulated and three upregulated; Figure 1). All six proteins were successfully identified by MALDI-TOF/TOF (Table II). Because of their involvement in lipid metabolism, an important feature of SCZ, we tested and validated by WB analysis the upregulated proteins — apolipoprotein E (ApoE: $P=0.0412$), apolipoprotein A1 (APOA1: $P=0.0042$) and prostaglandin-H2 D-isomerase (PTGDS: $P=0.0192$), detecting single bands in all experiments (Figure 2).

We found significant negative correlations between the PTGDS level and the PANSS negative subscore ($\rho=-0.774$; $P=0.0009$), PANSS general psychopathology subscore ($\rho=-0.788$; $P=0.0007$), and PANSS total score ($\rho=-0.732$; $P=0.016$), and significant positive correlations between the PTGDS level and DUI ($\rho=0.705$; $P=0.023$, Spearman rank correlations). No other significant associations were found between ApoE and APOA1 and clinical or sociodemographic parameters (PANSS positive subscore, CGI, GAF, antipsychotic dose in CPE, age, education). There was no significant influence of previous cannabis abuse on ApoE ($P=0.23$), APOA1 ($P=0.42$) or PTGDS ($P=0.065$) expression. Moreover, there was no significant influence of tobacco consume measured in cigarettes per day on ApoE ($\rho=-0.40$, $P=0.29$), APOA1 ($\rho=-0.25$, $P=0.49$) or PGTS ($\rho=-0.40$, $P=0.26$) expression.

Discussion

Although we used a very simple and quick method of protein extraction, which can also be used for peptidome analysis, we were able to find and validate CSF protein differences between SCZ and control samples. These results, taken together with previously published data, could improve comprehension of the disease mechanisms and help identify potential protein biomarkers.

Apolipoprotein E

ApoE is present in very low density (VLDL), intermediate density (IDL), and high density lipoproteins (HDL), and in chylomicrons. It plays a pivotal

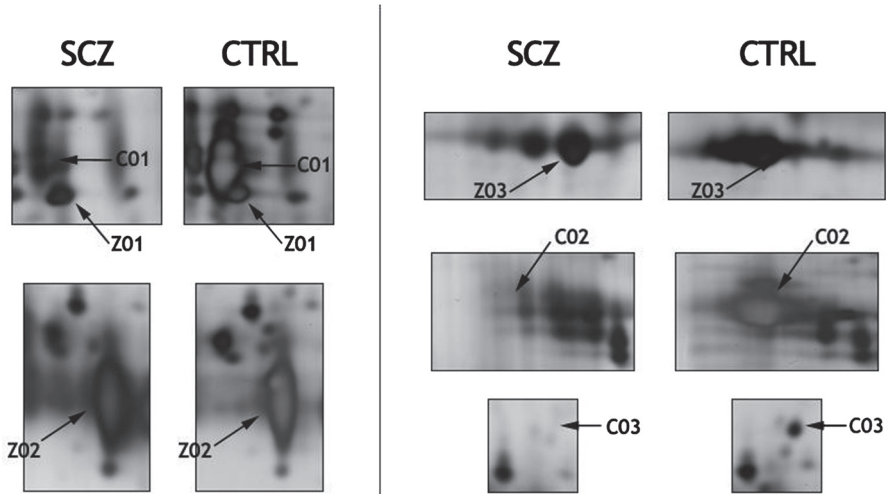

Figure 1. Enlarged sections of the 2DE profiles of the CSF from SCZ pooled samples and healthy control pooled samples. Differentially expressed proteins are indicated by arrows. "C" spots are proteins downregulated in SCZ while "Z" spots are proteins upregulated in SCZ.

Table II. Proteins up- or downregulated in SCZ samples compared to healthy control samples.

Spot in Figure 1	Fold change	Protein name	Gene name	UniProt	MW (th)	pI (th)	ID pept	Score
C01	4.12	Transthyretin precursor	TTR	TTHY_HUMAN	15887	5.52	13	420
C02	5.65	TGF-β receptor type-1 precursor	TGFBR1	TGFR1_HUMAN	55960	7.51	11	48
C03	Spot absent in SCZ	Coiled-coil domain-containing protein 3 precursor	CCDC3	CCDC3_HUMAN	30731	8.95	4	72
Z01	2.42	Apolipoprotein E precursor (Apo-E)	APOE	APOE_HUMAN	36154	5.65	21	362
Z02	1.97	Prostaglandin-H2 D-isomerase precursor (Prostaglandin-D2 synthase)	PTGDS	PTGDS_HUMAN	21029	7.66	7	186
Z03	2.01	Apolipoprotein A-I precursor (Apo-AI) (ApoA-I)	APOA1	APOA1_HUMAN	30778	5.56	29	552

Molecular weight (MW) and isoelectric point (pI) are theoretical; they were calculated by entering the protein sequences in the pI/MW prediction tool at http://pro-161-70.ib.unicamp.br/~itaraju/tools/pimw (Brum et al. 2009).

role in the metabolism of cholesterol and triglyceride-rich lipoproteins by anchoring these lipoproteins to the ApoE receptor cells. Deficiency or differential expression of ApoE may lead to disturbances of lipoprotein metabolism and thus increase the plasma levels of cholesterol and triglycerides. In the CNS, ApoE is synthesized in astrocytes and activated in microglia and its receptors are expressed in neurons (Pitas et al. 1987; Uchihara et al. 1995).

The ApoE receptor 2, which is exclusively expressed in brain and testis, is responsible for mediating cellular uptake of cholesterol and other lipids through interaction with the low density lipoproteins (LDL) family (Mahley 1988). In the brain, ApoE is mainly involved in the metabolism and homeostasis of cholesterol, which is part of glial and neuronal cell membranes and is essential for the formation of axonal myelin sheaths (Kim et al. 1996). Moreover, ApoE plays a critical role

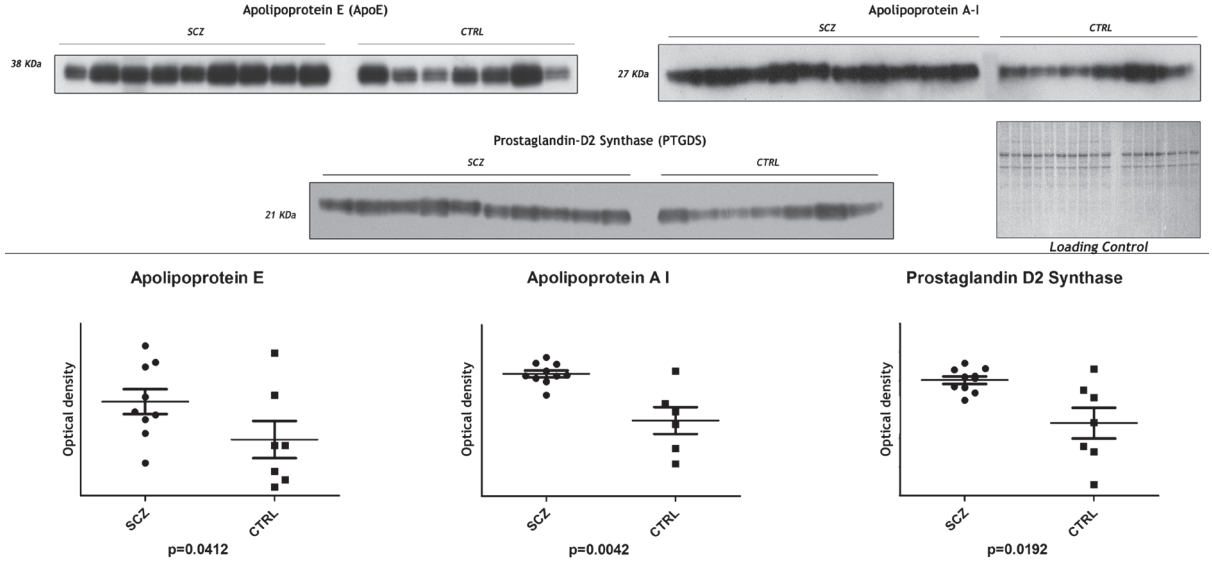

Figure 2. Western blot validation of ApoE, APOA1, and PTGDS expression differences in CSF from 17 patients and 10 healthy controls. In order to replicate the analysis, two rounds of WB were performed, each using 10 SCZ samples and seven control samples. One experiment is presented per protein (SCZ samples=10 and CTRL samples=7). In order to check the normalization of protein amounts, a loading control is also presented. In the graphs, bars are the mean with standard error of the mean.

in synaptogenesis, neurite outgrowth, and membrane repair and maintenance (Mauch et al. 2001; Nathan et al. 2002) which may be impaired in schizophrenia. It is known that lipid metabolism in the brain and adjacent fluids, such as CSF, are mandatory for perfect functioning of the CNS (Schmitt et al. 2004).

The well-described role of ApoE in Alzheimer's disease (AD) (Bu 2009), the presence of psychotic symptoms in some AD patients, and the probable role of some ApoE alleles as protective factors against the development of dementia in AD have led researchers to study the roles and potential involvement of ApoE in SCZ.

The epsilon 4 allele of ApoE indicates a strong genetic risk factor for AD (Laws et al. 2003). This allele has also been studied in SCZ samples, but the results are contradictory (Harrington et al. 1995; Jönsson et al. 1996; Lan et al. 1997; Pickar et al. 1997; Thibaut et al. 1998; Hong et al, 2000; Xu et al. 2006). Genotype studies have also found controversial data on the role of ApoE in SCZ (Arnold et al. 1997; Shinkai et al. 1998; Chen et al. 1999; Durany et al. 2000; Lee et al. 2001; Sáiz et al. 2002; Schürhoff et al. 2003) and found potential differences between ethnic groups. Interestingly, Liu et al. (2003) showed a potential environmental influence on the ApoE allele in a genetic association study in the Chinese population.

Facing the uncertain genetic association of ApoE with SCZ, researchers moved on to protein analysis. Dean et al. (2003) described the upregulation of ApoE in the prefrontal cortex in Brodmann's Area (BA) 9, and Digney et al. (2005) its upregulation in the prefrontal cortex in BA46. ApoE has been found to be downregulated (Dean et al. 2008) in SCZ plasma. Our finding of differential expression of ApoE in CSF is in line with previous *post mortem* protein findings and reinforces the potential role of ApoE in SCZ. Our data and previous findings might suggest that most probably ApoE differences are at the protein level and not at the genetic level.

The upregulation of ApoE in SCZ CSF found in our study (Figure 2) adds one more component to the picture of disturbed lipid metabolism in SCZ. The altered ApoE protein levels may have severe consequences for normal brain function since in the CNS ApoE regulates the homeostasis of cholesterol, which has pivotal functions in the brain. The ApoE upregulation in SCZ might lead to lower cholesterol levels, as previously described (Boston et al. 1996). Moreover, previous data have shown accelerated phospholipid turnover in the frontal lobe in SCZ, mediated through differential PLA2 activity (Gattaz et al. 1990), which together with lower cholesterol levels could lead to a disrupted formation of synapses and disturbed cellular membrane formation in SCZ.

Apolipoprotein A1

APOA1 is the major component of HDL in plasma, promotes cholesterol efflux from tissues for excretion in the liver and plays a role in the formation of plasma cholesteryl esters. APOA1 deficiencies are cause or consequences of a number of disorders such as Tangier disease, coronary disease, non-neuropathic amyloidosis and rheumatoid arthritis. Even though the functions of apolipoproteins in the CNS are not completely clear, they are known to be important players in the pathophysiology of neurological disorders such as AD.

We found APOA1 to be upregulated in the CSF of SCZ patients, whereas others found it to be downregulated in a different set of samples of CSF, serum, red blood cells, and liver (La et al. 2007; Prabakaran et al. 2007; Huang et al. 2008). APOA1 was found to be significantly increased in rats treated with chlorpromazine, suggesting that APOA1 is involved in the therapeutic action of this drug (La et al. 2007). Although we did not detect in our study correlations between daily dose or cumulative dose of antipsychotic medication in chlorpromazine equivalents or duration of medication, we analyzed samples from patients who had been treated for a short time with second generation antipsychotics, so that the APOA1 upregulation might have been a result of acute treatment that the statistical analysis could not predict. Another explanation could be that our findings might be related to a particular group of patients, and not a result of antipsychotic treatment. Even so, we hypothesize that the upregulation of APOA1 found in our study supports the hypothesis that SCZ is a disorder of phospholipids breakdown.

Prostaglandin-H2 D-isomerase

Prostaglandin D2 (PGD2) is highly abundant in the brain of humans, rats, and mice. It is a metabolite of arachidonic acid (AA) and synthesized by prostaglandin D2 synthase (PTGDS) via the cyclooxygenase (COX) pathway. AA production and degradation is essential to maintain the phospholipid structure of neuronal membranes, which is essential for normal functioning of the nervous system. Decreased levels of AA in red blood cell membrane phospholipids have been reported in SCZ patients with negative symptoms; this finding led to the conclusion that the altered membrane constitution in the CNS might be a plausible hypothesis for SCZ since overall changes in cellular membrane composition might have a wide range

of functional consequences (reviewed in Horrobin et al. 1994; Yao and Reddy, 2002). Moreover, the product of PTGDS conversion of PGH2, PGD2, functions as both a neuromodulator and a trophic factor in the CNS, and AA and its products, such as prostaglandins, are critical for various signaling pathways (Bosetti et al. 2003). Once more, the accelerated phospholipid turnover observed in SCZ brain tissue, caused by the differential PLA2 activity (Gattaz et al. 1990), and the decreased levels of other polyunsaturated fatty acids in SCZ peripheral tissue (Skosnik and Yao 2003), reinforce the idea that SCZ is a phospholipid/fatty acid disorder.

Despite the fact that genetic association studies in Portuguese and Brazilian families did not find a link between the PTGDS gene and SCZ (Ruano et al. 2007) we believe that our data and that of others cited above point to PTGDS as a potential player in the disturbed phospholipid metabolism in SCZ and a biomarker candidate for SCZ.

Downregulated proteins

Three of the six proteins we found to be significantly differentially expressed are downregulated in SCZ CSF. Coiled-coil domain-containing protein 3 precursor (CCDC3) has not previously been related to SCZ, but the coiled-coil domains are known to be important in genes such as disrupted-in-schizophrenia-1 (DISC1) (Leliveld et al. 2009). Recently, a genome-wide association study performed in 574 SCZ patients identified a SNP in the CCDC60 gene, highlighting coiled-coil domains importance in SCZ pathogenesis (Kirov et al. 2009). Although the downregulation of CCDC3 in SCZ is warranted, the absence of this protein spot in the 2DE profile of SCZ CSF means that the expression of CCDC3 is lower than the detectable range of 2DE. Shotgun proteomics methods may overlap this limitation.

Transforming growth factor-β (TGF-β) transduces signals of proliferation and cellular differentiation from the cell surface to the cytoplasm through membrane receptors such as TGF-β receptor type-1 (TGFBR1), which was downregulated in the CSF of the SCZ patients in our study. Interestingly, another member of the same family of receptors — TGFBR2 — was already described as a potential biomarker for SCZ (Numata et al. 2008), indicating that TGF-beta receptors might be potential targets of further studies in SCZ.

The main function of transthyretin (TTR) in the CSF is the transport of thyroid hormones. The downregulation of TTR we present here is supported by previous findings indicating that this protein may be a potential CSF biomarker for SCZ (Huang et al. 2006, 2007, 2008). Like CSF, TTR is also produced in the choroid plexus, a brain structure that was first found a long time ago to be linked to SCZ (Rudin 1979). Most recently, the calcification of the choroid plexus in patients — which is probably caused by the serotonergic hypofunction in SCZ (Sandyk 1993; Bersani et al. 1999) — has been claimed to be a neuroradiological hallmark of SCZ, especially because of the significant association between the size of the calcified choroid plexus and the presence of hallucinations in patients (Sandyk 1993). The differential expression of TTR and the calcification of the choroid plexus in patients might provide a basis for further studies in SCZ.

Clinical data and effects of antipsychotic medication

The correlation of PTGDS levels with DUI strengthens the hypothesis that increased PTGDS levels are a neurobiological consequence of the disease process, i.e. the disease process itself could lead to altered phospholipid metabolism. The metabolite PGD2 is involved in various pathophysiological events, such as regulation of the sleep/wake circle, pain response, hypoxia, seizure, and inflammation (Saleem et al. 2009). In SCZ, disturbances of the sleep/wake circle and pain response have been reported, as well as hypoxia or inflammation during neurodevelopment (Fendt et al. 2008, Li et al. 2009).

On the other hand, both ApoE and PTGDS may have been upregulated in the SCZ CSF samples because of compensatory and neuroplastic repair mechanisms. This speculation is supported by the negative association between the PTGDS level and the PANSS negative symptoms and general psychopathology subscores and total score. All of our first-episode patients had a paranoid subtype of SCZ at the time of investigation. However, the course of the syndrome may be different in individual patients. Even patients with a poor outcome and persistent negative and general symptoms develop progressive ventricular enlargement, which may be based on a neurodegenerative process (Lieberman et al. 2001). Thus, we hypothesize that patients with more severe negative symptoms who are not able to activate compensatory and repair mechanisms may suffer from more neurodegenerative aspects of SCZ. PTGDS, acting at its G-protein coupled receptor DP1, is known to protect the brain from neurodegeneration in transient and permanent cerebral ischemia (Saleem et al. 2007, 2009) and to protect motor neurons in an organotypic spinal cord model of amyotrophic lateral sclerosis. Agonists of the DP1 receptor and other prostaglandin receptor subtypes such as PGI2 and PGF2 additionally protect neurons from glutamate excitotoxicity (Liang et al. 2005, Wu et al. 2007), which also may be a pathophysiological event

in the development and progression of SCZ (Stone et al. 2007). An inhibitor of PTGDS, HQL-79, induced larger infarct size and reduced neuronal nuclei expression (Liu et al. 2009). Other neuroprotective substances such as acetyl-L-carnitine induce PTGDS expression (Traina et al. 2009), making PTGDS a suitable target for neuroprotection in chronic SCZ.

All of our patients had been treated with atypical antipsychotics, at least for a short time, which might have influenced our results. However, we did not detect any correlation between daily or cumulative antipsychotic dose, calculated in chlorpromazine equivalents, and protein levels. Further animal studies may elucidate the influence of neuroleptics on ApoE, APOA1 and PTGDS levels.

Conclusions

The simple, 2DE-based method presented here can quickly provide important information about a disease using only 50 µl of CSF and can be used for proteome and peptidome studies. The upregulation of ApoE, APOA1, and PTGDS described here confirms the disturbed cholesterol and phospholipid metabolism in SCZ proposed by previously experiments (Gattaz et al. 1990; Schmitt et al. 2004) but not clearly identified by genetic analysis. Moreover, the grouped analyses of ApoE, APOA1, and PTGDS protein expression might be a potential biomarker for SCZ.

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Statement of interest

The authors have no conflicts of interest to declare.

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ORIGINAL INVESTIGATION

Quality of life and subjective well-being in schizophrenia and schizophrenia spectrum disorders: Valid predictors of symptomatic response and remission?

REBECCA SCHENNACH-WOLFF[1], MARKUS JÄGER[1], MICHAEL OBERMEIER[1], MAX SCHMAUSS[2], GERD LAUX[3], HERBERT PFEIFFER[4], DIETER NABER[5], LUTZ G. SCHMIDT[6], WOLFGANG GAEBEL[7], JOACHIM KLOSTERKÖTTER[8], ISABELLA HEUSER[9], KAI-UWE KÜHN[10], MATTHIAS R. LEMKE[11], ECKART RÜTHER[12], STEFAN KLINGBERG[13], MARKUS GASTPAR[14], FLORIAN SEEMÜLLER[1], HANS-JÜRGEN MÖLLER[1] & MICHAEL RIEDEL[1,15]

[1]*Department of Psychiatry and Psychotherapy, Ludwig-Maximilians-University, Munich, Germany,* [2]*Psychiatric Clinic, District Hospital Augsburg, Germany,* [3]*Psychiatric Clinic, Inn-Salzach Hospital Wasserburg/Inn, Germany,* [4]*Psychiatric Clinic, Isar-Amper Hospital, Munich-Haar, Germany,* [5]*Department of Psychiatry and Psychotherapy, University of Hamburg, Hamburg, Germany,* [6]*Department of Psychiatry and Psychotherapy, University of Mainz, Mainz, Germany,* [7]*Department of Psychiatry and Psychotherapy, Heinrich-Heine-University of Düsseldorf, Düsseldorf, Germany,* [8]*Department of Psychiatry and Psychotherapy, University of Cologne, Cologne, Germany,* [9]*Department of Psychiatry and Psychotherapy, Charite Berlin, Campus Benjamin Franklin, Germany,* [10]*Department of Psychiatry and Psychotherapy, University of Bonn, Bonn, Germany,* [11]*Psychiatric Clinic, Alsterdrof Hospital, Hamburg, Hamburg, Germany,* [12]*Department of Psychiatry and Psychotherapy, University of Göttingen, Göttingen, Germany,* [13]*Department of Psychiatry and Psychotherapy, University of Tübingen, Tübingen, Germany,* [14]*Department of Psychiatry and Psychotherapy, University of Essen, Essen, Germany, and* [15]*Psychiatric Clinic, Vinzenz-von-Paul-Hospital, Rottweil, Germany*

Abstract

Objectives. To examine quality of life and subjective well-being as predictors of symptomatic treatment outcome. *Methods.* Biweekly PANSS ratings were performed in 285 inpatients with schizophrenia spectrum disorders within a multicenter trial by the German Research Network on Schizophrenia. Quality of life and subjective well-being were assessed using the Medical Outcomes Study-Short Form 36-Item Health Survey (SF-36), the Subjective Well-being Under Neuroleptic Treatment Scale (SWN-K) and the Adjective Mood Scale (AMS). Response was defined as an initial 20% PANSS total score reduction and remission according to the consensus criteria. Correlation analysis, logistic regression and CART-analysis were performed. *Results.* In total, 81% of the sample achieved symptom response and 48% symptom remission. The statistical analyses revealed early improvement within the first two treatment weeks in the SWN-K scale to be a significant predictor for symptomatic response. Concerning symptomatic remission the SF-36 and SWN-K baseline scores as well as SWN-K early improvement showed significant predictive value. *Conclusions.* These results highlight the importance of the patient's self-perception and especially of early improvement of quality of life and subjective well-being for symptomatic treatment outcome.

Key words: *Schizophrenia, response and remission, prediction, quality of life, subjective well-being*

Introduction

Schizophrenia is a severe and chronic disorder which affects multiple physical, functional and social domains. Since the introduction of antipsychotic medication in the 1950s assessing treatment outcome in schizophrenia has become more and more important. Remarkable attempts have been made to identify predictors for the long-term outcome and for the acute treatment response in schizophrenia (Gaebel 1996). Identifying specific outcome predictors has

Correspondence: Rebecca Schennach-Wolff, MD, Department of Psychiatry, Ludwig-Maximilians-University Munich, Nussbaumstr. 7, 80336 München, Germany. Tel: +49 89 5160 5511. Fax: +49 89 5160 5728. E-mail: rebecca.schennach-wolff@med.uni-muenchen.de

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considerable benefits in clinical practice. The early identification of poor outcome would allow timely adjustments to management programs and may provide specific treatment targets (Emsley et al. 2008).

However, factors determining the response to antipsychotic treatment in schizophrenia are not yet fully understood. Furthermore, study results in this regard have been inconclusive and inconsistent due to divergent methodologies, differing sample populations and most importantly different endpoint measures of treatment outcome (Emsley et al. 2006). Lately, in the hope of improving the assessment of treatment outcome, operational criteria defining remission were proposed by the Remission in Schizophrenia Working Group (Andreasen et al. 2005). The proposed criteria consist of a symptom-based criterion and a time criterion. This definition was considered as conceptually viable and recommended for implementation in clinical trials and clinical practice (Van et al. 2006).

In the meantime, several studies have applied these new consensus criteria to patients suffering from schizophrenia and have tried to evaluate outcome predictors. Duration of untreated psychosis or lack of early symptomatic treatment response have, amongst other factors, been associated with poor outcome (Emsley et al. 2007; Simonsen et al. 2007).

But not only sociodemographic, baseline clinical or early treatment variables are discussed as being able to predict outcome. Also, grade of individual autonomy and patient's well-being might be valuable predictors of treatment outcome (Caspi et al. 2007; Naber 1995). Against the background of the impressive symptomatology in schizophrenia, which in addition is easy to assess with a variety of well-validated instruments, subjective well-being is likely to be overlooked. Only few data exist on subjective influencing factors of patient's symptomatology. This might be due to the fact that well-being is hardly detectable by objective examinations. Moreover, the underlying theoretical conceptualization of well-being is poorly researched (Lambert and Naber 2004). Still, an increasing interest in analysing quality of life and subjective well-being of patients with schizophrenia can be detected, because the importance of the functional and social capability of schizophrenic patients has been repeatedly stated (Lambert et al. 2006; Narvaez et al. 2008). Identifying subjective influencing factors would have consequences for treatment, e.g., in taking more psychotherapeutic or sociotherapeutic approaches.

To our knowledge there is no study evaluating quality of life and subjective well-being as possible outcome predictors while applying the proposed criteria by the Remission in Schizophrenia Working Group.

On this background, response and remission criteria were applied to a large sample of inpatients who received treatment of schizophrenia under naturalistic conditions. The aims of the present study were:

(i) to examine what proportion of patients achieve symptom response and remission during inpatient treatment; and
(ii) to review the patients' quality of life and subjective well-being as predictor of response and remission.

Methods

Subjects

Data were collected in a multicenter follow-up programme (German Research Network on Schizophrenia) (Wolwer et al. 2003) at 11 psychiatric university hospitals (Aachen, Berlin, Bonn, Cologne, Düsseldorf, Essen, Göttingen, Hamburg, Mainz, Munich, Tübingen) and three psychiatric district hospitals in the Munich region (Augsburg, Inn-Salzach Klinikum, Isar-Amper-Klinikum Munich). All patients who were admitted between January 2001 and December 2004 to one of the above-mentioned hospitals with the diagnosis of schizophrenia paranoid, disorganized, catatonic or undifferentiated subtype), schizophreniform disorder, delusional disorder and schizoaffective disorder according to DSM-IV criteria were eligible for inclusion. Subjects were aged between 18 and 65 years. Exclusion criteria were a head injury, a history of major medical illness and alcohol or drug dependency. Only patients being able to understand the study design and to provide an informed written consent were included in the study. The study protocol was approved by the local ethics committees (Jager et al. 2007).

Assessments

DSM-IV diagnoses were established by clinical researchers on the basis of the German version of the Structured Clinical Interview for DSM-IV (American Psychiatric Association 1994). Using a standardized documentation system (Cording 1998) during interviews with patients, relatives and care providers sociodemographic variables (partnership, employment state) and course-related variables such as age at onset, age at first hospitalization, duration of untreated psychosis or episodes of illness were collected. Symptom severity was assessed using the Positive and Negative Syndrome Scale for Schizophrenia (PANSS) (Kay et al. 1988). Also, the PANSS cognitive index (conceptual disorganization (P2), difficulty with abstract thinking (N5), disorientation (G10), poor attention (G11), preoccupation (G15)) proposed by Lindenmayer et al. was examined (Lindenmayer et al. 2004).

The patients' adherence attitude was evaluated using the Compliance Rating Scale developed by Kemp and David (1996). [This scale is rated according to a seven-point rating scale: 1, complete refusal; 2, partial refusal; 3, accepts only because compulsory or very reluctant, persuasion or questions often needed; 4, occasional reluctance; 5, passive acceptance; 6, moderate participation, some knowledge and interest in medication and no promoting required; and 7, active participation. This clinician rating of adherence has been used in previous trials of compliance therapy (Kemp et al. 1996, 1998).]

Today, quality of life should be regarded as a multidimensional concept, combining three dimensions of non-disease aspects: (i) subjective well-being/satisfaction, (ii) functioning in daily life, including self-care and social roles, and (iii) material resources and social support (Katschnig 2000). Besides, quality of life and subjective well-being are regarded as an overall subjective measure of illness experience, antipsychotic treatment response, and life satisfaction (Lambert et al. 2003). Therefore, in order to cover the broad and complex construct of quality of life and subjective well-being three different self-rating scales (SF-36, SWN-K and AMS) were applied.

Subjective well-being was evaluated using the Subjective Well-being Under Neuroleptic Treatment Scale, short version (SWN-K) (Naber et al. 2001). [The SWN-K is a self-rating Likert scale with six response categories (absent to very much) covering 20 statements (10 positive and 10 negative) on five subscales with a minimum total score of 20 and a maximum total score of 120. Higher scores indicate better well-being.] This scale is supposed to evaluate illness experience and subjective well-being during antipsychotic treatment. Aspects on social roles and daily functioning are additionally included.

The AMS, designed by von Zerssen in the 1970s, is an internationally validated, self-rating instrument for assessing the current affective state and subjectively experienced general well-being of the individual (Bobon et al. 1981). [This scale consists of 28 pairs of antonymic everyday adjectives representing different dimensions of affects. The subject decides which word of each pair, e.g., satisfied-dissatisfied, happy-sad, most closely describes the current mood.] The AMS has been mostly used in depressed patients or patients with chronic somatic illnesses and pain states (Hofer et al. 2003; von Zerssen and Koeller 1976). However, the AMS has been applied to examine depressive symptoms and overall mood state in schizophrenic patients and was found to provide a good picture of the current state of the patient and to be in good agreement with psychiatrists' ratings (Moller and von 1982).

To mirror the patient's general quality of life the mental subscore of the Medical Outcomes Study-Short Form 36-Item Health Survey (SF-36) (Ware and Sherbourne 1992; Pukrop et al. 2003) was applied. This subscore subsumes the individual's energy/vitality, social functioning, general mental health and the extent to which health problems interfere with social activities (Ware 2000) and has been described as a valuable measure of disease burden (Tunis et al. 1999).

PANSS, SWN-K and AMS ratings were performed within the first three days after and biweekly during admission as well as at discharge, the SF-36 was rated at admission and discharge, the Compliance Rating Scale solely at admission. All raters had been trained using the applied observer scales. A high inter-rater reliability was achieved (ANOVA-ICC$>$0.8).

Statistical analysis

Response was defined as a 20% reduction of the PANSS total score from admission to discharge according to several other studies evaluating treatment response (Marder and Meibach 1994; Peuskens 1995). Remission was defined using the symptom-severity component of the standardized remission criteria (Andreasen et al. 2005) as a PANSS score of three or less of the following items: delusions (P1), unusual thought contents (G9), hallucinatory behavior (P3), conceptual disorganization (P2), mannerism/posturing (G5), blunted affect (N1), social withdrawal (N4) and lack of spontaneity (N6). Discharge was chosen as the final endpoint because we implied that clinicians would judge the mental state at the time point as stable.

Correlations at admission between the SF-36, SWN-K and AMS were evaluated with the Pearson correlations coefficient, whereas correlations between the patients' adherence and quality of life as well as subjective well-being was evaluated via the Spearman correlation coefficient. The prediction of symptom response and remission during treatment was examined with a mixed logistic regression model. This is a commonly applied model in multicenter studies when the outcome variables are binary and the independent variables include both numerical and nominal measures. A mixed model was chosen to filter the parameter multicenter trial with the study centre as random intercept effect. SF-36, SWN-K and AMS were regarded as possible quality of life and subjective well-being predictors. To analyse possible multicollinearity for SF-36, SWN-K and AMS the variance inflation factor (VIF) was applied. The VIF is a method of detecting the severity of multicollinearity which could be disregarded in this study (Fahrmeir et al. 2007).

Early improvement in SWN-K and AMS defined as a 20% reduction within the first two treatment

weeks was furthermore included in the mixed regression model as potential predictor (as the SF-36 scale was only rated at admission and discharge early improvement could not be evaluated). Additionally, the Classification and Regression Tree (CART) was used to confirm the results of the regression model. An improved algorithm to compute classification and regression trees was used that does not depend on purity measures as it combines recursive partitioning with a concept of conditional inference (Hothorn et al. 2006).

Predictive value, sensitivity and specificity levels depend on the cut-off point of the model. The discriminative ability of the regression model was also evaluated using a receiver-operating characteristic (ROC) curve. The area under the curve (AUC) is a measure of the overall discriminative power. A value of 0.5 for the AUC represents absence of discriminative power, whereas a value of 1.0 indicates perfect discrimination (Weinstein and Fineberg 1980).

All statistical analyses were performed using the statistical program R2.6.1 (R Development Core Team 2008).

Results

Patients

A total of 474 patients were enrolled in the entire multicentre study. Forty-six patients dropped out for different reasons (e.g., retrospective detection of violation of inclusion criteria, withdrawal of informed consent, incomplete information). Another 143 patients were excluded from analysis: 28 patients because they were discharged from the hospital within 7 days after admission and 115 patients had to be furthermore excluded due to missing data of the self-rating scales.

Therefore, the sample available for analysis comprised 285 subjects. A total of 160 of the patients were male, 125 were female. The mean age was 35.97 years (±11.31) and the mean duration of illness 8.55 years (±9.53). The mean number of clinical treatments was 3.51 (±4.42) and the length of current period was <1 month for 106 patients, <3 months for 46 patients, <6 months for 42 patients, <2 years for 31 patients, <10 years for 32 patients. For 18 patients length of current period was more than 10 years and 10 patients were not able to report on the duration of their current episode. The mean duration of current hospitalization was 64.96 days (±48.93) and the mean age at first treatment 27.52 years (±9.61). For DSM-IV diagnosis see Table I.

Patients were treated under naturalistic conditions: 28 patients (9.8%) received first-generation antipsychotics, 121 patients (42.5%) second-generation antipsychotics and 136 patients (47.7) received an overlapping or concurrent treatment of first- as well as second-generation antipsychotics. A total of 171 patients (60.0%) patients received tranquilizers. Fifteen patients (5.3%) were additionally treated with tricyclic antidepressants, 35 patients (12.3%) with selective serotonin reuptake inhibitors (SSRI) and 19 patients (6.7%) with other antidepressants. Fifteen patients (5.3%) were also treated with carbamazepine and 15 patients (5.3%) with valproate.

Table I. DSM-IV diagnoses of all study participants.

DSM-IV diagnoses	*N*	%
Schizophrenia:		
▪ disorganized type (295.1)	7	2.5
▪ paranoid type (295.3)	160	56.1
▪ residual type (295.6)	14	4.9
▪ undifferentiated subtype (295.9)	12	4.2
Schizophreniform disorder (295.4)	34	11.9
Schizoaffective disorder (295.7)	33	11.6
Delusional disorder (297.1)	4	1.4
Brief psychotic disorder (298.8)	19	6.7
Psychotic disorder nos (298.9)	2	0.7

Assessments

Time course of clinical assessments. The results of PANSS ratings as well as the well-being self-ratings at admission and discharge are displayed in Table II. A significant improvement from baseline to endpoint in all PANSS subscores as well as in the SF-36, SWN-K and AMS could be observed.

To evaluate a possible association between quality of life as well as subjective well-being and the patient's attitude towards adherence the Compliance Rating Scale was analysed. An attitude with complete refusal to antipsychotic treatment

Table II. Time course of clinical assessments.

	Admission	Discharge	*p* value[1]
PANSS positive subscore	18. 5 (±6.2)	10.3 (±3.4)	<0.01
PANSS negative subscore	17.2 (±7.3)	14.0 (±6.2)	<0.01
PANSS global subscore	33.9 (±9.6)	25.2 (±7.6)	<0.01
PANSS total score	69.6 (±18.2)	49.5 (±14.7)	<0.01
PANSS cognitive index[2]	12.05 (±4.2)	8.4 (±3.33)	<0.01
SF-36	32.0 (±11.6)	39.1 (±11.5)	<0.01
SWN-K	81.5 (±18.6)	88.2 (±18.5)	<0.01
AMS	26.1 (±14.7)	18.5 (±13.9)	<0.01

Admission and discharge scores are shown as well as significant improvements from admission to discharge.

[1]*P* value derives from a Wilcoxon test.

[2]The cognitive index by Lindenmayer et al. (2004) comprises the following PANSS items: conceptual disorganization (P2), difficulty with abstract thinking (N5), disorientation (G10), poor attention (G11), preoccupation (G15).

Table III. Correlation analysis of the patients' adherence behaviour and quality of life as well as subjective well-being.

Patient's adherence behaviour	Correlation coefficient[1]	*P* value[2]
SF-36	–0.0897	0.1415
AMS	0.0903	0.1289
SWN-K	–0.0185	0.7698

This table shows the correlation between the patient's adherence behavior and quality of life as well a subjective well-being.
[1]Spearman's correlation coefficient was applied.
[2]Tests based on Spearman's rho statistic revealed the *P*-value.

was scored for two (0.5%) patients. Partial refusal, e.g., refusing depot drugs or accepting only the minimum dose, was rated for nine (3%) patients. Eight (2.5%) patients were scored with an attitude of reluctant acceptance. A treatment attitude with occasional reluctance regarding treatment – questioning the need for treatment once a week – was scored for 24 (8%) patients. Passive acceptance was prevalent in 93 (33%) patients. An attitude of moderate participation, meaning some knowledge and interest in treatment, was scored for 93 (33%) patients, and an attitude of active participation with ready acceptance was rated for 56 (20%) patients (Kemp et al. 1996).

Applying the Spearman correlation coefficient no significant correlation was found regarding the patients' adherence and quality of life as well as subjective well-being (Table III).

Responder and remitter

Applying the above-mentioned response criteria 231 patients (81%) were treatment responder and 138 patients (48%) met symptom-severity criteria for remission at discharge.

Correlation matrix for SF-36, AMS and SWN-K

Regarding correlations between the applied scales, a high correlation was found between the AMS and the SWN-K. Moderate to high correlations were found for the AMS and SF-36 and the SWN-K and the SF-36, see Table IV.

Mixed models for prediction of response and remission

Predictors of response were the AMS baseline score as well as an early improvement in the AMS and SWN-K scale at week 2, whereas the only significant predictor was early improvement in the SWN-K scale. Receiver operating characteristics led to an AUC score of 0.69 indicating that this model had reasonable predictive power and reached statistical significance ($P=0.036$) (Table V, Figure 1). In this model, the best fitting decision rule (referred to as 'prediction') was found revealing a sensitivity of 93.1%, a specificity of 35.7%, a true positive rate of 88.2% and a true negative rate of 50.0%.

The strongest predictors of remission were the SF-36 and SWN-K baseline scores as well as early improvement in the SWN-K scale. Receiver operating characteristics revealed an AUC score of 0.67 indicating moderate predictability (Table V, Figure 2). In this model, the best fitting decision rule (referred to as 'prediction') was found revealing a sensitivity of 67.9%, a specificity of 71.4%, a true positive rate of 76.0% and a true negative rate of 62.5%. Again, the prediction model reached statistical significance ($P=0.002$).

CART-analysis for prediction of response and remission

The additionally performed CART-analysis revealed a similar pattern of outcome as the logistic

Table IV. Univariate tests of the medical treatment applied and its potential influence on quality of life and subjective well-being.

	AMS Change	SWN Change	SF-36 Change
Antipsychotics			
First-generation antipsychotics	–8.32 (±15.7)	6.59 (±12.21)	4.96 (±9.04)
Second-generation antipsychotics	–8.89 (±14.57)	7.36 (±14.37)	6.65 (±12.81)
First- and second-generation antipsychotics	–7.22 (±15.13)	6.77 (±16.74)	5.88 (±12.32)
P value	0.7001	0.9510	0.8885
Antidepressants			
Tricyclic antidepressants	–5.07 (±8.77)	3.13 (±16.61)	6.15 (±10.74)
SSRI	–13.66 (±19.93)	8.6 (±19.84)	8.15 (±14.91)
Others	–12.32 (±16.71)	13.26 (±12.94)	7.04 (±10.37)
P value	0.4914	0.2876	0.0705
Mood stabilizer			
Carbamazepine	–4.93 (±19.09)	4.2 (±19.19)	0.01 (±13.19)
Valproate	–6.8 (±12.85)	9.53 (±17.7)	2.31 (±10.59)
P value	0.756	0.4355	0.6871

This table shows univariate tests (ANOVA and t-tests) of the possible influence of the treatment applied on the patients' quality of life and subjective well-being. Changes in quality of life and subjective well-being refer to changes from admission to discharge.

Table V. Correlation analysis at admission.

	AMS	SWN-K
SF-36	–0.54 (<0.01)	0.60 (<0.01)
AMS		–0.76 (<0.01)

This table presents the correlation analysis at admission between all three self-rating scales applied. Values in bold print are the correlation coefficient and values in brackets the *P* values, respectively.
Pearson's correlation coefficient was applied and tests based on the Pearson's product moment correlation coefficient revealed the *P*-values.

regression analysis, however, without reaching statistical significance which can be attributed to the lower power of such classificatory analyses (0.05α criterion).

Discussion

Quality of life and subjective well-being in schizophrenia

In the present trial quality of life and subjective well-being were assessed by using self-rating scales, which is still controversial in schizophrenia patients. Some authors doubt as to whether patients with schizophrenia are capable of self-assessment of their auto-perception and quality of life because of their cognitive deficits, lack of insight into their illness and reduced levels of expectations, which especially holds true in chronic patients (Bobes et al. 2005). On the other hand convergent validity in the perception of well-being between patients and clinicians could be shown (Browne et al. 1996). Also, several authors stated that evaluating psychiatric patients' reports of well-being is desirable and demonstrated that schizophrenic patients feel, experience and report their personal deficits, supporting the thesis that well-being can be assessed subjectively in a valid way (Skantze et al. 1992).

Quality of life and subjective well-being as predictor

General aspects. Aim of this present study was to evaluate the predictive validity of quality of life and subjective well-being as predictor of symptomatic response and remission. High correlations were found between the AMS and SWN-K total and moderate to high correlations for the AMS and SF-36 and SWN-K and SF-36 (Table IV). However, despite the rather high correlations between the rating scales multicollinearity can be disregarded considering the variance inflation factors. Taken together, the quality of life and well-being rating scales seem to measure similar domains, yet without overlapping heavily.

The ROC curves of Figures 1 and 2 show sensitivity and specificity for every probability in terms

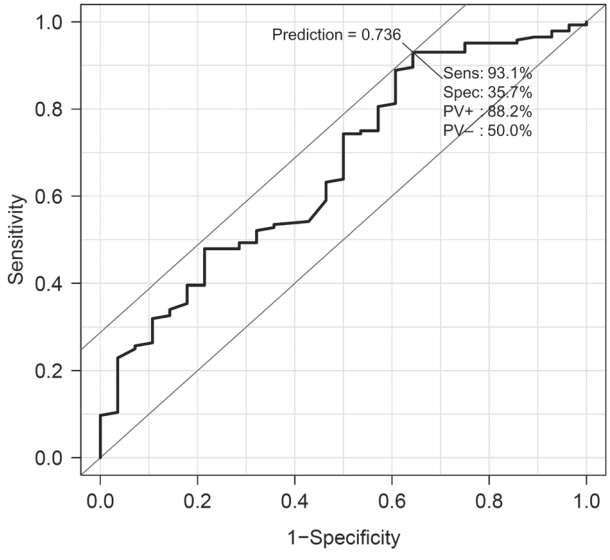

Figure 1. ROC curve for predicting response, AUC=0.69. The curve shows different probabilities (=different decision cut-points of the logistic model) of a patient to achieve/not achieve response. The best fitting decision rule regarding whether or not a patient will become responder is calculated by the maximum of sensitivity and specificity. In this model, the best fitting decision rule (referred to as "prediction") was found revealing a sensitivity of 93.1%, a specificity of 35.7%, a true positive rate of 88.2% and a true negative rate of 50.0%.

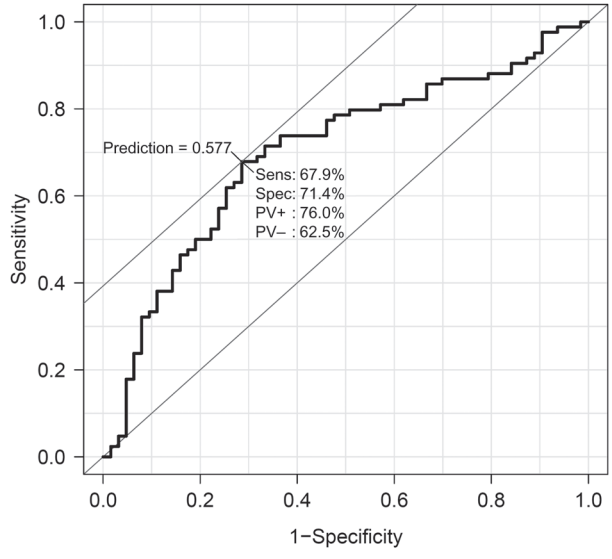

Figure 2. ROC curve for predicting remission, AUC=0.67. The curve shows different probabilities (=different decision cut-points of the logistic model) of a patient to achieve/not achieve remission, The best fitting decision rule regarding whether or not a patient will become remitter is calculated by the maximum of sensitivity and specificity. In this model, the best fitting decision rule (referred to as "prediction") was found revealing a sensitivity of 67.9%, a specificity of 71.4%, a true positive rate of 76.0% and a true negative rate of 62.5%.

Table VI. Mixed logistic regression model for the evaluation of quality of life and well-being as predictor of symptom response and remission.

	Effective coefficient (β)	Standard deviation (SD)	*z* value[1]	*P* value	CI-L[2]	CI-U[3]
Response						
AMS baseline	2.25	0.02	–1.83	0.07	1.09	4.63
Early improvement AMS	1.10	0.48	1.57	0.12	1.03	1.16
Early improvement SWN-K	1.06	0.47	–2.00	0.04	1.00	1.11
Remission						
Early improvement SWN-K	2.61	0.45	2.12	0.03	1.08	6.34
SF-36 baseline	0.94	0.02	–3.13	<0.01	0.90	0.98
SWN-K baseline	1.06	0.02	3.37	<0.01	1.02	1.09

This table shows the predictive validity of quality of life and subjective well-being regarding response and remission at discharge. A *P* value of <0.05 indicates the significant predictors of outcome at discharge.
[1]The *z* value is the corresponding test statistic of the effective coefficient leading to the *P* value.
[2]This abbreviation refers to the lower confidence interval of the effective coefficient.
[3]This abbreviation refers to the upper confidence interval of the effective coefficient.

of whether or not the patient will become a responder/remitter. The prediction value is the optimal cut-point of the calculated event probabilities of any new data put into the model. A prediction value of 0.738 in the curve of response means that a patient achieving any value below this cut-point in this model will be predicted as non-responder. The prediction value of remission was found to be 0.577, respectively. The mixed models revealed early improvement in the SWN-K to be a significant predictor of response, the SF-36 and SWN-K baseline scores as well as SWN-K early improvement were significantly predictive of remission. On the other side, CART-analysis was not able to find significant quality of life and subjective well-being variables that would best separate the patient sample in terms of achieving response and remission with the chosen significance level of $P<0.05$. However, this does not mean that these two statistical methods disqualify each other, but they provide a broad basis to evaluate the predictive validity of quality of life and subjective well-being.

The importance of quality of life for short- and long-term outcome has been repeatedly stated. In a recent 5-year follow-up study on improvement in subjective well-being and enduring symptomatic remission de Haan et al. (2008) found early improvements of quality of life to be significantly related to enduring symptomatic remission and thereby to the long-term course of the disease. Other authors have stated the important role of quality of life and well-being regarding treatment compliance with patients suffering from an impaired quality of life to feature less compliance resulting in illness relapses and re-hospitalizations (Donohoe et al. 2001; Naber et al. 2001). Interestingly, we did not find a significant correlation between quality of life/subjective well-being and the patient's attitude towards adherence in the present study. One explanation might be that most patients were treated with atypical antipsychotics, which are known to cause fewer stigmatizing side effects having an impairment on quality of life, and thus not influencing adherence that much (Valenstein et al. 2006). Another aspect might be that in the present study adherence was measured solely on the basis of the physicians' judgement and was not controlled via objective measurements.

Baseline quality of life and subjective well-being as predictor. The patient's baseline quality of life and subjective well-being were found to significantly predict symptomatic remission, regarding response the predictive validity did not reach the level of significance. The influence of the patient's baseline quality of life and well-being for outcome is in agreement with findings from other studies. Lambert et al. (2009) followed 2842 patients with schizophrenia over 3 years and found baseline subjective well-being cut-off points to be sufficient predictors of outcome underlining the importance of early treatment adaptations. In another analysis the authors examined the prediction of remission as a combination of symptomatic and functional remission in 2960 patients with schizophrenia detecting that among the most relevant predictors of symptomatic remission was a better subjective well-being at baseline (Lambert et al. 2006). The influence of quality of life seems to be important for treatment outcome already in early phases of schizophrenia, because when examining 559 patients with a first-episode of schizophrenia Emsely et al. (2007) found patients in remission to perform significantly better regarding quality of life.

Early improvement of quality of life and subjective well-being as predictor. Early improvement significantly predicted both, response and remission, in this study.

The importance of an early improvement of well-being has been discussed before when stating the results by de Haan et al. (2008) finding early improvement in subjective well-being to be a significant predictor of enduring symptomatic remission in a 5-year follow-up, whereas they were not able to find the same results regarding early symptomatic improvement. This underlines the importance of a subjective experience of improvement during early treatment. Other studies evaluated different outcome domains and also found that achieving complete remission, which included subjective well-being early in the course of treatment, was a robust predictor of subsequent outcome (Lambert et al. 2007). In the same study early response in well-being was revealed to be predictive for later well-being and was closely linked to overall symptomatic improvement.

Strengths and limitations

Patients were treated under naturalistic conditions and such a design does not allow a sufficient control of study results for the effect of different pharmacological and psychological treatments. Also, the Remission in Schizophrenia Working Group developed the symptomatic remission criteria solely for schizophrenic patients. In this study the criteria were used in a wider spectrum of schizophrenia related disorders. Other authors examined the consensus criteria in schizophrenia and other psychiatric disorders simultaneously (Lasser et al. 2005; Sethuraman et al. 2005). The time criterion was likewise not considered, for the criteria were proposed to define remission as the absence of relevant symptoms for at least 6 months and the mean duration of current hospitalization was only 65 days (Andreasen et al. 2005). Furthermore, it should be kept in mind that 189 patients were excluded after recruitment and only patients that accepted to complete questionnaires were included in statistical analyses.

Strengths of this study include the rather large sample in the evaluation of quality of life and subjective well-being as possible predictor of symptomatology. In addition, due to the liberal inclusion and exclusion criteria, findings of this study on treatment seeking patients might be more generalizable and exhibit higher external validity. This is of special importance as randomized controlled trials tend to include patients less impaired and with better functioning leading to an underestimation of true rates of impaired quality of life and subjective well-being.

Conclusion

In addition to previous findings, we are able to demonstrate the clinical relevance of quality of life and subjective well-being as a subjective measure of illness experience and overall life satisfaction. The significant influence of early improvements of quality of life and subjective well-being for the patient's outcome underlines the importance of early treatment interventions and the need for psychotherapeutic as well as sociotherapeutic actions besides the psychopharmacological treatment to improve the patient's symptomatic status.

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Statement of interest

The authors have no conflict of interest with any commercial or other associations in connection with the submitted article.

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ORIGINAL INVESTIGATION

The association of infectious agents and schizophrenia

DANIELA KRAUSE, JUDITH MATZ, ELIF WEIDINGER, JENNY WAGNER, AGNES WILDENAUER, MICHAEL OBERMEIER, MICHAEL RIEDEL & NORBERT MÜLLER

Department of Psychiatry and Psychotherapy, Ludwig-Maximilians University, Munich, Germany

Abstract

Objectives. The influence of infectious agents on the pathogenesis of psychiatric disorders has been discussed for decades. Pre- and postnatal infections are risk factors for schizophrenia. This may be explained by chronic infections or an altered immune status. However most of the studies have only focused on one single pathogen and not on the impact of different infectious agents. We investigated the association between schizophrenia and various neurotophic infectious agents. *Methods.* A total of 31 schizophrenic patients and 30 healthy matched individuals were included. Antibody titres of cytomegalovirus, herpes simplex virus, Epstein-Barr virus, mycoplasma, chlamydia and toxoplasma were evaluated. For statistical analysis we used Fisher's exact and Wilcoxon test. *Results.* Significantly elevated positive antibody titres within schizophrenic patients were found only for *Chlamydia trachomatis* ($P=0.005$) and a trend to significance for herpes simplex virus ($P=0.055$). Combining the different agents, schizophrenics had a significantly higher rate of positive titres to infectious agents as compared to controls ($P=0.04$). *Conclusions.* The higher prevalence of antibodies within schizophrenic patients emphasizes a possible role of infectious agents in the pathogenesis of schizophrenia. Our data indicates that not one specific agent might be responsible for schizophrenic symptoms but the resulting immune response in the central nervous system.

Key words: *Schizophrenia, infection, chlamydia, herpes simplex, immune system*

Introduction

Several causative factors have been identified in the pathophysiology of schizophrenia. However, the results of extensive research to identify schizophrenia-causing genes remains fragmentary (Tomppo et al. 2009). Epidemiological studies have revealed different environmental factors such as winter and spring birth, birth in an urban area, and complications during labour and delivery (Machon et al. 1983; Wright et al. 1995).

Moreover it has been suggested that environmental factors, such as infections, are involved in the aetiology of some cases of schizophrenia (Brown 2006). Serious viral CNS infections during childhood appear to be associated with the later development of schizophrenia (Dalman et al. 2008). One indication for this assumption is that a substantial number of encephalitis cases is manifested in symptoms simulating classical psychotic or mood disorders (Caroff et al. 1998).

While until now different microbial agents have been proposed as risk factors for schizophrenia, many recent studies have focused on members of the viral family of Herpesviridae (Niebuhr et al. 2008), Borna virus (Nunes et al. 2008) intracellular bacteria like *Chlamydia* (Fellerhoff et al. 2007) as well as the protozoan organism *Toxoplasma gondii* (Yolken et al. 2001). Reasons for the focus on these agents include their ability to establish persistent infections within the central nervous system as well as the occurrence of neurological and psychiatric symptoms in some individuals infected with these agents (Quinn et al. 2000).

In the meantime there is evidence that even a prenatal infection influences the prevalence of schizophrenia. It was shown that maternal exposure to herpes simplex virus type 2 is associated with an increased risk for psychoses among adult offspring (Buka et al. 2008). In addition to this, a 7-fold increased risk of developing schizophrenia has been

Correspondence: Daniela Krause, Department of Psychiatry and Psychotherapy, Ludwig-Maximilians University, Munich, Germany. Tel: +49 51605528. Fax: +49 51605348. E-mail: Daniela.Krause@med.uni-muenchen.de

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reported after exposure to influenza during pregnancy of the mother (Brown et al. 2004). But not only viruses are related to psychiatric diseases, it is also assumed that maternal exposure to toxoplasmosis may be a risk factor for schizophrenia (Brown et al. 2005). Results of a Finnish epidemiological study showed that an infection of the CNS in childhood increases the risk by 5-fold of becoming psychotic later (Koponen et al. 2004).

This indicates that an infection during early childhood is in accordance with the assumption that an infection-triggered disturbance within brain development might play a key role in the aetiology of schizophrenia.

Furthermore, studies of adults with recent onset of schizophrenia have revealed that these patients have increased levels of serum cerebral spinal fluid IgG antibodies to cytomegalovirus and *Toxoplasma gondii* (Leweke et al. 2004).

Considering all these findings, it still remains controversial which of the neurotrophic infectious agents are involved in the pathogenesis of schizophrenia.

Regarding treatment and prevention, it is the focus of this research as to whether viruses can eventually give rise to psychiatric diseases or also contribute to the maintenance of psychiatric symptoms.

In this study we investigated whether there is a higher prevalence of infectious agents that establish a persistent central nervous system infection in adult schizophrenic patients.

Methods and materials

Characterization of the patient and control population

For this study 31 patients with schizophrenia (age 36.7±13.6 years; 58.1% male) were diagnosed by two experienced psychiatrists according to the diagnostic criteria, as defined by the Diagnostic and Statistical Manual – IV edition (DSM-IV). As a structured tool the positive and negative symptoms scale (PANSS) was used to rate the patients (mean value 92.06; σ=20.25). Patients were recruited through the Department of Psychiatry of the Ludwig-Maximilians University Munich and were hospitalized. Healthy control subjects were recruited via advertisement. The 30 people of the control population (age 33.7±16.1 years; 60% male) were matched to the schizophrenic group considering gender, ethnicity and age. All study participants gave their informed consent prior to study inclusion. For this study, the responsible authorities (the university's ethics committee) approved the procedure for sample collection and analysis, in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. A concomitant organic disease or acute general or genito-urinary infections were exclusion criteria. In addition the control group did not meet the criteria for DSM-IV diagnosis of mental illness. In the schizophrenic group, antipsychotic treatment had to be stopped at least 4 weeks prior to study inclusion. This means that patients where either drug-naïve or had their antipsychotic medication stopped for some other reasons than study inclusion. Blood samples were taken from schizophrenic individuals before antipsychotic medication was administered. Patients and volunteers were all Caucasians and living in Munich or the periphery of Munich, Bavaria Germany. So patients and controls were recruited from the same rather homogeneous region with regard to socioeconomic status: (1) education: a mean of 30.5% reached a diploma qualifying for university education; (2) income: the annual income was €19.700 per year in 2007; and (3) occupation: the unemployment rate was 4.5% in Bavaria in October 2007 (according to the German federal statistical office and the Bavarian ministry of economy). Table I summarizes the characteristics of the study population.

Measurement of antibodies

Serum blood samples were obtained from the participants by venipuncture. The samples were stored at –80°C and tested for antibodies using enzyme-linked immunosorbent assay (ELISA) at Max von Pettenkofer Institute for Hygiene and Microbiology. Serum IgG and IgM antibodies were measured

Table I. Characterisation of the study collective.

	Schizophrenic patients N=31	Mean	Standard deviation (SD)	Healthy controls N=30	Mean	Standard deviation (SD)
Sex						
Female	13 (41.9%)	36.7	4.9	12 (40%)	36.9	15
Male	18 (58.1%)	37.2	13.2	18 (60%)	34.0	16.1
Age		36.7	13.6		33.7	16.1
Age of onset		31.9	12.9			
BMI (kg/m²)	30 (96.8%)	25.4	4.32	29 (93.6%)	23.3	3.85
Smoking status	18 (58%)			6 (19%)		

of cytomegalovirus (CMV), herpes simplex virus (type 1 and 2 were not distinguishable) (HSV-1/2), Epstein–Barr virus (EBV) and *Mycoplasma pneumoniae*. For *Toxoplasma gondii* (Toxo), *Chlamydia trachomatis* and *Chlamydia pneumoniae* antibody titres of IgG, IgM and IgA were evaluated. For CMV and HSV samples were pipetted automatically on the BEP 3, which is an ELISA machine from Siemens. EBV ELISA results were generated with tests from Diasorin on BEP 3. In addition, for *Toxoplasma gondii* IgG and IgM the test "Enzygnost" from Dade Behring was used. *Toxoplasma gondii* IgA was measured with "PLATELIA" from BIO RAD. Antibodies against *Mycoplasma pneumoniae* were evaluated with "SERION ELISA classic" from virion\serion. For *Chlamydia trachomatis* and *pneumoniae* "SeroCT" from savyon DIAGNOSTICS was applied.

Data analysis

For statistical analysis of categorical variables the exact test of Fisher was applied. In order to test a not normally distributed population the Wilcoxon test was used.

Results

Antibody titres of seven different infectious agents were measured from 31 patients with schizophrenia and 30 healthy controls.

Bacterial and protozoan antibody prevalence in schizophrenic patients and controls

We found that the group of schizophrenic patients had a significantly higher number of positive IgG antibody titres of *Chlamydia trachomatis* ($P=0.005$) as compared to healthy controls. No statistical differences could be measured for the other bacteria and toxoplasma. These results are shown in Table II.

Table II. Percentage of positive bacterial and protozoan antibody titres in comparison of schizophrenic patients and controls.

Bacteria/Protozoan	Prevalence of positive antibodies in %: Schizophrenia controls	Prevalence of positive antibodies in %: Healthy	Fisher's exact test
Chlamydia trachomatis IgG	25.8	0.0	$P=0.005$
Chlamydia trachomatis IgA	29.0	10.0	$P=0.106$
Chlamydia pneumonia IgG	54.8	43.3	$P=0.446$
Chlamydia pneumonia IgA	29.0	36.7	$P=0.592$
Mycoplasma IgG	80.0	86.7	$P=0.211$
Mycoplasma IgM	12.9	13.3	$P=1$
Toxoplasma gondii IgG	38.7	20.0	$P=0.161$
Toxoplasma gondii IgM	3.2	0.0	$P=1$
Toxoplasma gondii IgA	3.2	0.0	$P=1$

Table III. Percentage of positive viral antibody titres in comparison of schizophrenic patients and controls.

Virus	Prevalence of positive antibodies in %: Schizophrenia controls	Prevalence of positive antibodies in %: Healthy	Fisher's exact test
Herpes simplex virus IgG	80.6	54.8	$P=0.0558$
Herpes simplex virus IgM	3.2	0.0	$P=1$
Epstein–Barr virus IgG	90.3	86.6	$P=0.707$
Epstein–Barr virus IgM	12.9	0.0	$P=0.113$
Cytomegalovirus IgG	48.4	43.3	$P=0.798$
Cytomegalovirus IgM	9.7	0.0	$P=0.238$

Viral antibody prevalence in schizophrenic patients and controls

The investigation of the viral antibodies showed a tendency towards an association of more viral infections in the group of schizophrenic patients. For Herpes simplex virus, half the occurrence of a positive antibody titre was nearly significantly elevated in schizophrenic patients ($P=0.0558$). In total, the schizophrenic group had a higher prevalence of antibodies for all analyzed viruses. Table III summarizes the results.

Comparison of all antibody titres within schizophrenic patients and controls

We evaluated the number of positive antibody titres per person. So the frequency of all viral, bacterial and protozoan antibodies (IgG, IgM and IgA) was counted for each study participant. An "infectious index" was created for the group of schizophrenic patients and controls. A calculation with the Wilcoxon test

Table IV. Numbers of positive antibody titres per person.

Number of positive antibody titres	Schizophrenic patients	Healthy controls
1	0	1
2	3	4
3	6	9
4	7	8
5	8	7
6	6	1
7	1	0
Total	31	30
Wilcoxon test: $P=0.04$		

The first column shows the possible numbers of positive antibody titres. Looking at the first row, there were no schizophrenic patients that had only one positive titre and one control individual had one positive titre.

revealed that the group of schizophrenic patients had a significantly higher rate of positive antibodies to infectious agents as compared to healthy controls ($P=0.04$). Results are shown in Table IV.

Correletion of clinical data and infection rates

For the severity of schizophrenic symptoms measured with the positive and negative symptom scale, no correlation to the prevalence of antibodies to infectious agents could be found (data not shown).

Discussion

The present study revealed that the schizophrenic patients had a significantly higher rate of positive antibodies to infectious agents as compared to healthy controls. Especially antibodies to *Chlamydia trachomatis* and herpes simplex virus were overrepresented in schizophrenic patients.

Our findings are in accordance to previous studies that concluded that chlamydia infection represents one risk factor for schizophrenia (Fellerhoff et al. 2007). Other investigations also showed an association between herpes family viruses and schizophrenia (Niebuhr et al. 2008). However, it is still controversial whether infectious agents play a causal role in psychotic symptoms. Proving causality is one of the major limitations of studies about the association of schizophrenia and infections. As schizophrenia is constituted of a number of inhomogeneous symptoms, it seems more probable that infections might just be one contributing factor among others like genetic disposition (Tomppo et al. 2009). The interactions between infectious agents and host factors resulting in complex disorders such as schizophrenia generally do not follow the classical rules. These rules, known as Koch's postulates, require that there be a one-to-one correspondence between an infectious agent and a disease process. In the case of schizophrenia, one possible causality could be that infectious agents do not directly cause psychiatric symptoms but influence the immune balance via the status of a chronic infection.

The present study represents an exploratory investigation. Therefore it is difficult to draw substantial conclusions. Though we have seen mainly IgG and not IgM antibodies to be elevated in schizophrenia, this could indicate that the infections are not acute any more, but have progressed to dormant infections with a persistent immune response. As IgG antibodies are involved in secondary immune response, IgM antibodies appear early in the course of an infection and usually reappear. In our study, we just showed the rates of antibody titres, the infectious agents were not investigated directly with PCR. Though, there is no evidence from this study that patients with schizophrenia have an increased prevalence of acute infections, but clearly these patients have had more infections in the past and/or are suffering from a chronic infectious condition.

There are also some limitations to our study: the sample size was rather small and therefore the statistical power might be restricted. This could also mean that we might have missed some increased antibody titre differences because of the low subject numbers. Another explanation why only certain infectious agents were elevated could be that just specific bacteria and viruses are associated with schizophrenic symptoms. The fact that all control subjects did not meet the criteria of a psychiatric illness could be a further limitation. Therefore the control population could eventually be healthier than the whole general population. Also it was not possible to evaluate the rate of sexual activity in patients and controls. This seems important as *Chlamydia trachomatis* is a sexually transmitted disease. There are studies showing that schizophrenic patients suffer from sexual dysfunction and of less social activity and have therefore an impaired sexual life (Hariri et al. 2009). It also remains possible that schizophrenic patients might show riskier sexual behaviour.

The precise underlying mechanism of the connection between an infection and the aetiology of schizophrenia remains to be determined. As we have found in this study that for schizophrenic patients antibodies of various infectious agents were overrepresented, we assume that these different infections have an impact on the immune system and therefore might contribute to psychiatric symptoms. Several studies have identified the important role of immunological parameters in schizophrenia (Miuller [*sic*] and Schwarz 2007). Also in other psychiatric conditions, immunological parameters (e.g., soluble interleukin-2 receptor and interleukin-6) are being discussed as biological markers for the disease (Mossner et al. 2007). An immune disturbance in schizophrenia was suggested because of elevated levels of e.g., interleukin-1β, tumour necrosis factor-α and a higher nuclear factor-kappaB (regulates the cytokine system) activation have been observed (Song et al. 2009). Furthermore, an increase in CSF levels of cytokines like interleukin-2 in schizophrenic patients was described (McAllister et al. 1995) and signs of inflammation were found in the brains of schizophrenic patients (Korschenhausen et al. 1996). Recently, these proinflammatory cytokines that could enhance the activity of the enzyme indoleamine 2,3-dioxygenase (IDO) play an important role in the pathophysiology of schizophrenia. IDO increases tryptophan degradation into kynurenine and decreases tryptophan availability in the brain to synthesize neurotransmitters. (Myint et al. 2009).

In summary, we showed a higher prevalence of infections within the schizophrenic group. These findings suggest that the elevated rate of infectious agents within the schizophrenic patients could provoke an immunological disturbance that might influence the cerebral neurotransmitter balance. A deeper insight into the precise mechanism of how neurotrophic infectious agents influence the immune system, tryptophan metabolism and the resulting neurotransmitter availability could help finding new therapeutic strategies for psychiatric diseases. Further studies investigating the association between the infection status and immune parameters are needed.

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ORIGINAL INVESTIGATION

Associations between satisfaction with life, burnout-related emotional and physical exhaustion, and sleep complaints

SERGE BRAND[1], JOHANNES BECK[1], MARTIN HATZINGER[2], ALEXANDER HARBAUGH[3], WILLIBALD RUCH[4] & EDITH HOLSBOER-TRACHSLER[1]

[1]*Psychiatric Hospital of the University of Basel, Depression and Sleep Research Unit, Basel,* [2]*Psychiatric Out-Patient Clinic of the University of Basel, Switzerland,* [3]*Swissburnout, Berne, and* [4]*Department of Psychology, Personality and Diagnostics, University of Zurich, Switzerland*

Abstract

Objectives. Burnout is a state of work-related emotional and physical exhaustion. Burnout is related to sleep complaints. By contrast, people with optimistic attitude seem to be less vulnerable to stress and burnout. Therefore, the present study aimed at investigating the relation between burnout, depressive symptoms, satisfaction with life, and sleep complaints. *Methods.* A total of 2231 participants (age [years]: M=40.8; 1183 females and 1048 males) took part in the study. Participants completed a series of questionnaires such as the Tedium Measure, the Insomnia Severity Index, and the Satisfaction with Life-questionnaire. For statistical analyses, a Structural Equation Model (SEM) was applied. *Results.* Pessimism, emotional and physical exhaustion, depressive symptoms, and low satisfaction with life were interrelated. Emotional and physical exhaustion was related to sleep complaints, whereas sleep complaints were not related to depressive symptoms and pessimism. Satisfaction with life was related to low sleep complaints, though mediated via low emotional and physical exhaustion, and low pessimism. *Conclusions.* Results suggest that among burnout symptoms emotional and physical exhaustion, but not depressive symptoms, are related to sleep complaints. Satisfaction with life, via low emotional and physical exhaustion, and low pessimism, further contributes to favourable sleep.

Key words: *Burnout, satisfaction with life, depressive symptoms, sleep complaints, emotional and physical exhaustion*

Introduction

Physical and mental wellbeing (Banks and Dinges 2007), and academic (Curcio et al. 2006) and emotional (Killgore et al. 2008) performance are highly associated with restoring and satisfying sleep. In the occupational area, disrupted and non-restorative sleep increases the risk of accidents or "headline hitting" disasters (Folkard et al. 2005). Individuals who do not sleep well tend to have impaired work productivity and to consume more medical resources (Nishikitani et al. 2005), and employees suffering from insomnia have been shown to have significantly higher rates of absenteeism and to cause increased costs for both employers and the community (Godet-Cayre et al. 2006; Metlaine et al. 2005). They have also been shown to have a 3-fold greater risk of having poor self-esteem at work, less job satisfaction and decreased efficiency at work (Leger et al. 2006). Taken together, these concepts indicate that unfavourable sleep conditions may lead to impaired performance at work.

Complementary to these concepts, there is a growing body of research evidence showing that the direction of the relation between sleep patterns and working conditions may be inverted: Adverse working conditions may also cause and maintain sleep disturbances. First, with respect to working structures, sleep disturbances may be associated with or mediated by irregular working schedules (cf. Caruso 2006), and lifestyle changes have increased the demand for a 24/7 service (Bohle et al. 2004). Second, with respect to psychological factors, results from an investigation of 8,700 Japanese local government and transit company employees showed that occupational stress

Correspondence: Serge Brand, PhD, Psychiatric Hospital of the University of Basel, Depression and Sleep Research Unit, Wilhelm Klein-Strasse 27, 4025 Basel, Switzerland. Tel: +41 61 3255 114. Fax: +41 61 3255 513. E-mail: serge.brand@upkbs.ch

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was a possible risk factor for insomnia (Utsugi et al. 2005). At 1-year follow-up, Jansson and Linton (2006) revealed that high work demands increased the risk of developing insomnia one year later. Taken together, these findings hold that work-related psychological factors have adverse impact on sleep.

Among the adverse psychological consequences associated with job-related stress, burnout (BO) demands particular attention, because, for instance in Europe, epidemiological studies have shown work-related burnout rates of approximately 7% (Hallsten 2005), with higher rates in professionals such as teachers and physicians (Thomas 2004).

The term burnout is multifaceted. Generally, burnout is understood as a negative affective state, which comprehends feelings of emotional exhaustion, physical fatigue, cognitive weariness, and chronic depletion of energetic resources resulting from cumulative exposure to chronic work and life stresses (Melamed et al. 2006). Maslach and Jackson (1981) defined burnout as a set of attitudes and behaviour focused on emotional exhaustion, depersonalization, and low personal accomplishment, whereas the ICD-10 refers to burnout as a state of complete exhaustion (Z73.0#; WHO 1992). Thus, the term burnout covers a broad variety of unfavourable change in emotion, cognition, and behaviour.

Burnout is related to physical complaints (e.g., cardiovascular disease: Schuitemaker et al. 2004; metabolic syndromes such as increased fasting glucose levels, total cholesterol, low-density lipoprotein: Melamed et al. 1992, dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis: Griep et al. 1998), but most importantly, burnout has also been linked to poor sleep, lack of feeling refreshed in the morning, increased daytime sleepiness and fatigue (Grossi et al. 2003). In this view, the association of sleep and burnout was also shown in a cohort study with 240 physicians: They had to fill out the Shirom-Melamed-Burnout-Questionnaire (SMBQ) and the Pittsburgh Sleep Quality Index Questionnaire. Insomnia was more often diagnosed for individuals with high burnout scores (21.1%) than for individuals with low burnout scores (6.9%) (Grossi et al. 2005; Vela-Bueno et al. 2008). Higher burnout scores were also associated with greater sleepiness during the day and more awakenings during the night (Grossi et al. 2003). In addition, low sleep quality for people with high burnout scores could also be shown by polysomnographic sleep measures: High burnout scores were associated with increased number of arousals, less recovery and more diurnal sleepiness (Söderström et al. 2004). Additionally, if burnout is left untreated, it also has the effect of disturbing sleep, presumably by contributing to new sources of fatigue. This could predispose to less successful treatment course and increased chronicity (Armon et al. 2008). Moreover, Armon et al. mentioned that burnout predicted subsequent cases of insomnia (OR=1.93) and changes in levels of insomnia (β=0.05), and that insomnia predicted subsequent cases of burnout (OR=1.64) and changes in levels of burnout (β=0.07, P<0.05).

In clinical burnout, that is in an advanced stage of mental, emotional, and physical exhaustion, sleep was also found to be disturbed (Ekstedt et al. 2006; Mommersteeg 2004); most importantly, sleep was already disturbed in the early stages of the burnout process (Äkerstedt et al. 2002; Melamed et al. 1999; Söderström 2004).

In sum, there is empirical evidence that burnout and poor sleep are related; however, given that there is not a single burnout symptom (cf. Maslach and Jackson, 1981; Melamed et al. 2006), but rather a broad variety of different dimensions of emotion, cognition and behaviour, surprisingly, no study has addressed the issue so far, which of the burnout dimensions is more closely related to poor sleep. One of the aims of the present study was therefore to explore which burnout dimension may better predict sleep complaints.

By contrast, people with optimistic attitude seem to be less vulnerable to stress and burnout. In a cross-sectional study with 736 men and women, positive affect and purpose in life, sleep problems and psychosocial risk factors were assessed (Steptoe et al. 2008). There could be shown that people with optimistic attitude had a better sleep quality. Negative psychosocial factors and psychosocial distress were related to sleep problems. This association was less strong if positive affect and positive purpose in life were taken into account. Thus, an optimistic attitude seemed to be like a buffer for psychosocial risk factors, stress and sleep disorders (Schuitemaker et al. 2004).

For short, poor sleep quality, poor recovery, impaired performance at work, more strain at work, emotional and physical exhaustion and burnout seem to be interdependent. Each factor may amplify the others as in a vicious circle. On the other hand, a factor which seems to be protective is a positive attitude towards life. Therefore our study aimed at investigating the relation between different dimensions of burnout, satisfaction with life, and sleep complaints.

The following hypotheses were formulated. First, we assumed that dimensions of burnout and sleep were highly interrelated. Second, given the high overlap between poor sleep and depressive symptoms (cf. Buysse et al. 2008; Hatzinger et al. 2004), we expected that among different dimensions of burnout, depressive symptoms predicted best poor sleep. Third, we expected that satisfaction with was negatively related to sleep complaints.

Table I. Participants' descriptive and statistical overview related to demography, employment and social context.

			Group divided by gender		
		Total group	Female	Male	Statistical comparison
N		2231	1183	1048	Binomial distribution $P<0.005$
Age (years) M (SD)		40.77 (10.30)	39.56 (10.27)	42.14 (10.18)	$T(2229)=5.93$, $P<0.001$
Employment		2184	1163	1021	χ^2 (4)=165.76, $P<0.001$
	Big concern	704	295	409	
	Small- and medium-sized company	533	241	292	
	Self-employed	254	126	128	
	Education	226	141	85	
	Social employment and health care	467	360	107	
	No answer	47	20	27	
Position	Higher management	475	170	305	χ^2 (5)=123.59, $P<0.001$
	Lower management	441	213	228	
	Employed	836	527	309	
	Self-employed	172	76	96	
	Free-lancer	78	55	23	
	Others	182	122	60	
	No answer	47	20	27	
Social context	Single	524	308	216	χ^2 (4)=40.17, $P<0.001$
	Relationship without children	800	452	348	
	Relationship with children	788	350	438	
	Apartment-sharing community	78	52	26	
	Family of origin	41	21	20	

Method

Sample

A total of 2231 participants took part in the study (mean age in years: 40.77, SD=10.30; 1183 females (53%); 1048 males (47%)).[1] Table I gives the descriptive and statistical overview related to demography, employment, position, and social context.

Female participants were about 2.5 years younger, they were rather employed in education, social employment, and health care. Males were rather employed in big concerns and small- and medium-sized companies. Female participants were prevalently employed or free-lancers, whereas male participants' position was rather in the higher management or self-employed. Then, females were rather singles or lived in a relationship without children, or lived in an apartment-sharing community. Male participants were more likely to live in a relationship with children.

Procedure

The study was performed in German as an internet-based study.[2] Commercially available software (Globalpark®; www.globalpark.com) was used to run the study via internet. The software is an easily applicable tool for the creation of internet-based studies. The software provider guarantees that all data are stored on a server not accessible to who is running a study, that is: the user of the software receives the data related to the questionnaires, but not related to the IP-address of a participant. Thus, data security and anonymity of the participants were ensured. Moreover, to avoid repeated participation,

[1]A total of 2418 people did click on the first introductory page of the internet-based study. Of those, 2,375 proceeded to complete the questionnaire. Of those, 144 had missing values higher than 5% and following Schafer and Graham (2002), these participants were excluded from further analyses. Thus, from 2,418 potentially interested people, 2375 (98%) proceeded, and data were further analysed from 2,231 participants (93.94%).

[2]There is growing evidence that internet-based questionnaires do provide as reliable data as p-a-p version do (cf. Mangunkusumo et al. 2005; Vereecken and Maes, 2006; Wang et al. 2005).

the software blocked participation with an IP-address already used. From March 2007 to November 2007, the study was posted on the homepage of the Swiss Burnout Society (www.swissburnout.ch). Moreover, ads were electronically posted on so-called "electronic market places" of three concerns and variously sized companies and two supermarket chains in the German-speaking part of Switzerland.

Participants were informed about the purpose of the study and about the voluntary basis of the participation. They were also assured of the confidentiality of their responses, and informed consent was obtained on the first page of the questionnaire. Moreover, participants could stop or withdraw from the study without giving any further explication. To enhance compliance, participants could take part in a drawing, though in this case they had to provide an email-address. As a token, 10 iPod® Shuffles® were raffled. On average, 9 min (range: 4–14 min) were needed to answer to the questions.

Data were automatically gathered in an excel-file® and afterwards converted into an SPSS®-file for further analysis.

Instruments

Sleep complaints. To assess sleep complaints, the Insomnia Severity Index (Bastien et al. 2001) was applied. It consists of seven items. Typical items are: "In the last two weeks, how much did you suffer from the following disturbances: difficulty to fall asleep, difficulties to maintain sleep, early morning awakening, increased daytime sleepiness?" Answers were given on a five-point rating scale ranging from 0="not at all" to 4="very much". "How satisfied are you with your sleep?" Answers were given on a five-point scale ranging from 0="very satisfied" to 4="very dissatisfied" Or: "How much does sleep disturbance have a negative impact on your daily performance?" Answers were given on a five-point scale ranging from 0="not at all" to 4="very much". The higher the sum score, the more the person believes to suffer from insomnia (Cronbach's α=0.92).

Satisfaction with Life Scale. To assess satisfaction with life, the German translation of the Satisfaction with Life Scale (Diener et al. 1985) was applied. It consists of five items. Typical items are "I am satisfied with my life", or "The actual conditions to life are best". Answers were given on a seven-point scale ranging from 1="not at all" to 7="definitively true". The higher the sum score, the more the person believes to be happy and satisfied with her or his life.

Burnout. To assess burnout and its factorial components, the German translation of the Tedium Scale (Pines et al. 1988) was applied. It consists of 21 items, and typical items are: "I feel being emotionally exhausted", "I feel being worthless", or "I am feeling rejected". Answers were given on a seven-point scale ranging from 1=never to 7=always. The higher the sum score, the more a person subjectively experiences burnout. Whereas the internal validity of the Tedium Scale has been proven (Burisch 2006), the scale is criticized because of its low external validity and because it may not well distinguish between tedium, depression, self-worth or anxiousness. For this reason, a factor analytical approach was chosen, as described below.

Social context. To assess actual social condition, a self-administered questionnaire was applied. The entry question was: "What is your actual living condition? I am living…", followed by the items "… still at home with my parents", "… together with my family and children", "… together with my partner", "… in an apartment with another person", "… alone". Answers were yes or no.

Employment and position

To assess employment and positions, a self-administered questionnaire was applied. Participants had to indicate one of the items as displayed in Table I.

Statistical analyses

For correlations, Pearson's r was used. First, a correlation matrix was computed. It turned out that gender, age, employment, position, and social context were not related with any other psychological dimensions (r values <0.13). Thus, these dimensions were not introduced as further variables.

Second, the Tedium Scale covers several psychological dimensions (cf. Pines et al. 1988). Thus, composite variables were calculated. To this end, exploratory factor analyses (EFA) were performed (cf. Brown 2006). Factor analyses (always principal component analysis with Varimax factor rotation) of the 21 variables yielded 21 factors; the first three had Eigenvalues higher than 1, together accounting for 71.2% of the overall variance. The Eigenvalue of the first factor, labeled "Emotional and physical exhaustion", was 12.04; the Eigenvalue of the second factor, labeled "Pessimism and low mood", was 2.04; the Eigenvalue of the third factor, labeled "Depressive

symptoms", was 1.39.[3] All these calculations were performed using SPSS 15.0.

To enter all psychological dimensions and sleep simultaneously, confirmatory factor analyses (CFA) and structural equation modeling (SEM) were conducted using AMOS 6.0 (Arbuckle and Wothke 2005). Parameter estimation was conducted using maximum-likelihood (ML). As generally recommended, multiple goodness-of-fit indices were considered to examine how well the theoretical model fitted the empirical data (Hu and Bentler 1999; McDonald and Ho 2002): AGFI should be ≥ 0.95, PClose>0.50, CFI>0.90, RMR<0.08, and RMSEA≤ 0.05.

Results

Age, gender, employment, professional position, and social context related to the items related to Satisfaction with life, Insomnia, Emotional and physical exhaustion, Depressive symptoms, and Pessimism and low mood

Age did not correlate with Satisfaction with life, Insomnia, Emotional and physical exhaustion, Depressive symptoms, or Pessimism and low mood (all rs<0.19). No differences between females and males were observed (separate t-tests for the factor Gender: Insomnia: $t(2229)=1.62$, $P=0.11$, $d=0.03$; Satisfaction with life: $t(2229)=2.23$, $P=0.026$, $d=0.09$; Emotional and physical exhaustion: $t(2229)=1.70$, $P=0.09$, $d=0.02$; Depressive symptoms: $t(2220)=1.59$, $P=0.11$, $d=0.00$, Pessimism and low mood: $t(2229)=1.91$, $P=0.06$, $d=0.03$). No differences were observed for the factors Employment, Professional position, and Social context: Separate ANOVAs with the dependent variables Insomnia, Satisfaction with life, Emotional and physical exhaustion, Depressive symptoms, and Pessimism and low mood were performed: Employment: $F(5, 2225)=0.12$–2.23, $P>0.1$, $\eta^2<0.004$. Position: $F(6, 2224)=0.09$–1.26, $P>0.1$, $\eta^2<0.002$; Social context: $F(5, 2225)=0.10$–2.56, $P>0.1$, $\eta^2<0.003$[4]). Thus, age, gender, employment, professional position, and social context were not introduced as covariates.

Intercorrelations between the items related to Satisfaction with life, Insomnia, Emotional and physical exhaustion, Depressive symptoms, and Pessimism and low mood

Tables IIa and b provide the correlation matrix of all items extracted from the questionnaires. The general pattern of results suggested high correlations between the items of the applied questionnaires, specifically, positive correlations between the dimensions related to burnout (that is: Emotional and physical exhaustion; Pessimism and low mood; Depressive symptoms) and poor sleep, and negative correlations between satisfaction with life and poor sleep. To disentangle possible underlying associations, a structural equation model (SEM) was applied.

Structural equation model to predict sleep complaints as a function of psychological functioning

Figure 1 shows the structural equation model (SEM). Compared to the criteria established by Hu and Bentler (1999), and McDonald and Ho (2002) (criteria indicated in [squared brackets]) the model presented an excellent fit: $\chi^2/df=3.61$, AGFI$=0.981$ [>0.95], PClose$=0.99$ [≥ 0.95], CFI$=0.965$ [>0.90], RMR$=0.035$ [<0.08], and RMSEA$=0.032$ [≤ 0.05].

The model revealed that the dimension Emotional and physical exhaustion was the strongest variable to predict Sleep complaints, whereas the dimensions Depressive symptoms and Pessimism and low mood were of poor predictive value. The dimension Emotional and physical exhaustion, Pessimism and low mood, and Depressive symptoms were highly interrelated. The dimension Satisfaction with life was negatively related to the dimensions Emotional and physical exhaustion, Pessimism and low mood, and Depressive symptoms. Moreover, an increased Satisfaction with life and Sleep complaints were directly and indirectly related, that is to say: Satisfaction with life predicted decreased sleep complaints directly ($\beta=-0.14$, $P<0.001$), and indirectly via the dimension Emotional and physical exhaustion.

However, as Tables IIa and b and Figure 1 show, the dimensions related to burnout (that is: Emotional and physical exhaustion, Pessimism and low mood, and Depressive symptoms) were interrelated, and therefore, the pattern of results as depicted in Figure 1 might potentially reflect spurious results. Therefore, to substantiate the present pattern of results, two further SEMs were performed. In the

[3]In Table II only those 15 items were reported without high loadings on more than two factors (i.e. cross-loadings) and without small loadings on all factors (i.e. low communalities; cf. Brown 2006).

[4]Effect sizes (d) for t-tests were performed following Cohen (1988, 1994), with $0.49\geq d\geq 0.20$ indicating small (i.e. negligible practical importance), $0.79\geq d\geq 0.50$ indicating medium (i.e. moderate practical importance), and $d\geq 0.80$ indicating large (i.e. crucial practical importance) effect sizes. Effect sizes for ANOVAs (partial eta squared [η^2]) were performed following Cohen (1988, 1994), with $0.059\geq\eta^2\geq 0.01$ indicating small (i.e. negligible practical importance), $0.139\geq\eta^2\geq 0.06$ indicating medium (i.e. moderate practical importance), and ($\eta^2\geq 0.14$ indicating large (i.e. crucial practical importance) effect sizes. The main pattern of results is that effect sizes were very small.

Table IIa. Intercorrelations among primary variables, Part 1 (N=2231).

	Satisfaction with life						Insomnia Severity Scale						
	1	2	3	4	5	6	7	8	9	10	11	12	13
Satisfaction with life													
1. Ideal	–												
2. Excellent conditions	0.68***	–											
3. Satisfaction	0.71***	0.71***	–										
4. Realization	0.58***	0.52***	0.57***	–									
5. Same life again	0.46***	0.40***	0.42***	0.46***	–								
Insomnia Severity Scale													
6. Diff falling asleep	0.22***	0.24***	0.28***	0.21***	0.19***	–							
7. Diff maintaining sleep	0.21***	0.23***	0.28***	0.19***	0.17***	0.38***	–						
8. Early awakening	0.14***	0.17***	0.22***	0.11***	0.11***	0.23***	0.55***	–					
9. Sleepiness	0.34***	0.34***	0.22***	0.28***	0.20***	0.34***	0.38***	0.28***	–				
10. Satisfaction with sleep	0.23***	0.24***	0.39***	0.19***	0.15***	0.45***	0.57***	0.44***	0.41***	–			
11. Impact on performance	0.33***	0.33***	0.29***	0.28***	0.22***	0.47***	0.48***	0.37***	0.60***	0.54***	–		
12. Other's judgement	0.21***	0.22***	0.37***	0.21***	0.15***	0.32***	0.31***	0.24***	0.42***	0.35***	0.56***	–	
13. Worrying about sleep	0.27***	0.29***	0.35***	0.22***	0.17***	0.50***	0.57***	0.47***	0.46***	0.62***	0.68***	0.52***	
Tedium Scale													
Emotional and physical exhaustion													
14. Phys exhaustion	0.32***	0.31***	0.38***	0.22***	0.20***	0.31***	0.36***	0.25***	0.53***	0.38***	0.50***	0.32***	0.42***
15. Tired	0.32***	0.30***	0.36***	0.23***	0.21***	0.31***	0.34***	0.22***	0.56***	0.40***	0.51***	0.34***	0.41***
16. Dead beat	0.39***	0.37***	0.46***	0.28***	0.25***	0.36***	0.36***	0.27***	0.52***	0.39***	0.48***	0.33***	0.44***
17. Burnout	0.43***	0.40***	0.50***	0.30***	0.26***	0.39***	0.42***	0.33***	0.52***	0.45***	0.52***	0.35***	0.51***
18. Worn out	0.37***	0.36***	0.44***	0.25***	0.23***	0.32***	0.40***	0.30***	0.47***	0.40***	0.47***	0.31***	0.46***
19. Emot exhausted	0.40***	0.39***	0.48***	0.29***	0.25***	0.33***	0.38***	0.28***	0.47***	0.40***	0.45***	0.31***	0.43***
Depressive symptoms													
20. Rejection	0.43***	0.42***	0.48***	0.34***	0.29***	0.28***	0.27***	0.21***	0.34***	0.25***	0.33***	0.25***	0.32***
21. Worthless	0.48***	0.43***	0.56***	0.38***	0.32***	0.31***	0.30***	0.25***	0.38***	0.30***	0.36***	0.28***	0.36***
22. Tedious	0.46***	0.43***	0.54***	0.35***	0.30***	0.32***	0.33***	0.28***	0.42***	0.33***	0.42***	0.29***	0.40***
23. Hopeless	0.51***	0.48***	0.60***	0.39***	0.33***	0.33***	0.36***	0.28***	0.42***	0.34***	0.43***	0.31***	0.43***
24. Worried	0.46***	0.42***	0.52***	0.34***	0.31***	0.34***	0.33***	0.26***	0.41***	0.32***	0.42***	0.30***	0.41***
Pessimism and low mood													
25. Pessimistic	0.47***	0.44***	0.54***	0.41***	0.34***	0.24***	0.27***	0.19***	0.31***	0.23***	0.34***	0.24***	0.32***
26. Powerless	0.44***	0.42***	0.50***	0.39***	0.30***	0.25***	0.28***	0.20***	0.38***	0.26***	0.39***	0.26***	0.35***
27. Sad	0.57***	0.56***	0.66***	0.44***	0.35***	0.25***	0.24***	0.18***	0.33***	0.24***	0.34***	0.23***	0.32***
28. Starting a bad day	0.49***	0.45***	0.56***	0.38***	0.30***	0.28***	0.27***	0.19***	0.37***	0.27***	0.38***	0.25***	0.36***

Note. Diff = difficulties; Phys = physical. The items Optimistic, Vigorous, Happy, and Starting a good day were inverted to Pessimistic, Powerless, Sad, and Starting a bad day. $^{*}P<0.05$. $^{**}P<0.01$. $^{***}P<0.001$. Only those 15 items were reported without high loadings on more than two factors (i.e., cross-loadings) and without small loadings on all factors (i.e., low communalities; cf. Brown 2006).

Table IIb. Intercorrelations among primary variables, Part 2 (N=2231).

	Tedium Scale													
	Emotional and physical exhaustion						Depressive symptoms					Pessimism and low mood		
	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Tedium Scale														
Emotional and physical exhaustion														
14. Phys exhaustion	–													
15. Tired	0.69***	–												
16. Dead beat	0.70***	0.65***	–											
17. Burnout	0.68***	0.63***	0.81***	–										
18. Worn out	0.67***	0.61***	0.71***	0.78***	–									
19. Emot exhausted	0.70***	0.62***	0.73***	0.75***	0.69***	–								
Depressive symptoms														
20. Rejection	0.38***	0.36***	0.47***	0.50***	0.45***	0.49***	–							
21. Worthless	0.41***	0.40***	0.52***	0.54***	0.49***	0.51***	0.66***	–						
22. Tedious	0.48***	0.45***	0.59***	0.64***	0.58***	0.59***	0.59***	0.68***	–					
23. Hopeless	0.49***	0.45***	0.61***	0.65***	0.56***	0.61***	0.68***	0.70***	0.70***	–				
24. Worried	0.49***	0.46***	0.58***	0.63***	0.57***	0.60***	0.57***	0.62***	0.72***	0.68***	–			
Pessimism and low mood														
25. Pessimistic	0.32***	0.32***	0.39***	0.41***	0.35***	0.41***	0.41***	0.45***	0.46***	0.54***	0.43***			
26. Powerless	0.40***	0.38***	0.42***	0.45***	0.40***	0.44***	0.35**	0.41***	0.44***	0.48***	0.41***	0.70***	–	
27. Sad	0.33***	0.31***	0.42***	0.45***	0.40***	0.45***	0.44***	0.47***	0.49***	0.55***	0.48***	0.59***	0.55***	–
28. Starting a bad day	0.38***	0.35***	0.45***	0.46***	0.41***	0.44***	0.40***	0.44***	0.46***	0.51***	0.46***	0.54***	0.55***	0.66***

Note. Diff = difficulties; phys = physical. The items Optimistic, Vigorous, Happy, and Starting a good day were inverted to Pessimistic, Powerless, Sad, and Starting a bad day. *P<0.05. **P<0.01. ***P<0.001. Only those 15 items were reported without high loadings on more than two factors (i.e., cross-loadings) and without small loadings on all factors (i.e., low communalities; cf. Brown 2006).

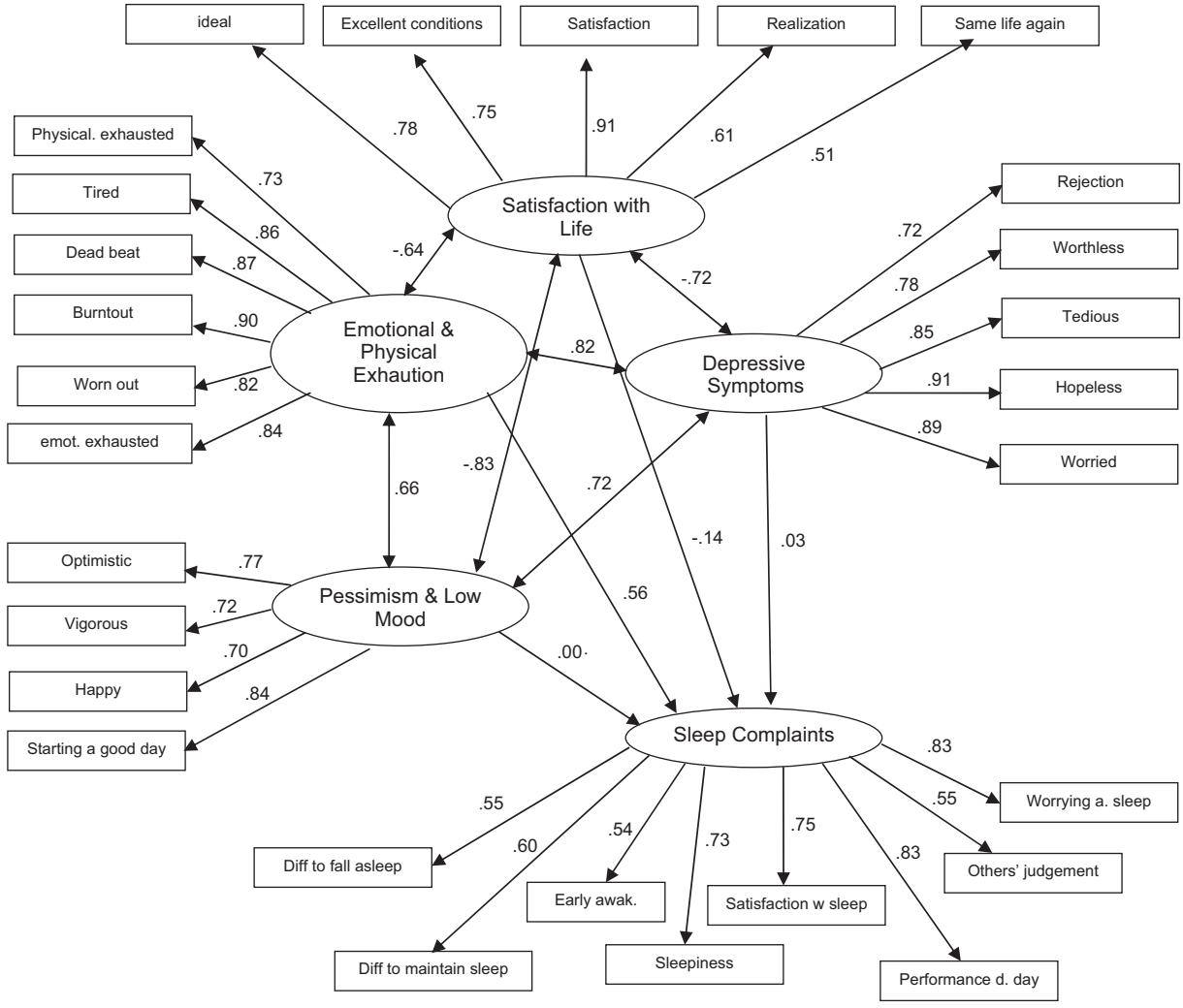

Figure 1. Full structural equation model (SEM) to show the relations between Satisfaction with life, Emotional and physical exhaustion, Pessimism and low mood, Depressive symptoms, and Sleep complaints. Arrows pointing in two directions indicate intercorrelations; arrows in one direction indicate that independent variables predict the dependent variable.

first alternative SEM, the pathway from Emotional and physical exhaustion to Sleep complaints was set to 0; on doing so, we tested whether the dimension Emotional and physical exhaustion did suppress possible favourable pathways from Pessimism and low mood and Depressive symptoms to Sleep complaints. Compared to the first model, the first alternative SEM fit decreased (χ^2/df=5.75, AGFI=0.92 [>0.95], PClose=0.949 [≥0.95], CFI=0.862 [>0.90], RMR=0.11 [<0.08], and RMSEA=0.052 [≤0.05]); the β-weight did not vary for Pessimism and low mood to Sleep complaints (β=0.00 did not alter); the β-weight did increase for Depressive symptoms to Sleep complaints: from β=0.03 to β=0.25, P<0.01. In the second alternative SEM, the pathways from Pessimism and low mood and Depressive symptoms to Sleep complaints were set to 0; on doing so, we tested whether the dimensions Pessimism and low mood and Depressive symptoms did confound the pathway from Emotional and physical exhaustion to Sleep complaints. The model fit increased (χ^2/df=2.75, AGFI=0.982 [>0.95], PClose=0.99 [≥0.95], CFI=0.971 [>0.90], RMR=0.028 [<0.08], and RMSEA=0.029 [≤0.05]) and the β-weight between Emotional and physical exhaustion and Sleep complaints further increased (from β=0.56 to β=0.69, P<0.001). Thus, the results from the two further SEMs could further substantiate that rather Emotional and physical exhaustion and not Pessimism and low mood or Depressive symptoms was related to Sleep complaints.

Discussion

The key findings of the present study are that among a sample of working adults, sleep complaints were

not related to depressive symptoms, but sleep complaints were positively related to emotional and physical exhaustion and negatively related to satisfaction with life. Moreover, neither age, gender, position, employment, nor social context were related to sleep complaints or any other psychological dimension.

Three hypotheses were formulated and each of these is now considered in turn. With the first hypothesis we assumed that dimensions of burnout and sleep were highly interrelated, and data do support this assumption (see Tables IIa and b). Insofar, our data do fit well within the wealth of studies which emphasized that burnout was related to poor sleep (Äckerstedt et al. 2002; Armon et al. 2008; Grossi et al. 2003, 2005; Ekstedt et al. 2006; Melamed et al. 1999; Mommersteeg et al. 2004; Söderström et al. 2004; Vela-Bueno et al. 2008). Moreover, in a larger context, the present data do also fit within those studies showing the association between unfavourable working conditions and poor sleep (cf. Bohle et al. 2004; Caruso 2006; Folkard et al. 2005; Godet-Cayre et al. 2006; Jansson and Linton et al. 2006; Leger et al. 2006; Metlaine et al. 2005; Nishikitani et al. 2005; Utsugi et al. 2005).

However, given the heterogeneity of the term burnout (cf. Burisch 2006; Maslach and Jackson 1981; Melamed et al. 2006; WHO 1991), it remained unclear so far, if burnout per se, or subdimensions of it were related to poor sleep. To answer this question, we assumed with the second hypothesis that among different dimensions of burnout, depressive symptoms predicted best poor sleep. This assumption was based on research in the area of sleep and psychiatry, where a high overlap between poor sleep and depressive symptoms are commonly observed. However, against, prediction, the present results suggest that among the variety of dimensions related to burnout, Emotional and physical exhaustion, but not Depressive symptoms, nor Pessimism and low mood, do predict sleep complaints. Thus, the present pattern of result is at odds with those data suggesting a close relationship between depressive symptoms and poor sleep (cf. Buysse et al. 2008; Hatzinger et al. 2004). Rather, the present results are in line with one previous study (Sonnenschein et al. 2007) which underlined that in burnout, impaired recovery from sleep was related to the severity of exhaustion, but not to the severity of depressive mood. As a consequence, future studies may take into account that assessment and treatment of burnout should rather focus on emotional and physical exhaustion, and not on depressive symptoms.

Most importantly, no relation could be found between the dimensions related to burnout and sleep and demographic variables (age; gender; social context) and dimensions related to work (employment; position), suggesting that dimensions related to burnout and sleep do not depend on social and work-related context. These findings are in line with a previous study that evidenced that burnout can evolve in all kinds of employees and in all kinds of vocational groups (Ahola et al. 2006). On the other hand, the present findings are at odds with previous studies (e.g., Thomas 2004) which emphasized increased risks to suffer from burnout among teachers and physicians. The reason why in contrast to other studies no differences in burnout related to the profession could be found remains unclear. However, one might assume that previous studies were mainly conducted within and not between different professions. Moreover, it is also possible that the studies conducted so far assessed employees within the same company (e.g., Jansson and Linton 2006; Utsugi et al. 2005), whereas the present study was not related to such constraints.

With the third hypothesis we expected that satisfaction with life was negatively related to sleep complaints, and data suggest that an increased satisfaction seems to protect directly and indirectly from sleep complaints, thus, confirming previous studies (Schuitemaker et al. 2004; Steptoe et al. 2008). Moreover, the data fit well with the wealth of studies which underlined the association between well-being, happiness, and sleep (e.g., Brand et al. 2007), or, in other terms, which underlined the association between psychological stress and poor sleep (cf. Nil et al. 2009), an association treated best with psychological and behavioural therapies (Morin et al. 2006).

The implications of the present results need to be balanced against the following study limitations. First, the term burnout lacks of a homogeneous definition (cf. Maslach and Jackson 1981; Melamed et al. 2006; WHO 1992); however, we applied the Tedium Scale which assumes that burnout is a state of physical, emotional, and mental exhaustion (cf. Arthur 1990). Based on this inventory, we deduced the factorial dimensions of Emotional and physical exhaustion, Pessimism and low mood, and Depressive symptoms, though the external validity of these factors remains to be tested. Second, the present study is a cross-sectional design, which does not allow conclusive judgments about cause and effect. Even if applying SEM, different pathways and interpretations of cause and effect are possible. To illustrate, there is evidence that poor sleep leads to low work performance (Godet-Cayre 2006; Metlaine et al. 2005; Nishikitani et al. 2005) and that by contrast, low working conditions also lead to poor sleep (Jansson and Linton 2006; Utsugi et al. 2005). Third, sources of work-related stress were not assessed. As evidenced, the combination of high effort-low reward and high demand-low control seemed to lead to

insomnia in the long term (cf. Ota et al. 2009). Fourth, the applied dimensions Emotional and physical exhaustion, Pessimism and low mood, and Depressive symptoms were all dimensions labelled and extracted from a factor analytical approach; therefore, comparing for instance the factor Depressive symptoms with results carried out from other research with validated depression-related self-report questionnaires needs caution. Finally, only adults willing and able to participate in the study and to complete the questionnaires were included, and consequently the possibility of systematic sample-related biases cannot be excluded.

Conclusion

Results suggest that among burnout symptoms emotional and physical exhaustion, but not depressive symptoms, are strongly related to sleep complaints. Moreover, satisfaction with life, via low emotional and physical exhaustion and low pessimism, further contributes to favourable sleep.

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Statement of interest

All authors declare no conflict of interest.

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BRIEF REPORT

Positive effects of repetitive transcranial magnetic stimulation on attention in ADHD Subjects: A randomized controlled pilot study

Y. BLOCH*, E.V. HAREL*, S. AVIRAM, J. GOVEZENSKY, G. RATZONI & Y. LEVKOVITZ

Laboratory for Cognitive and Affective Research, Shalvata Mental Health Center, Hod-Hasharon, Israel (affiliated to the Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel)

Abstract

Objectives. Repetitive transcranial stimulation (rTMS) affects dopaminergic secretion in the prefrontal cortex. Attention deficit hyperactivity disorder (ADHD) had been suggested to involve dopaminergic prefrontal abnormalities. *Methods.* In this crossover double-blind randomized, sham-controlled pilot study, patients diagnosed as having adult ADHD received either a single session of high-frequency rTMS directed to the right prefrontal cortex (real rTMS) or a single session of sham rTMS. *Results.* A total of 13 patients (seven males, six females) who fulfilled the criteria for adult ADHD, according to DSM-IV criteria gave informed consent and were enrolled. There was a specific beneficial effect on attention 10 minutes after a real rTMS course. The post-real rTMS attention score improved significantly (M=3.56, SD=0.39) compared to the pre-real rTMS attention score (M=3.31, SD=0.5) [$t(12)=2.235$, $P < 0.05$]. TMS had no effect on measures of mood and anxiety. The sham rTMS had no effect whatsoever. *Conclusions.* Our findings should encourage future research on the possibility of amelioration of attention difficulties in patients suffering from ADHD by using high frequency rTMS directed to the right dorsolateral prefrontal cortex. (NIH registry NCT00825708)

Key words: *Neuroimaging, rTMS, ADHD, attention, right dorsolateral prefrontal cortex*

Introduction

Attention deficit hyperactivity disorder (ADHD) is a highly prevalent condition that impacts the affected individual throughout life (Acosta 2000; Castellanos and Acosta 2002; Arnsten 2006). Neuroanatomic and neuroimaging studies in patients with ADHD point to fronto-striatal circuit abnormalities, mainly in the right hemisphere (Castellanos and Acosta 2002; Arnsten 2006). Stimulants of the nervous system through mediation of the dopamine system comprise evidence-based therapy for ADHD (Mészáros et al. 2009). Stimulants, however, have multiple side effects that limit usage and adherence in many cases (Kociancic et al. 2004). Transcranial magnetic stimulation (TMS) is a non-invasive tool that had been developed for studying the nervous system and showed promising findings of having the capability of favorably affecting neural plasticity (Acosta et al. 2002; Hallett 2001; Siebner and Rothwell 2003; Strafella et al. 2001). Recent studies have shown that repetitive TMS (rTMS) can produce effects on the dopaminergic system in healthy subjects similar to the effect of D-amphetamine (Strafella et al. 2001; Pogarell et al. 2007). TMS has also been found useful in increasing the understanding of ADHD pathophysiology (Ucles et al. 2000; Moll et al. 2000). The published literature contains only one single case report that showed a beneficial effect of 1 Hz rTMS on attention in ADHD (Niederhofer 2008).

The aim of the present pilot study was to examine a possible amelioration in ADHD symptoms by stimulating the right prefrontal cortex with a course of rTMS.

Methods

This study was approved by the local IRB and registered in the NIH (NIH registry NCT00825708). Subjects were recruited by advertisements in Tel Aviv University and Shalvata Mental Health Center. Screening included a thorough clinical interview by a psychiatrist experienced in adult ADHD diagnosis

Correspondence: Yuval Bloch, MD, Shalvata Mental Health Center, P.O.B. 94, Hod-Hasharon, Israel. Tel: +972 9 7478510. Fax: +972 3 5496872. E-mail: aviva100@bezeqint.net; yuvalbl@clalit.org.il
*Contributed equally to this work.

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assisted by the Adult ADHD Self Report Scale (ASRS) and the Wender-Utah adult ADHD scale (WUAAS).

The study methodology had a crossover double blind randomized design. It consisted of two visits (Visit 1 and Visit 2) that took place one week apart. Patients were randomized to either a single real rTMS session or a single sham rTMS session at Visit 1 and they were crossed over at Visit 2. Evaluations were conducted at the beginning of each day and 10 minutes after the administration of the real/sham rTMS. The physician giving the treatment was responsible for randomization based on pre-set numbers. He was in contact with the subject only during the treatment itself.

The evaluations included:

1. The Positive and Negative Affect Schedule (PANAS) questionnaire for assessing the subjective experience at a given time (Watson et al. 1988). This is a 20-item self-report measure with subjects rating the extent to which they feel a particular emotion on a five-point scale (1 = "not at all" to 5 = "strongly"). We divided the PANAS questionnaire into four subgroups with three measures in each group (validated by Cronbach's α) as follows: the *attention* score included concentration, detachment and attention (0.734), the *hyperactivity* score included nervousness, impulsiveness and irritability (0.763), the *anxiety* score included feeling worried and frightened (0.798), and the *mood* score included feeling happy, sad and enthusiastic (0.705). We averaged the attention and hyperactivity scores in order to establish an overall "ADHD score". The reliability test of the six items (mentioned above) reached a Cronbach α of 0.788 (i.e. internal consistency). The attention, mood and "ADHD score" measures were calculated so that higher scores represented better condition, while the hyperactivity and anxiety measures were calculated so that higher scores represented worst condition. Findings from the PANAS were defined as primary outcome measures.
2. Visual analogue scales (VASs) for attention and mood. The current attention and mood states were self-reported on a scale of 1–10 (Wewers and Lowe 1990).
3. Neuropsychological battery of tests using the Cambridge Neuropsychological Test Automated Battery CANTAB testing system (Morris et al. 1987), defined as secondary outcome measures.

A Magstim super rapid stimulator and a figure 8 coil with an internal loop diameter of 7 cm were used to deliver the rTMS. The session at each of the two visits included 42 2-s, 20-Hz stimuli at a 100% motor threshold intensity, with a 30-s inter-stimulus interval. The motor threshold was measured according to the common practice of using the visible movement of the left abductor pollicis brevis muscle. The stimulation site was the right dorsolateral prefrontal cortex located by measuring 5 cm anterior to the motor threshold. The sham condition was administered using the same stimulation parameters with one wing of the figure 8 coil in contact with the scalp and at a 45° angle with respect to the head.

Data analysis

Descriptive statistics were carried out to show the distribution of demographics and clinical variables. A paired *t*-test was used for control testing of differences between the two pre-rTMS evaluations during the two visits (baseline/control), and an independent *t*-test was used for testing differences in the pre-post delta of the TMS (real/sham) sessions between the two order groups (order effect control). Analysis of repeated measures with two within-subject variables was suitable for the crossover design of the study (1, real/sham rTMS; 2, pre/post rTMS).

Results

A total of 24 subjects were screened between May 2007 and March 2009. Five subjects were excluded for not fulfilling the ADHD criteria and another five subjects were excluded due to co-morbidity (depression = 2, post-traumatic distress syndrome = 1 and substance abuse = 2). One subject withdrew consent after Visit 1 because he perceived TMS as being painful, leaving a total of 13 consenting subjects (seven males, six females) who fulfilled the criteria for adult ADHD according to DSM-IV criteria and who were entered into the study. None of these 13 patients took any stimulant agents during the study period. Five of them had been taking methylphenidate in the last year, four on a regular daily basis. Another two had taken methylphenidate in childhood. Six of the study patients had never taken stimulants.

Real rTMS was found to improve attention as evaluated by the PANAS attention score. There were significant interactions (real/sham rTMS X pre/post rTMS) [$F(1,12) = 6.516$, $P < 0.05$]. Further analysis of the interactions revealed a significantly higher attention score post-real rTMS

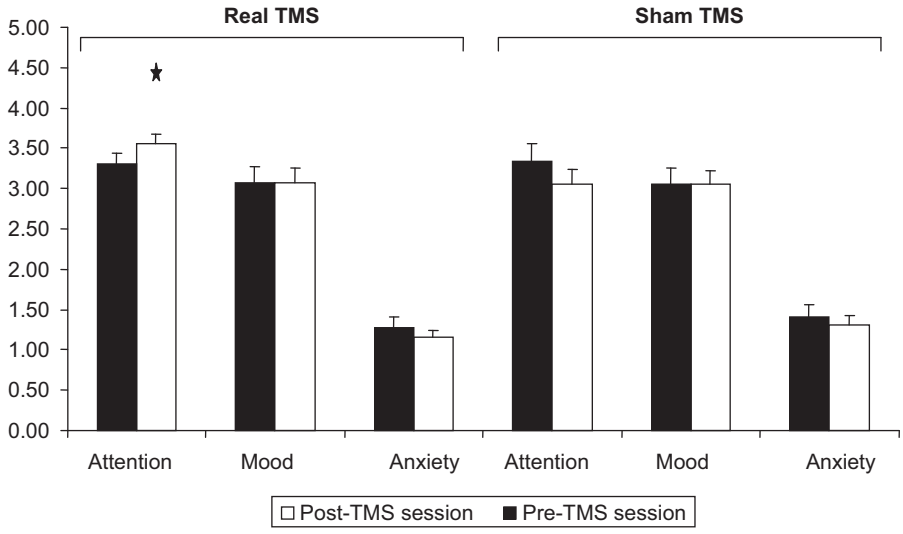

Figure 1. Improvement in attention but not in mood or anxiety after real transcranial magnetic stimulation (TMS) according to PANAS.

(M=3.56, SD=0.39) compared to pre-real rTMS (M=3.31, SD=0.5), [$t(12)=2.235$, $P < 0.05$]. No difference was found for the attention score when the pre- and post-sham rTMS results were compared. Similarly, there was no difference in the effect on mood, anxiety or hyperactivity PANAS scores between post-real/sham rTMS compared to pre-real/sham rTMS (Figure 1).

When the attention and hyperactivity scores were combined to comprise the overall "ADHD score", the interaction reached a level of significance (real/sham rTMS X pre/post rTMS), $F(1,12)=6.857$, $P < 0.05$. The "ADHD score" improved significantly following real rTMS [(M=3.96, SD=0.36) compared to pre-real rTMS (M=3.58, SD=0.46), $t(12)=3.746$, $P < 0.01$]. Sham rTMS had no effect whatsoever on the "ADHD score".

Similar findings were found on the VAS scores for attention. There was a significant interaction between real/sham rTMS and pre/post rTMS, $F(1,12)=7.57$, $P < 0.05$. The VAS score for attention improved only after real rTMS and not after sham rTMS [post rTMS session (M=7.61, SD=1.38) compared to pre-rTMS session (6.42, SD=1.85), $t(12)=2.934$, $P < 0.05$]. No such interaction or any effect was found for the VAS score for mood, indicating no change in mood following either real or sham rTMS.

There was no difference on the WUAAS and the ASRS between the subjects who had been randomized to receive real or sham rTMS on Visit 1. There was, however, a significant difference in the baseline PANAS hyperactivity score between the subjects who received real or sham rTMS on Visit 1 [$t(11)=5.66$, $P < 0.01$; $t(11)=-3.61$, $P < 0.01$, respectively], with a higher score in hyperactivity for the group that received real rTMS at Visit 1 and a lower "ADHD score" for the group that received sham rTMS at Visit 1.

Further analysis revealed that there was no difference in the pre-rTMS clinical evaluation on both visits between the randomized subjects. The cognitive neuropsychological test results (CANTAB) showed no specific profile in this group of subjects, with standard deviations at baseline that did not allow any further analysis of the effects of rTMS on these measures.

Discussion

This pilot study sought to discern whether there is a possible effect of rTMS in subjects diagnosed as having adult ADHD. The findings revealed a positive effect, albeit a modest one with questionable clinical relevance (mean change of 0.25 on a scale of 1–5), in measures of attention (as evaluated by the PANAS questionnaire and the VAS for attention) following a single session of real rTMS, using a high-frequency stimulation protocol to the right prefrontal cortex. Mood and anxiety (as measured by the PANAS mood and anxiety scores and the VAS for mood) were not affected by either sham or real rTMS, further supporting the effect of our rTMS protocol being specific to attention.

ADHD is defined as a clinical entity that is diagnosed and evaluated by means of questionnaires and clinical assessments. Results of cognitive tests on ADHD patients are heterogeneous (Willcutt et al. 2005), and so it is not surprising that cognitive functions, as assessed by a computerized battery, were too variable for systematic analysis in our small sample. We chose the dorsolateral prefrontal cortex as

the stimulation site based on previous findings that described its having a major role in the pathophysiology of ADHD (Mészáros et al. 2009; Castellanos et al. 1996). There is substantial evidence from both animal and human imaging studies that rTMS has an effect on the modulation of neurotransmitters, specifically dopamine and its metabolites (e.g., homovanillic acid), mainly after prefrontal cortex stimulation (Pogarell et al. 2007; Ucles et al. 2000; Shimamoto et al. 2001). Thus, prefrontal dopaminergic stimulation is a reasonable physiological explanation for our findings.

The effect exclusive to attention and not on mood or anxiety caused by stimulating the right prefrontal cortex also adds credence to our hypothesis. We recommend the conducting of studies on larger populations to evaluate the effects of stimulation in this area, and then to compare them to the effects of stimulation in other brain regions (i.e. the left prefrontal cortex). We consider this study as being a preliminary step towards the evaluation of rTMS as a possible tool in the treatment of ADHD.

The limitations of our study are that it includes a small group of patients, is based on a subjective report and that it has a crossover design. Another limitation is that the difference in the somatosensory experience of real rTMS and sham limits the true blinding.

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Statement of interest

No author has any biomedical function interests or potential conflicts of interest.

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LETTER TO THE EDITOR

Hyperprolactinaemia and acute psychosis: Prolactinoma or medication-induced phenomenon?

PETER HÖFER[1], FABIAN FRIEDRICH[1], BENJAMIN VYSSOKI[1], CHRISTOPH SPINDELEGGER[1], MARIA SCHMOOK[2], CHRISTIAN BARNAS[1], OTTO M. LESCH[1] & HENRIETTE WALTER[1]

[1]*Department of Psychiatry and Psychotherapy, Medical University of Vienna, Vienna, Austria, and* [2]*Department of Radiology, Medical University of Vienna, Vienna, Austria*

Dear Editors,

About 10% of the normal adult population have pituitary abnormalities on MRI scans that are compatible with the diagnosis of asymptomatic pituitary adenomas (Hall et al. 1994). In contrary, the prevalence of hyperprolactinaemia (0.4%) in the general population is low (Hummer et al. 2004). Hyperprolactinaemia is a common side-effect of first-generation anti-psychotics (FGAP) used in the treatment of schizophrenia; moreover, even newer second-generation anti-psychotics (SGAP) tend to elevate prolactin (PRL) plasma levels, especially amisulpride (prevalence rate 89%) (Bushe et al. 2008a). Both first and second generation anti-psychotics drugs achieve their anti-psychotic effect by blocking the D2- and D3-receptors in the limbic region (Mendhekar et al. 2008). Typical hyperprolactinaemia associated side-effects include galactorrhea, sexual dysfunction, decreased bone mineral density and behavioural effects (Hamner et al. 2002; Hummer et al. 2004). However, it remains unclear if amisulpride can cause pituitary adenomas and if this process is reversible after switching medication (Perroud et al. 2004; Szarfman et al. 2006; Akkaya et al. 2008; Bushe et al. 2008a,b). In this report the authors present a case of prolactinoma in a patient treated with amisulpride.

A 52-year-old married woman, with galactorrhea and amenorrhea, was admitted to our psychiatric ward for clarification and an operative cure of a pituitary adenoma which had been detected in a prior magnetic resonance imaging (MRI) performed 2 months earlier. At the time of admission, she suffered from acoustic and optic hallucinations (with delusional interpretations) and showed symptoms of anxiety and inner restlessness; furthermore, signs of depression and reduced drive were observed. The CGI-score (Clinical Global Impression) was 5.

MRI scans (using a 1.5-Tesla machine) showed a moderately enlarged adenohypophysis (Figure 1a) and a left-sided circumscribed hypointensity sized 7 mm (Figure 2a); a microadenoma was suspected as well.

Anamnestically, the patient had been treated with amisulpride for over a year prior to admission to our department (daily dosage of 400 mg for the last 10 months).

The medication was switched to olanzapine, initial dose 10 mg, and a significant decrease of PRL (from 185 to 70 ng/ml; a PEG-precipitation was performed to rule out the possibility of measuring macroprolactin) was observed over the next 3 weeks. In addition, a thyrotropin releasing hormone (TRH)-test was performed in order to differentiate between the diagnosis of an autonomous microprolactinoma and a reactive secondary hyperprolactinaemia through amisulpride. The TRH-test provided the first hint of a regulatory disturbance of hormonal circulation. Other causes for hyperprolactinaemia like, e.g., renal failure, hypothyroidism or acromegaly were excluded.

Shortly before discharge (after 23 days) and 3 months after the initial scan, follow-up imaging of the pituitary gland was performed on a 3-Tesla MRI-machine. The moderate enlargement and superiorly convex margin of the adenohypophysis was unchanged, compatible with pituitary hyperplasia (Figures 1b and 2b). Even in direct comparison

Correspondence: Peter Höfer, MD, Medical University of Vienna Department of Psychiatry and Psychotherapy, Währinger Gürtel 18-20; A-1090 Vienna, Austria. Tel: +43 40400 3526; Fax: +43 40400 3472. E-mail: peter.hoefer@meduniwien.ac.at

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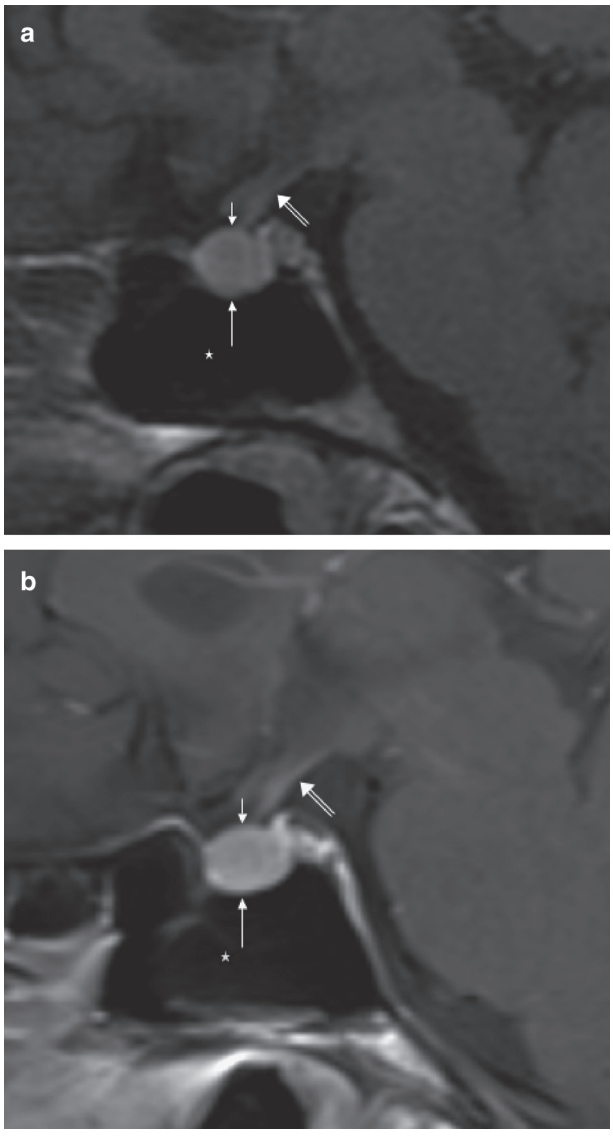

Figure 1. MRI of the sellar region. Sagittal contrast enhanced T1 weighted image at the intitial scan (a) and at the 3-month follow-up (b). Note the diffuse and strong contrast enhancement of the enlarged pituitary gland. The short arrow points at the superiorly convex margin of the adenohypophyis, the long arrow at the sellar floor, the open arrow at the pituitary stalk. The asterix marks the air filled sphenoid sinus.

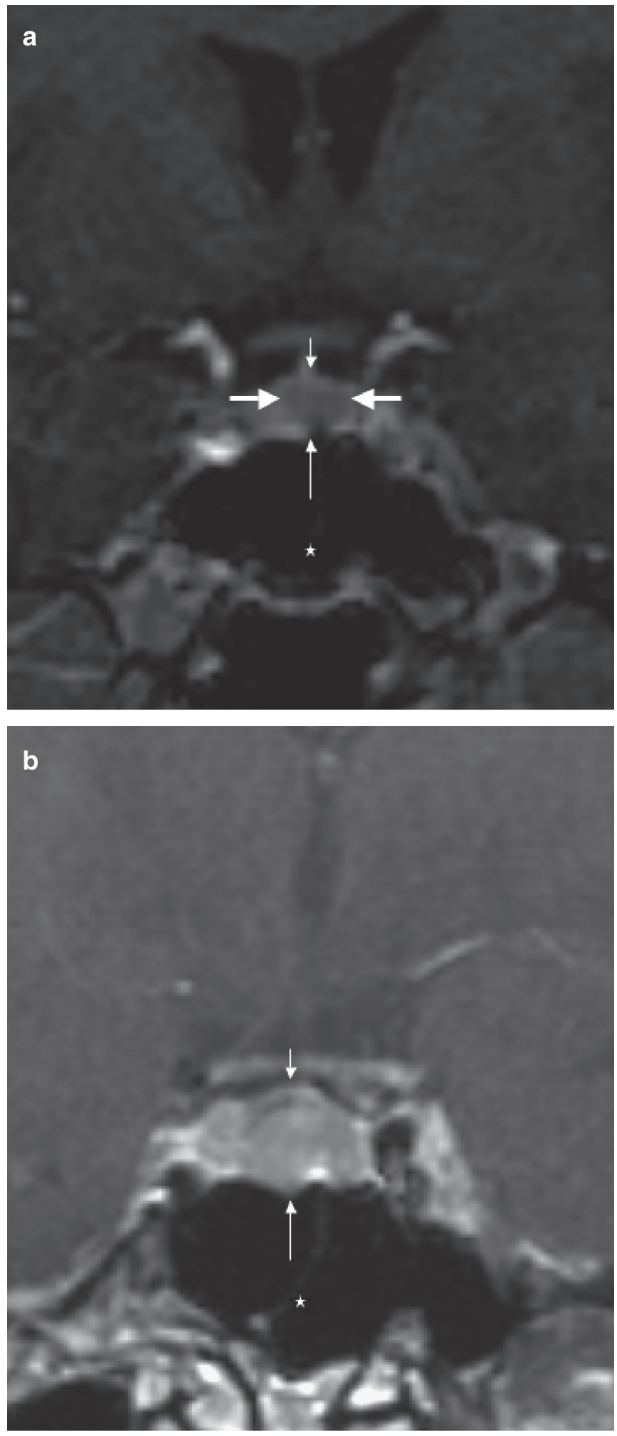

Figure 2. Coronal contrast enhanced T1 weighted MR image at the initial scan (a), showing a circumscribed hypointense intrapituitary lesion (thick arrows). Three-month follow-up showed only slight inhomogeneities, demonstrated on a coronal contrast enhanced T1 weighted image (b). The long arrow points at the superiorly convex margin of the adenohypophyis, the short arrow at the sellar floor. The asterix marks the air filled sphenoid sinus.

to the images of the prior examination, the 7-mm sized lesion could not be confirmed on the follow-up images. Again, the differential diagnosis of a (very small) microadenoma could not be entirely ruled out due to the mentioned inhomogeneous signal within the pituitary gland. Consequently, one of these focal inhomogeneities could theoretically represent a minuscule microadenoma.

At the point of discharge the hyperprolactinaemia associated symptoms including amenorrhea had disappeared indicating that the use of amisulpride in this patient correlated to the elevation of PRL plasma levels and had possibly induced the development of a reversible prolactinoma of the pituitary gland. Psychopathologically, the patient's hallucinations,

anxiety and restlessness symptoms had disappeared; Furthermore, an improvement of the patient's mood was observed, causing a deacrease of the CGI-score from 5 to 3.

In the past years, prolactinoma had repeatedly diagnosed in schizophrenic patients, and their occurrence were correlated with treatment using anti-psychotic medication (especially amisulpride) (Perroud et al. 2004; Akkaya et al. 2008).

On the one hand, hyperprolactinaemia can result from an existing autonomous prolactinoma, on the other hand, it can develop during treatment with selected anti-psychotics. Furthermore, prolactinoma can be induced by anti-psychotic therapy (Pal et al. 2000; Mendhekar et al. 2003).

For differential diagnosis of prolactinoma or medication-induced hyperprolactinaemia, a TRH-Test seems to be useful, followed by an MRI. In the case of normal TRH test results, a disturbance caused by anti-psychotic medication can be assumed and a switch to medication that rarely causes hyperprolactinaemia should be considered. One limitation of this case report is the lack of an MRI imaging prior to the first anti-psychotic treatment.

In conclusion, this case highlights the necessity of considering medication induced enlargement of the pituitary gland and of avoiding surgery of a medication induced adenoma of the hypophysis, especially in patients with a psychiatric case history.

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Statement of interest

The authors have no conflict of interest with any commercial or other associations in connection with the submitted article.

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EDITORIAL

Dear Colleagues,

It is my pleasure to welcome you to the sixth issue of the year 2010.

Alterations of BDNF signaling in **major depression** (MD) are supported by studies demonstrating decreased levels of the neurotrophin serum and plasma content in MD patients. Luisella Bocchio-Chiavetto and colleagues from Italy conducted a replication study and performed two meta-analyses on studies analysing serum and plasma BDNF levels in MD patients. The study included 489 patients/483 controls for the meta-analysis on serum and 161 patients/211 controls for that on plasma levels. The results provide evidence of alteration in MD of peripheral BDNF levels and support the importance of further investigation aiming to the identification of biomarkers for differential diagnosis and personalization of therapies in this disorder.

Orestes Vicente Forlenza and Brazilian colleagues present a study with 160 varying degrees of **cognitive impairment** (30 patients with Alzheimer's disease (AD), 71 with Mild Cognitive Impairment (MCI), and 59 healthy controls). The patients were longitudinally assessed for up to 60 months. The authors investigated whether abnormalities in neurotrophic systems and decreased serum brain-derived neurotrophic factor (BDNF) levels which have been reported in AD, can also be detected in patients with MCI. The authors concluded that decreased neurotrophic support, as indicated by a reduced systemic availability of BDNF, may play role in the neurodegenerative processes that underlie the continuum from MCI to AD. The presence of Met-BDNF allele, particularly in association with APOE*E4, may predict a worse cognitive outcome in patients with MCI.

Depression, a disease usually accompanied by a serotonergic deficit, has been observed in about 40% of patients suffering from **Parkinson's disease** (PD). Thus, a serotonergic dysfunction in PD can be assumed. Jan Beucke and German colleagues studied the interaction between serotonergic (5-HT) and dopaminergic activity in early PD. The study included 9 unmedicated PD patients before and twelve weeks after L-dopa treatment and 9 healthy subjects. The results support the hypothesis that serotonergic neurotransmission is decreased in untreated PD and suggest that a low serotonergic activity may be related to the dopamine pathology in PD. This could be related to the high prevalence of depression in PD.

Daniel O'Connor and colleagues from Australia present a clinical file review in regards to **Clozapine** use in 3 aged psychiatry services in Melbourne to compare its safety and tolerability with findings reported in the literature. The review period spanned the intervals from 2008 to the services' origins between 11 to 15 years earlier. The result of this review was that most of the adverse events leading to treatment cessation occurred within the first month, emphasising the need for slow titration. Strict monitoring procedures ensured that there were no fatal haematological adverse events.

In an original article by Bih-Fen Lin and colleagues from Taiwan, the long-term behavioral and neurochemical consequences of **toluene** exposure during adolescence was studied. Male NMRI mice received one injection per day of either toluene (600 mg/kg) or corn oil during postnatal day (PN) 35-37 and (750 mg/kg) during PN38-39 and PN42-46. A variety of psychiatric disorder-relevant behavioral tests were examined at PN56-P84. The results outline the social deficits and cognitive impairment at adulthood as well as neurochemical dysfunction in mice when exposed to toluene during adolescence. The findings correlate to the symptoms observed in patients suffering from **solvent-induced psychosis**.

Tardive dyskinesia (TD) is a severe and potentially irreversible motor side effect linked to **long-term antipsychotic exposure**. Changes in dopamine neurotransmission have been implicated in the etiology of TD, and Catechol-O-Methyl-Transferase (COMT) is an enzyme that metabolizes dopamine. Clement Zai and colleagues investigated five single-nucleotide polymorphisms in addition to the functional Val158Met variant spanning the COMT gene for association with TD. The results suggest that the COMT gene may be playing a small effect in explaining TD, although sex-stratified studies with additional markers in larger clinical samples should be performed.

Elena Tenconi and colleagues from Italy studied study set-shifting abilities, central coherence, and handedness in subjects with lifetime **anorexia nervosa** (AN), in a sample of unaffected sisters and in healthy controls, in order to explore their suitability as endophenotypes of AN. 153 subjects with lifetime AN, 28 unaffected sisters and 120 healthy controls were administered. The investigation provides further evidence that AN is associated with an impairment of set-shifting abilities and central coherence.

There is mixed evidence of association of serotoninergic genes with **anorexia nervosa** (AN), but substantial evidence for the involvement of serotonergic mechanisms in appetite control. Kirsty Kiezebrink and colleagues from the UK investigated possible associations between the two subtypes of AN (Restricting-RAN, and Binge-purging–BPAN) and polymorphisms within 5 genes encoding for proteins involved in the serotoninergic system. 226 females meeting the criteria for AN, and 678 matched healthy females were included for this study. The outcome indicates a substantial and complex inter-relationship between serotoninergic genes and AN.

In a brief report Hannu Koponen and colleagues from Finland investigated the correlation between **depression** and the increase of fatal and nonfatal **cardiovascular (CV) events**. The prevalence of increased depressive symptoms was measured with the Beck Depression Inventory (BDI), and the SCORE and Framingham risk functions were calculated in a middle-aged population-based sample (N=923). For metabolic syndrome (MetS), the modified National Cholesterol Education Program – Adult Treatment Panel III criteria were employed. The results emphasise the importance of screening and effective treatment of depression in the primary and secondary prevention of cardiovascular events, especially in males.

Yours sincerely,

Siegfried Kasper, MD
Chief Editor

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ORIGINAL INVESTIGATION

Serum and plasma BDNF levels in major depression: A replication study and meta-analyses

LUISELLA BOCCHIO-CHIAVETTO[1], VINCENZO BAGNARDI[2,3], ROBERTA ZANARDINI[1], RAFFAELLA MOLTENI[4], MARIA GABRIELA NIELSEN[5], ANNA PLACENTINO[5], CATERINA GIOVANNINI[5], LUCIANA RILLOSI[5], MARIACARLA VENTRIGLIA[6,7], MARCO A. RIVA[4] & MASSIMO GENNARELLI[8,9]

[1]*Neuropsychopharmacology Unit, IRCCS San Giovanni di Dio Fatebenefratelli, Brescia, Italy,* [2]*Department of Statistics, University of Milan-Bicocca, Milan, Italy,* [3]*Division of Epidemiology and Biostatistics, European Institute of Oncology, Milan, Italy,* [4]*Center of Neuropharmacology, Department of Pharmacological Sciences, Universita' degli Studi di Milano, Milan, Italy,* [5]*Psychiatric Rehabilitation Unit, IRCCS San Giovanni di Dio Fatebenefratelli, Brescia, Italy,* [6]*Department of Neuroscience, AFaR-Hosp. Fatebenefratelli, Rome, Italy,* [7]*Neurology, University "Campus Biomedico", Rome, Italy,* [8]*Genetic Unit, IRCCS San Giovanni di Dio Fatebenefratelli, Brescia, Italy, and* [9]*Division of Biology and Genetics, Department of Biomedical Science and Biotechnologies, University of Brescia, Italy*

Abstract

Objectives. Alterations of BDNF signalling in major depression (MD) are supported by studies demonstrating decreased levels of the neurotrophin serum and plasma content in MD patients. We conducted a replication study and we performed two meta-analyses on studies analysing serum and plasma BDNF levels in MD patients. *Methods.* The samples were composed by 489 patients/483 controls for the meta-analysis on serum and by 161 patients/211 controls for that on plasma levels. We performed also subgroup analyses to examine whether the decrease in BDNF levels in MD was influenced by gender. *Results.* In the replication study we found decreased serum BDNF levels in MD patients ($P<0.01$) and we demonstrated that is down-regulated the mature form of the neurotrophin (mBDNF). No significant difference was evidenced for plasma BDNF levels. The meta-analyses showed a reduction of both BDNF serum ($P<0.0001$) and plasma levels ($P=0.02$) in MD. No difference in the effect size on serum BDNF was observed between males and females ($P=0.18$). *Conclusions.* In conclusion, our results provide evidence of peripheral BDNF alteration in MD and support the rationale for further investigation aiming to the identification of biomarkers for differential diagnosis and personalization of therapies in this disorder.

Key words: *BDNF, major depression, meta-analysis, gender, mBDNF*

Introduction

Major depression (MD) is a severe mental disease and one of the most disabling for incidence and chronic course. Understanding the molecular bases of this disorder and identifying the biological risk factors is crucial in the effort to discover new targets for pharmacological treatment and to identify biomarkers for differential diagnosis and treatment response.

The neurotrophin brain-derived neurotrophic factor (BDNF) contributes to a variety of neural processes ranging from neurodevelopment to the survival and homeostatic maintenance of central and peripheral nervous system in adulthood and in ageing. During development, BDNF plays a key role in neurogenesis, differentiation and maturation of neurotransmitter systems known to be altered in mental disorders (Guillin et al. 2004; Carvalho et al. 2008; Martinowich et al. 2008), while in the adult brain its action is associated with the modulation of synaptic plasticity and is essential for memory-related mechanisms (Poo 2001; Lu et al. 2008).

Correspondence: Luisella Bocchio-Chiavetto, Neuropsychopharmacology Unit, IRCCS San Giovanni di Dio Fatebenefratelli, Via Pilastroni 4, 25125 Brescia, Italy. Tel. +39-030-3501453. Fax +39-030-3533513. E-mail: lbocchio@fatebenefratelli.it

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Compelling evidences in animal models have indicated that BDNF is involved in the pathogenetic mechanism of MD (Duman and Monteggia 2006) as well as in the behavioural response to antidepressant drugs (Calabrese et al. in press; Molteni et al. 2009). Moreover, postmortem studies evidence an altered expression of BDNF in the hippocampus and prefrontal cortex of depressed patients (Dwivedi et al. 2003; Molnar et al. 2003; Karege et al. 2005a).

BDNF is present in large quantities also in the blood where it is mostly stored in platelets (Fujimura et al. 2002). In the recent years, the involvement of BDNF in depression aetiology and treatment has been also supported by a series of biochemical studies in humans. Interestingly, since the first evidence of Karege and colleagues (2002a), studies on serum samples have consistently reported a decreased content in MD patients (Sen et al. 2008), suggesting that in vivo peripheral levels might reflect alterations already observed in post-mortem brains. More recently, low BDNF levels have been observed also in plasma of depressed subjects (Karege et al. 2005b). Some of these studies report differential effects on peripheral BDNF levels in female (Gervasoni et al. 2005; Karege et al. 2005b; Huang et al. 2008) or male (Başterzi et al. 2009) depressed subjects pointing out a specific role of gender in the alteration of the neurotrophin regulation observed in MD. This hypothesis is supported also by studies in BDNF knockout mice showing gender differences in depression-related behaviors (Monteggia et al. 2007; Autry et al. 2009).

Potentially, the identification of peripheral biomarkers, measurable in vivo with non-invasive methods, might be of great valuable to facilitate the differential diagnosis of mood disorders and the development of individual drug-tailored therapies (Mössner et al. 2007). The first step in the way to the identification of an illness marker is the validation of the results reported by single independent studies in a meta-analysis that could also clarify differential effects in subpopulations.

In order to analyse the alterations of serum and plasma BDNF levels in depression and to evaluate a putative role of gender, we perform a replication study in an independent sample of depressed patients and controls and a meta-analyses of data on both blood compartments.

Methods

Replication study

Subjects. From July 2005 to January 2006, 116 consecutive patients attending the Biological Psychiatry Unit of IRCCS Centro S. Giovanni di Dio FBF, Brescia, Italy were screened.

The inclusion criteria were as follows: meets either ICD-10 or DSM-IV criteria for at least moderate severity of depression, age 18–65 years, male or female, parents of caucasoid European ethnicity, and inpatients or outpatients. The exclusion criteria were: family history of bipolar affective disorder or schizophrenia in first-degree relatives, a personal psychiatric history of bipolar affective disorder, schizophrenia, mood incongruent psychotic symptoms, primary substance misuse, or primary organic disease, current or actively seeking of pregnancy, and current treatment with an antidepressant, antipsychotic or a mood stabiliser, or any regular treatment for a medical condition. Only treatments with benzodiazepines were allowed.

Twenty-five MD patients were enrolled in the study after signing the informed consent approved by the local Ethical Committee. Each patient was assessed by interview using the Schedules for Clinical Assessment in Neuropsychiatry (SCAN version 2.1, WHO, 1999), as having a MD as defined by the ICD-10 or DSM-IV. The Montgomery–Asberg Depression Rating Scale – MADRS (Montgomery and Asberg 1979) was administered at the baseline to assess severity of illness. Before entering the study, the wash-out period was at least 2 weeks for 13 patients and 1 week for five patients, while seven patients were at their first episode of depression.

In parallel, a control group of volunteers with a negative personal and familiar anamnesis for any Axis I disorder confirmed with the Mini International Neuropsychiatric Interview – MINI (Sheehan et al. 1998) has been enrolled for the study after signing informed consent approved by the local Ethical Committee. The study has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

BDNF serum and plasma dosage. Venous blood samples for patients and controls were collected in the morning after an overnight fast (between 08:00 and 09:00 h) in anticoagulant-free tubes (for serum preparation) and in EDTA tubes (for plasma preparation). Serum tubes were kept at room temperature for 2 h followed by 1 h at 4°C before serum separation by centrifugation (3000 rpm for 15 min). Plasma tubes were centrifuged at 3000 rpm for 15 min. Serum and plasma samples were stored at –80°C till the time of assay. BDNF levels were measured by an ELISA method (BDNF Quantikine kit R&D system Minneapolis, USA). BDNF content was expressed as equivalent of the human recombinant protein, the detection limit was 20 pg/ml and data were expressed

as ng of protein/ml of serum or pg of protein/ml of plasma.

Additional analysis of serum BDNF protein levels was performed by Western blotting. The same volume of serum sample was run under reducing conditions on 14% SDS–polyacrylamide gel and then electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad, Milan, Italy). Blots were blocked with 10% nonfat dry milk and then incubated with the primary anti-BDNF polyclonal antibody (1:1000, 4°C, overnight; Santa Cruz Biotechnology) able to recognize both the mature form of the neurotrophin (mBDNF; 14 kDa) and its precursor (proBDNF; 32 kDa). Membranes were then incubated for 1 h at room temperature with a peroxidase-conjugated anti-rabbit IgG (1:5000, Cell Signaling) and immunocomplexes were visualized by chemiluminescence utilizing the ECL Western blot kit (Amersham Life Science, Milan, Italy) according to the manufacturer's instructions.

Statistical analysis. Demographic and clinical characteristics in case and control samples were described either in terms of mean ± SD if quantitative or in terms of proportions. Serum and plasma BDNF levels in antidepressant drug-free patients versus controls after checking for normality were compared using student t test. Differences in BDNF levels were also tested for other covariates, such as age, gender, BMI and smoking status, using *t*-test for dichotomous variables or a simple linear regression model for continuous variables. Qualitative variables were tested by means of χ^2 and Fisher exact tests. All statistical evaluations were performed using the SPSS version 13.0 software package (website: http://www.spss.com).

Meta-analyses

Literature screening. To identify eligible studies for meta-analysis, MEDLINE citations (1980–2009) were surveyed on the National Library of Medicine's PubMed online search engine, using the keywords "Major Depression", "Depression", "MD", "mood disorder", "Brain derived neurotrophic factor", "BDNF", "serum", "plasma", "blood", "neurotrophins", and their combinations. Studies were also identified by cross-referencing of included studies. Only studies published in a peer-reviewed journal, written in English and presenting original results were considered for inclusion in the meta-analysis.

The abstract of each extracted citation was screened in order to select studies that explicitly analysed BDNF serum and plasma levels in MD patients and in a control population. The papers satisfying this criterion were further evaluated by two independent readers, who assessed suitability for inclusion in the meta-analyses. Each reference cited in the articles was also reviewed to identify additional papers not indexed by MEDLINE.

Inclusion criteria were as follows: (1) BDNF serum or plasma levels were measured in adult subjects with a depression diagnosis according to DSM-IV criteria, (2) the study had a cross-sectional design and comprised a control group of healthy volunteers screened for negative past and present history of Axis I disorders; (3) BDNF concentration was measured with standard methods. Manuscripts had to present mean BDNF levels and standard deviations for patient and control groups. In the case of sample overlapping between studies, only the results of the publication with the highest number of participants were included. Studies were excluded when serum BDNF was measured only in patients under antidepressant drug treatments.

Statistical analysis. For each study identified, the standardized mean difference (SMD) in serum and in plasma BDNF levels between drug free depressed patients and the control group was calculated using Hedges' correction for small sample (Hedges and Olkin 1985), along with their associated variance and 95% Confidence Interval (CI). SMD was favoured over weighted mean difference as outcome measure in order to reduce the impact of different measurement related to sampling conditions. The analyses were conducted separately for serum and plasma BDNF data. In the main analysis data from all the studies were included and random effect models were fitted using inverse variance weighting to obtain pooled estimates of SMD and their corresponding 95% CIs. The *Q*-test was performed to assess between-study heterogeneity, and the I^2 statistics, which express the percentage of the total observed variability due to heterogeneity, was also calculated. In the presence of heterogeneity, no attempt to evaluate publication bias was done (Ioannidis and Trikalinos 2007). An influential analysis was conducted and the pooled SMD were computed with the omission of one study at a time to identify whether the results were influenced by single studies.

Subgroup analyses were performed to examine whether the difference in serum BDNF levels between patients and controls varied by gender. Results of the subgroup analyses were presented for each level of the factor as pooled SMD together with 95% CI, and the null hypothesis that in the population the difference in SMD between the levels of the factor is zero was tested using a *z*-test (Altman and Bland 2003).

Results

Sample characteristics

All MD patients recruited (20 females, five males; mean age 43.36±9.97 years, range 23–64 years) underwent routine blood clinical examinations: all values, including platelet count (mean 273.28±65.29×1000/μl reference range 150–450) were inside normal ranges. Mean Body Max Index (BMI) was 24.09±3.12 and 17 patients were smokers while eight were non-smokers. Baseline illness severity in the MD sample (measured by the MADRS scale) was 22.72±4.65.

The control group consisted in 59 subjects (48 females, 11 males; mean age 42.59±10.07 years, range 19–69 years; mean BMI= 24.02±4.39). Ten subjects were smokers while 49 were non-smokers. No differences in demographic and morphometric characteristics (sex, age, BMI) were observed comparing patient to control sample.

Replication study

A significant decrease in BDNF serum content was observed in drug-free MD patients (P) towards controls (C) (P: 29.60±12.41 ng/ml, C: 40.78±11.34 ng/ml, F=16 P=0.00013, Figure 1). Univariate analysis of variance did not report a significant effect in the whole sample of the covariate gender (P=0.15), age (P=0.18) and smoke (P=0.78) on serum BDNF levels while a main effect was evidenced for the BMI (F=4.09, P=0.046). Multiple linear regression, taking into account the covariate BMI confirmed the decrease of BDNF serum levels in the patient group (P=0.000087). In the patient sample no correlation

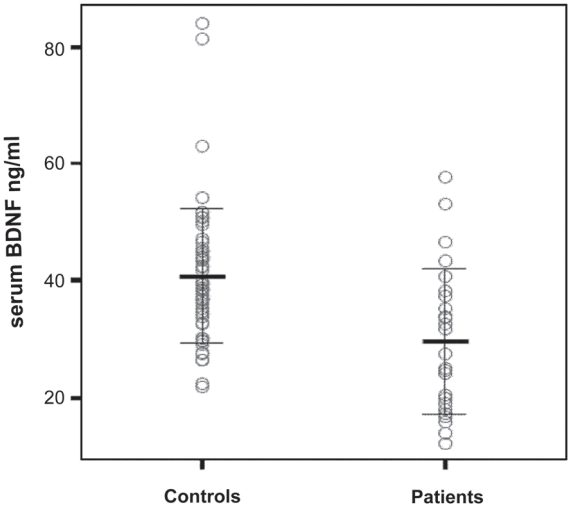

Figure 1. BDNF serum levels: differences between depressed patients and healthy controls.

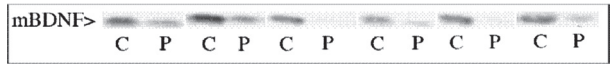

Figure 2. Representative bands from western blot analysis showing reduced levels of the mature form of BDNF (mBDNF) in the serum of depressed patients (P) with respect to control subjects (C).

was found between baseline BDNF serum levels and platelet content (P=0.79) or baseline MADRS scores (P=0.07). Significant differences in BDNF serum levels between drug-free patients (T_0) and controls (C) were evidenced also after normalization for serum total protein content (BDNF ng /proteins μg) (data not shown).

These results were also confirmed by western blotting analysis, as we found a significant down-regulation of the mature form of the neurotrophin (mBDNF) in the serum of MD patients with respect to controls (Figure 2). Although we used an antibody able to recognize also BDNF precursor, we were not able to detect a consistent and reproducible band, probably due to its low levels in serum samples.

Univariate analysis of variance did not report significant differences in BDNF plasma levels between MD patients and controls (P: 3.224±3.185 ng/ml, C: 3.233±3.040 ng/ml, P=0.99). Similarly, no effect for covariates gender (P=0.22), age (P=0.92), smoke (P=0.77) and BMI (P=0.94) were observed on plasma BDNF content.

No correlations were observed between serum and plasma levels in the whole sample (P= 0.259), nor in patient or control sub-samples.

Meta-analysis

Fifteen and five studies, including the data of our replication study, fulfilled the inclusion/exclusion criteria, respectively, for serum and plasma BDNF determinations. Three studies were reporting data on both the outcomes. The whole sample was composed by 489 depressed patients and 483 controls for the meta-analysis on serum levels and by 161 depressed patients and 211 controls for the meta-analysis on plasma levels.

The study characteristics are shown in Table 1 (Shimizu et al. 2003; Gonul et al. 2005; Aydemir et al. 2006; Yoshimura et al. 2007; Matrisciano et al. 2008; Piccinni et al. 2008).

Studies on serum BDNF

The trial sample size ranged from 20 (Aydemir et al. 2005) to 218 (Huang et al. 2008) subjects. Patients illness symptomatology was measured with HDRS (Hamilton Depression Rating Scale) in 11 studies

Table I. Study characteristics: first author name and publication year, ethnic origin and size of the sample (patient and controls), gender composition and mean age, original serum and plasma BDNF means and *P* values reported in the papers, CV% in the control samples for serum and plasma studies, SMD and 95%CI used in the meta-analyses.

Reference	Origin	Sample size*	% of females*	Age (years)*	Symptomatology	BDNF patients	BDNF controls	*P* value	SMD	95%CI
Studies on serum						ng/ml:	ng/ml:			
Karege et al. 2002	Switzerland	30:30	50%:50%	36±8 : 38±9	(MADRS) 34±5	22.6±3	26.5±7	$P<0.01$	–0.71	[–1.24;–0.19]
Shimizu et al. 2003	Japan	16:50	25%:48%	40.8±13.6 : 41.9±15.9	(HDRS) 27.8±10.2	17.9±9.6	27.7±11.4	$P=0.002$	–0.88	[–1.46;–0.30]
Aydemir O. et al. 2005	Turkey	10:10	80%:80%	31.8±14.3 : 39.8±7.1	(HDRS) 23.2±4.6	17.9±9.1	31.6±8.6	$P=0.007$	–1.48	[–2.50;–0.47]
Gervasoni et al. 2005	Switzerland	26:26	57%:50%	40.5±10.7 : 39.6±12.2	(MADRS) 32.8±4.9	22.6±3.6	26.4±3.6	$P=0.0002$	–1.04	[–1.62;–0.46]
Karege et al. 2005b	Switzerland	43:35	63%:49%	36±10 : 31±11	(MADRS) 32±4	10.1±2.3	12.2±2.4	$P=0.006$	–0.89	[–1.36;–0.41]
Gonul et al. 2005	Turkey	28:18	75%:67%	35.5±8.1 : 35.7±5.8	(HDRS) 27.3±3.5	20.8±6.7	26.8±9.3	$P=0.015$	–0.76	[–1.37;–0.14]
Aydemir C. et al. 2006	Turkey	20:20	100%:100%	35.6±7.6 : 34.6±7.9	(HDRS) 39.8±7.4	27.7±13.7	41.2±15.1	$P=0.01$	–0.92	[–1.57;–0.26]
Aydemir O. et al. 2007	Turkey	24:26	71%:77%	33.9±15.7 : 31.4±5.9	(HDRS) 21.0±3.6	21.2±11.3	31.4±8.8	$P=0.002$	–1.00	[–1.59;–0.41]
Yoshimura et al. 2007	Japan	42:30	62%:67%	47±19 : 45±15	(HDRS) 23.5±6.5	9.6±7.7	23.4±10.1	$P=0.02$	–1.55	[–2.08;–1.01]
Piccinni et al. 2008	Italy	15:15	87%:80%	47.0±10.8 : 36.9±9.2	(HDRS) 22.8±5.3	19.3±8.8	33.6±8.6	$P<0.001$	–1.60	[–2.44;–0.76]
Huang et al. 2008	Taiwan	111:107	82%:61%	36.0±10.1 : 28.9±5.1	(HDRS) 35.1±4.9	10.9±7.1	14.1±7.0	$P=0.007$ females $P=0.765$ males	–0.45	[–0.72;–0.18]
Monteleone et al. 2008	Italy	35:22	80%:63%	48.1±13.0 : 40.1±16.4	(HDRS) 8.5±4.7§	29.3±13.2§	42.5±12.5§	$P<0.05$	–0.99	[–1.58;–0.40]
Matrisciano et al. 2008	Italy	21:20	48%:55%	42.5± 8.3 : 31.8±5.9	(HDRS) 17.6±5.2	35.4±14.3	64.1±13.1	$P<0.0001$	–2.05	[–2.82;–1.28]
Ba terzi et al. 2009	Turkey	43:15	70%:60%	31.5±12.5 : 36±10	(HDRS) 26.1±4.2	42.0±12.6	47.7± 7.7	$P=0.105$	–0.49	[–1.08; 0.11]
Present study	Italy	25:59	80%:81%	43.4±10.0 : 42.6±10.1	(MADRS) 22.7±4.65	29.6±12.4	40.7±11.3	$P=0.00013$	–0.95	[–1.44;–0.46]
				Weighted mean coefficient of variation (CV) in the control samples			0.33			
Studies on plasma						pg/ml:	pg/ml:			
Karege et al. 2005b	Switzerland	12:12	50%:50%	36±10 : 31±11	(MADRS) 32±4	1685±243	2165±349	$P<0.001$	–1.54	[–2.47;–0.61]
Kim et al. 2007	Korea	32:30	57%:69%	47.3±14.5 : 41.0±7.8	(HDRS) 28.66±5.73	875.8±663.0	889.4±611.3	$P=0.996$	–0.02	[–0.52; 0.48]
Lee et al. 2007	Korea	77:95	65%:61%	35.3±11.6 : 34.4±9.4	(HDRS) 27.7±5.9	579.5±414.2	819.2±345.0	$P<0.01$	–0.63	[–0.94;–0.32]
Piccinni et al. 2008	Italy	15:15	87%:80%	47.0±10.8 : 36.9±9.2	(HDRS) 22.8±5.3	2900±1900	5400±2300	$P=0.003$	–1.15	[–1.93;–0.37]
Present study	Italy	25:59	80%:81%	43.4±10.0 : 42.6±10.1	(MADRS) 22.7±4.65	3224±3184	3233±3040	$P=0.990$	0.00	[–0.47; 0.46]
				Weighted mean coefficient of variation (CV) in the control samples			0.59			

*patients:controls; §data provided by the authors.

(range mean HDRS: 8.5–39.8) and with MADRS in four studies (range mean MADRS: 22.7–34).

Twelve studies included a patient samples with a percentage in females >50%. Mean age in the patient group ranged from 31.8 (Ba terzi et al. 2009) to 48.1 (Monteleone et al. 2008).

Thirteen studies individually reported overall significant differences between patient and controls ($P<0.05$), whereas one study reported negative findings in the whole sample (Başterzi et al. 2009) and a significant decrease in BDNF levels only in male patients ($P=0.041$). Finally, one study (Huang et al. 2008) did not report an overall analysis but only estimates in the sample stratified by gender reporting a significant difference only in women ($P=0.007$ in females, $P=0.765$ in males).

Since mean serum BDNF values in control groups significantly varied between studies ($P=0.005$), our meta-analysis was conducted on SMDs. SMDs and 95% Confidence Intervals (CI) used in the meta-analyses are shown in Table 1.

Figure 3A shows a forest plot of the SMD of BDNF serum levels in MD patients relative to controls in each of the 15 studies included in the meta-analysis, along with the random effect pooled estimate. Overall, MD patients showed a significant decrease in BDNF serum levels (Pooled random effect SMD=–0.98; 95%CI=–1.20, –0.76, $P<0.0001$).

A significant heterogeneity between single estimates was observed (Q-test, $P= 0.004$), with a 56% of total variability due to heterogeneity. Influential analysis showed that the heterogeneity was mainly due to one study (Huang et al. 2008) (data not shown).

Moreover, seven studies (Gervasoni et al. 2005; Karege et al. 2005b; Aydemir et al. 2005, 2007; Huang et al. 2008; Ba terzi et al. 2009, and the present study) reported specific estimates for females and males. Figure 4 shows a forest plot of the SMDs of BDNF serum levels in female and male MD patients relative to sex-matched controls along with the random effect pooled estimates. Pooled random effect SMDs were significant both in females (–0.81, 95% CI –1.09, –0.53 $P<0.0001$) and in males (–0.56; 95% CI –0.90, –0.21, $P=0.0013$).

No significant heterogeneity was observed between gender-specific estimates ($P=0.18$)

Studies on plasma BDNF

Trial sample size ranged from 24 (Karege et al. 2005b) to 172 (Lee et al. 2007) for plasma BDNF data. Patients illness symptomatology was measured with HDRS in three studies (range mean HDRS: 22.8–28.6) and with MADRS in two studies (range mean MADRS: 22.7–32).

Four studies included a patient sample with a percentage in females >50%. Mean age in the patient group ranged from 35.3 (Lee et al. 2007) to 47.5 (Kim et al. 2007).

Three studies individually reported overall significant differences between patient and controls ($P<0.05$), whereas two studies (Lee et al. 2007 and the present study) reported negative findings.

Similarly to what observed in the serum analysis, BDNF plasma levels in control samples were significantly different between studies ($P<0.01$).

SMDs and 95% Confidence Intervals 95% CI were used in the meta-analysis (Table I).

Figure 3B shows a forest plot of the SMD of BDNF plasma levels in MD patients relative to controls in each of the five studies included in the meta-analysis, along with the random effect pooled estimate. Overall, patients showed a significant decrease in BDNF plasma levels when compared to controls (Pooled random effect SMD=–0.57; 95%CI=–1.04, –0.09, $P=0.02$). A significant heterogeneity between single estimates was observed (Q-test, $P=0.003$), with a 75% of total variability due to heterogeneity. No subgroup analyses were conducted for this outcome due to the small numbers of studies considered.

Discussion

The aims of this study were: (1) to replicate previous findings on MD patients in an independent sample where the concomitant dosage of serum and plasma BDNF levels was available; (2) to resume and quantify data supporting serum/plasma BDNF alterations in MD in two meta-analyses; and (3) to investigate putative gender-related effects.

In the replication study, the simultaneous BDNF content assessment in serum and plasma of the same subject, indicates decreased BDNF serum levels in drug-free MD patients, conversely no alterations were observed in the neurotrophin plasma content. Our data on plasma are discordant from those by other studies reporting a parallel decrease in serum and plasma BDNF in depression (Karege et al. 2005b; Piccinni et al. 2008) and from those of Lee and colleagues (2007). This inconsistency might derive from methodological differences between studies that bring to a wide interindividual variability in plasma levels (see Table I: the mean coefficient of variation (CV) of plasma BDNF in the control groups is twice as that observed for the serum content). In fact, while the accuracy and the reproducibility of BDNF determination in serum is validated

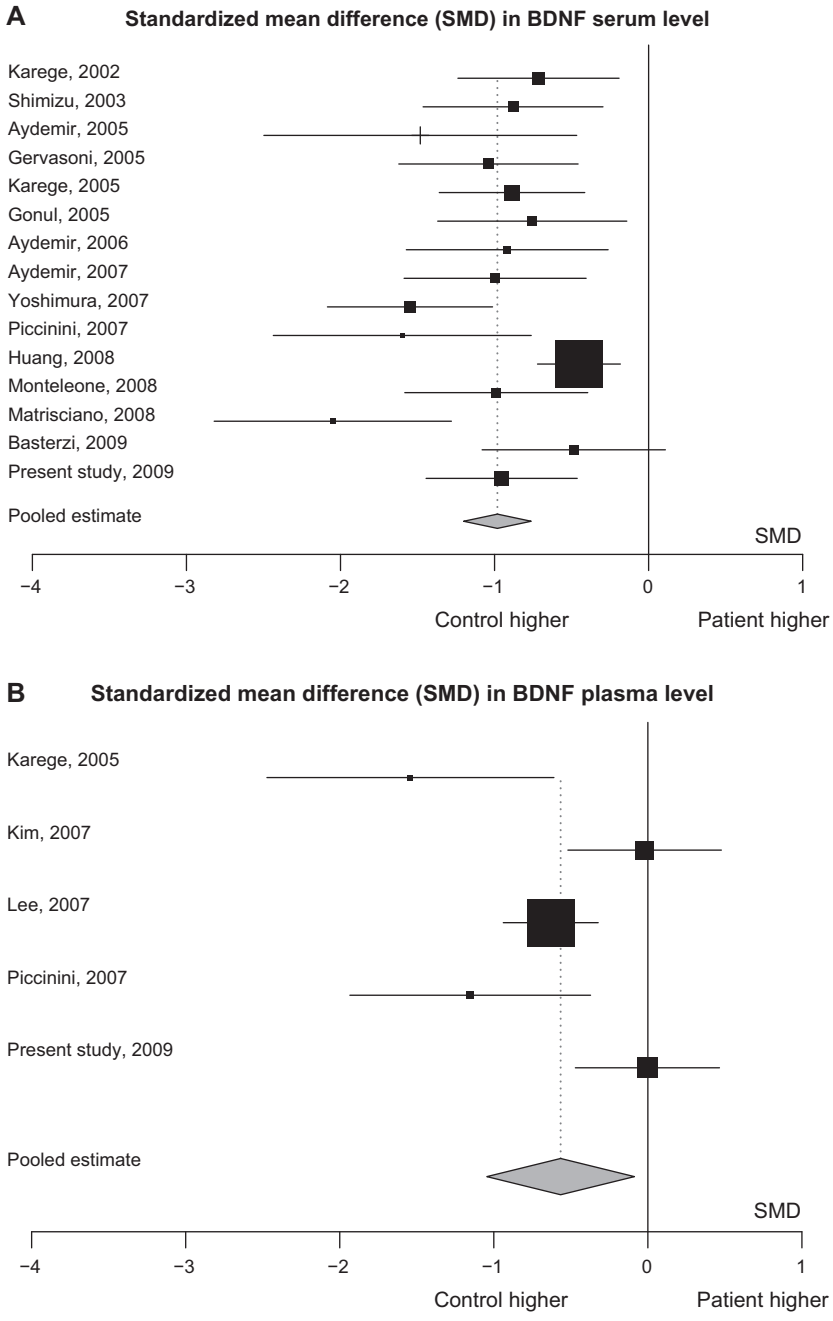

Figure 3. Standardized mean difference (SMD) in BDNF serum level (panel A) and in BDNF plasma level (panel B). The squares represent the SMD between patients and controls. The size of the squares is proportional to the sample size of the study, the whiskers represent the 95% confidence interval. The diamond represents the pooled estimate based on random effects model, with the centre representing the point estimate and the width representing the associated 95% CI.

(Trajkovska et al. 2007), standardized protocols for plasma collection and BDNF dosage have to be developed. At this regard, in previous studies plasma was collected in lithium heparinised tubes (Kim et al. 2007; Lee et al. 2007) or in tubes for poor-plateled plasma sampling (Karege et al. 2005b) and we choose to use EDTA tubes since a recent study showed that heparin, but not EDTA, may interfere with BDNF assay (Begliuomini et al. 2007). Furthermore, also centrifugation conditions, often not reported in the studies, may vary a lot and may likely influence the quantities of platelets suspended in plasma and consequently the amount of BDNF in plasma (Lee and Kim 2009). Finally, we could not exclude that our study was underpowered to evidence the difference in BDNF plasma levels esti-

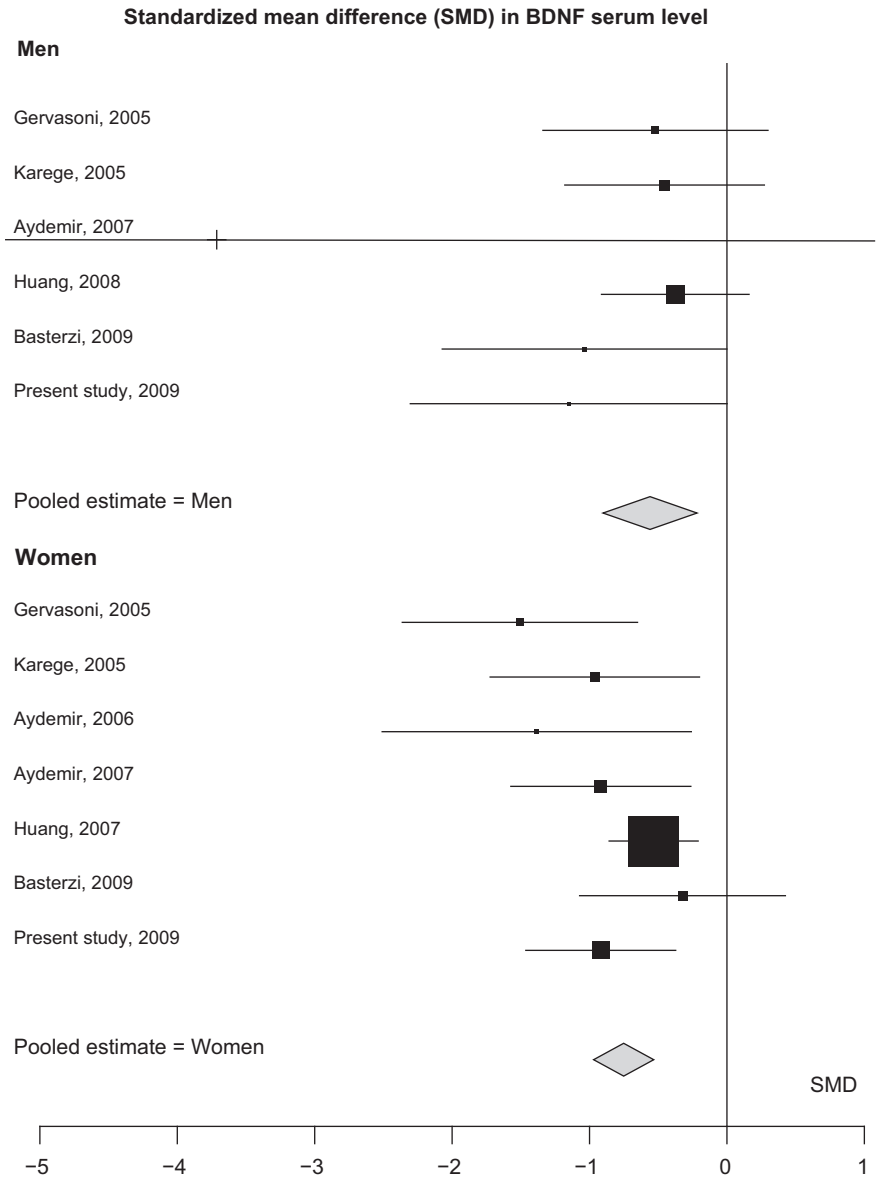

Figure 4. Standardized mean difference (SMD) in BDNF serum level in the studies reporting specific estimates for male and female subjects (five and six studies, respectively). The squares represent the SMD between patients and controls. The size of the squares is proportional to the sample size, the whiskers represent the 95% confidence interval. The diamond represents the pooled estimate based on random effects model, with the centre representing the point estimate and the width representing the associated 95% CI.

mated in the meta-analysis since a post hoc analysis on our data revealed a sample power of 66%.

Moreover, it must be noticed that no correlation between plasma and serum BDNF levels of the same subject was found in our sample, indicating that these measures may be independent.

However, the results of the meta-analyses showed a significant decrease of BDNF levels in both blood compartments. These findings extend the evidence provided by a recent meta-analysis (Sen et al. 2008) on serum BDNF and supply new information on plasma BDNF in depression. The results of the sub-analysis in male and female subjects did not evidence any significant differences in the effect sizes, although the reduction of BDNF levels in MD female patients seems to be larger.

The results obtained confirmed a decrease in peripheral BDNF levels in MD supporting the potential role of the neurotrophin in the disease pathogenesis. How peripheral BDNF levels may reflect the expression in the brain is still an open question: beyond its cerebral production, many systemic BDNF sources may synthesize and release the neurotrophin in blood as epithelial cells, vascular endothelial cells,

muscle cells, activate macrophages and lymphocytes. Peripheral levels of BDNF might be linked to the brain neurotrophin content since BDNF might bidirectionally cross the blood–brain barrier through an active transport system (Pan et al. 1998; Pan and Kastin 1999), and it has been demonstrated that serum levels parallel cerebral protein expression during neurodevelopment and aging in animal models (Karege et al. 2002b). Moreover, recent evidences by functional magnetic resonance investigations in human subjects indicated that BDNF serum concentration is associated with cerebral cortex integrity, a brain region involved in mood disorders (Lang et al. 2007). Therefore, the reduction of serum and plasma BDNF levels in major depression, confirmed by our meta-analyses, might reflect the alterations observed in the brain of MD patients. It cannot be excluded, however, that decreased peripheral BDNF levels might be the outcome of a deregulation in BDNF expression or release by peripheral cells (Karege et al. 2005b). In particular, platelets play a central role in the controlling of blood circulating BDNF since they bind, store and release it constitutively (Fujimura et al. 2002) and alterations in such blood cells have been commonly observed in depression (Parakh et al. 2008). Beside reflecting what happens in the brain, the blood neurotrophin content may have an effect on CNS functions. In this regard, recent evidences found that peripheral infusion of BDNF may increase brain neurogenesis and produce anxiolytic and antidepressant effects in rodents (unpublished data cited in Sen et al. 2008), suggesting new potential therapeutic strategies for mood disorders. However, another important aspect to investigate is related to the form of the neurotrophin present in blood. Indeed, it is known that BDNF protein in the brain is present as mature protein (mBDNF) and as its precursor (proBDNF), which undergoes proteolytic cleavage to generate mBDNF (Mowla et al. 2001; Martinowich et al. 2007; Matsumoto et al. 2008). While mBDNF signals through its high-affinity TrkB receptor leading to a cascade of intracellular signalling promoting cell survival, proBDNF binds to the p75 receptor and might activate a "death" cascade (Lu et al. 2005; Martinowich et al. 2007). Our data indicate that the reduction of the neurotrophin observed in the serum of depressed subjects may be ascribed to decreased levels of mBDNF. Since we were not able to detect a consistent and reproducible immunoreactive band for proBDNF, we cannot rule out the possibility that its levels are also affected in depressed subjects. However, given its very low expression in the human serum (Katoh-Semba et al. 2007), it is feasible to hypothesize a major role of mBDNF in this compartment. It may also be inferred that the absence, or extremely low levels, of proBDNF in serum is due to the fact that only mBDNF may be effectively released from producing cells.

Regarding the potential usefulness of peripheral BDNF levels as biomarkers for differential diagnosis of depressive disorders it must be noted that the serum and plasma BDNF reduction is not specific for depression since it has been observed also in other mental illnesses that share a depressive symptomatology as bipolar disorder (Machado-Vieira et al. 2007; Monteleone et al. 2008), schizophrenia (Buckley et al. 2007; Ikeda et al. 2008), eating disorders (Nakazato et al. 2006), obsessive-compulsive disorder (Maina et al. in press) and Alzheimer dementia (Leyhe et al. 2008) or in healthy subjects exposed to stress at work (Mitoma et al. 2008). BDNF peripheral levels rather than be an illness biomarker in MD might be an indicator of the underlying neuropathogenetic mechanism common to different mood and anxiety disorders.

In conclusion, the results of our study confirm a reduction in serum and plasma BDNF levels in depression and provide a rationale for further investigations aiming to the identify the role and the putative function of the blood neurotrophin content in the pathogenesis and in the treatment of depressive disorders.

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Statement of interest

All authors declare no conflicts of interest.

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ORIGINAL INVESTIGATION

Effect of brain-derived neurotrophic factor Val66Met polymorphism and serum levels on the progression of mild cognitive impairment

ORESTES VICENTE FORLENZA[1*], BRENO SATLER DINIZ[1*], ANTONIO LUCIO TEIXEIRA[2], ELIDA BENQUIQUE OJOPI[1], LEDA LEME TALIB[1], VANESSA AMARAL MENDONÇA[2], GISELLE IZZO[1] & WAGNER FARID GATTAZ[1]

[1]*Laboratory of Neuroscience – LIM 27, Department and Institute of Psychiatry, Faculty of Medicine, University of Sao Paulo, Brazil, and* [2]*Group of Neuroimmunology, Laboratory of Immunopharmacology, Institute of Biological Sciences and School of Medicine, Federal University of Minas Gerais, Belo Horizonte, Brazil*

Abstract

Objectives. Abnormalities in neurotrophic systems have been reported in Alzheimer's disease (AD), as shown by decreased serum brain-derived neurotrophic factor (BDNF) levels and association with BDNF genetic polymorphisms. In this study, we investigate whether these findings can be detected in patients with mild cognitive impairment (MCI), which is recognized as a high risk condition for AD. We also address the impact of these variables on the progression of cognitive deficits within the MCI-AD continuum. *Methods.* One hundred and sixty older adults with varying degrees of cognitive impairment (30 patients with AD, 71 with MCI, and 59 healthy controls) were longitudinally assessed for up to 60 months. Baseline serum BDNF levels were determined by sandwich ELISA, and the presence of polymorphisms of BDNF and apolipoprotein E (Val66Met and APOE*E4, respectively) was determined by allelic discrimination analysis on real time PCR. Modifications of cognitive state were ascertained for non-demented subjects. *Results.* Mean serum BDNF levels were reduced in patients with MCI and AD, as compared to controls (509.2±210.5; 581.9±379.4; and 777.5±467.8 pg/l respectively; $P<0.001$). Baseline serum BDNF levels were not associated with the progression of cognitive impairment upon follow-up in patients with MCI (progressive MCI, 750.8±463.0; stable MCI, 724.0±343.4; $P=0.8$), nor with the conversion to AD. Although Val66Met polymorphisms were not associated with the cross-sectional diagnoses of MCI or AD, the presence of Met-BDNF allele was associated with a higher risk of disease-progression in patients with MCI (OR=3.0 $CI_{95\%}$ [1.2–7.8], $P=0.02$). We also found a significant interaction between the APOE*E4 and Met-BDNF allele increasing the risk of progression of cognitive impairment in MCI patients (OR=4.4 $CI_{95\%}$ [1.6–12.1], $P=0.004$). *Conclusion.* Decreased neurotrophic support, as indicated by a reduced systemic availability of BDNF, may play role in the neurodegenerative processes that underlie the continuum from MCI to AD. The presence of Met-BDNF allele, particularly in association with APOE*E4, may predict a worse cognitive outcome in patients with MCI.

Key words: *Brain-derived neurotrophic factor polymorphisms, APOE, mild cognitive impairment, Alzheimer's disease, neurotrophic cascade*

Introduction

Brain-derived neurotrophic factor (BDNF) is one of the most important and widely distributed neurotrophic factors within the brain (Tapia-Aranciba et al. 2008). BDNF may exert substantial protective effects on crucial neuronal circuitry involved in neurodegenerative diseases. Abnormalities in the BDNF system may be related to the pathophysiology of Alzheimer's disease (AD), since there is evidence of a bi-directional interplay between BDNF homeostasis and the amyloid cascade. Intra-hippocampal injections of the amyloid-β_{1-42} peptide in rats reduce the expression of BDNF, resulting in decreased serum and pre-frontal cortex BDNF levels (Christensen et al. 2008). In a rodent AD model, the infusion of BDNF in the entorhinal cortex reversed synapse loss, partially normalized aberrant gene expression, improved cell signaling and restored learning and memory independently of local β-amyloid load (Nagahara et al.

Correspondence: Orestes Vicente Forlenza, MD, PhD, Laboratory of Neuroscience, Institute of Psychiatry, Rua Dr. Ovidio Pires de Campos 785, 3th floor, Jardim America, São Paulo – SP, CEP 05403-010, Brazil. Tel: +55 11 3069 7267. Fax +55 11 3069 8010. E-mail: forlenza@usp.br

*These authors contributed equally to this work.

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2009). Finally, in aged primates, there is evidence that BDNF may reverse neuronal atrophy and ameliorate age-related cognitive decline (Nagahara et al. 2009).

Post-mortem studies of the AD brain showed reduced intracerebral expression of BDNF, with decreased BDNF mRNA levels (Holsinger et al. 2000) and lower levels of its precursor form (pro-BDNF) in the parietal cortex (Peng et al. 2005). In addition, serum BDNF levels have been found to be decreased in patients with AD (Laske et al. 2006), but not in patients with vascular dementia (Yasutake et al. 2006). A recent study identified reduced levels of BDNF in the serum of subjects with amnestic mild cognitive impairment (MCI), in addition to a positive correlation between BDNF levels and cognitive performance, especially in episodic memory tests (Yu et al. 2008). Finally, genetic studies suggested that BDNF polymorphisms (e.g., Val66Met) may confer higher risk for AD (Kunugi et al. 2001), and recent neuroimaging studies have shown that the presence of the Met-BDNF allele is associated with progression of brain atrophy in cognitively unimpaired older adults (Nemoto et al. 2006) and in patients with MCI, particularly in the presence of the APOE*E4 allele (Hashimoto et al. 2009).

There is limited information regarding the relationship between serum BDNF levels and the Met-BDNF genotype in the MCI-AD continuum. Thus, the aim of the present study was to determine serum BDNF levels in a cross-section of older adults with varying degrees of cognitive impairment, including subjects with MCI, AD and healthy controls. We further examined whether BDNF polymorphisms were associated with baseline diagnoses of MCI or AD, and with the progression of cognitive impairment in patients with MCI and controls upon follow-up.

Methods

Clinical assessment

The study was conducted at the Institute of Psychiatry, Faculty of Medicine, University of Sao Paulo, Brazil. A total of 160 elderly subjects were included in this study, being 30 patients with mild or moderate AD, 71 with MCI, and 59 cognitively healthy older adults (controls). Participants were recruited from an ongoing cohort dedicated to the study of cognitive ageing, and assessed at a multidisciplinary memory clinic after providing informed consent. Detailed information regarding the recruitment strategy, clinical and cognitive assessment as well as diagnostic procedures and criteria can be found in a previous publication from our group (Diniz et al. 2008a). In brief, all participants underwent a comprehensive clinical and cognitive evaluation including the administration of the CAMDEX semi-structured interview (Roth et al. 1986), which yields a cognitive sub-scale (CAMCOG), the Mini-Mental State Examination (Folstein et al. 1975). Neuropsychological assessment included the Rivermead Behavioral Memory Test, the Fuld Object Memory Evaluation, the Trail Making Test (TMT) A and B, and the Short Cognitive Test (SKT). The diagnosis of probable or possible AD was established according to the NINCDS-ADRDA criteria (McKhann et al. 1984), and the diagnosis of MCI and its subtypes was made according to the Mayo Clinic criteria (Petersen et al. 2001). Older subjects with normal cognitive function and no evidence of concomitant psychiatric disorders were regarded as controls. All participants were living in the community and were physically healthy at the time of clinical and laboratorial assessments, i.e. patients were adequately treated for concurrent clinical co-morbidities (such as hypertension or diabetes mellitus). All AD patients were on stable doses of cholinesterase inhibitors at the time of blood sampling, as opposed to subjects with MCI and controls.

Patients with MCI and elderly controls were longitudinally assessed at 12-month intervals (mean duration of follow-up: 22.2 ± 12.3 months). Patients with MCI were re-classified according to their outcome: those with no evidence of additional cognitive decline were regarded as "stable MCI" (MCI-S); patients with demonstrable worsening of cognitive deficits, albeit not sufficient to meet the criteria for dementia, were regarded as "progressive MCI" (MCI-P); otherwise, MCI patients who converted to AD-type dementia upon follow-up were regarded as "converters" (MCI-AD). With respect to subjects who were cognitively unimpaired at baseline (controls), cognitive outcomes comprised either the maintenance of normal cognitive state (stable controls) or the development of cognitive impairment, rendering the re-allocation of this subset of individuals in the MCI group (incident MCI).

Serum BDNF determination

In the morning following the conclusion of clinical and neuropsychological assessments, blood samples were aseptically collected from a peripheral vein of the forearm. A 10-h fasting was required for all participants. Serum was then prepared and stored at -70°C until experimentation, all samples being analyzed at the same time. BDNF concentrations were determined with the aid of commercially available sandwich ELISA kits, under detection limits of 10 pg/l (DuoSet, R&D Systems, Minneapolis, MN, USA). All samples were assayed on duplicate, according to the procedure supplied by the manufacturer. The capture antibody was diluted in phosphate-buffered saline (PBS), added to each well and left overnight at 4°C. Plates were washed four times in

PBS with 0.05% Tween-20 (Sigma, St. Louis, MO, USA), blocked with 1% bovine serum albumin and incubated for 2 h at room temperature before washing four times with PBS-Tween solution. Samples and standards were dispensed to plate wells and incubated overnight at 4°C. After washing, detection antibody (concentration provided by the manufacturer) diluted in PBS was added. The plates were incubated for 2 h at room temperature. After a final wash, streptavidin (DuoSet R&D Systems, Minneapolis, MN, USA) was added and plates incubated for 30 min, after which the colour reagent *o*-phenylenediamine (Sigma,) was added to each well and the reaction was allowed to develop in the dark for 15 min. The reaction was stopped with the addition of 1 M H_2SO_4 to each well. The absorbance was read on a plate reader at 492 nm wavelengths (Emax, Molecular Devices, Minneapolis, MN, USA). The coefficient of variability inter- and intra-assay was <5% (Reis et al. 2008).

APOE genotyping

Genomic DNA was isolated from whole blood from each subject and the APOE genotyping was performed using the TaqMan® 5'-exonuclease allelic discrimination assay obtained from Applied Biosystems (Foster City, CA, USA) with primers and probes sets from inventoried assays. This methodology uses two PCR assays to screen for single nucleotide polymorphisms (rs429358, rs7412) within the exon 4 of APOE gene. Results from the individual assays were used to determine the ultimate *APOE* genotype (Livak 1999).

BDNF genotyping

One-step PCR (Polymerase chain reaction) was performed for the amplification of the gene BDNF (rs6265) by use of the primers F 5′-AAACATC CGAGGACAGGTG-3′ and R 5′-AGAAGAGGAG GCTCCAAAGG-3′. PCR was performed in 10 μl reactions containing 5 ng DNA, 1× Buffer (LGC Biotecnologia, Cotia, SP, Brazil), 2.25 mM $MgCl_2$, 0.125 mM each dNTP, 200 pM of each primer, and 0.05 U *Taq* polymerase. The PCR reaction was carried out on a PTC-200 MJ Research Thermal Cycler. Initial denaturation at 94°C for 5 min was followed by 37 cycles of denaturation at 94°C for 45 s, annealing at 61°C for 40 s, and extension at 72°C for 30 s, with a final extension step of 5 min at 72°C. The amplified DNA was submitted to electrophoresis on 1% agarose gels. PCR products were directly sequenced in both directions using the BigDye® Terminator v3.1 (Applied Biosystems, Foster City, CA) sequencing ready reaction kit and with ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). Subsequent sequence similarity search was performed using BLAST (Altschul et al. 1990), and target SNP was analyzed by visual inspection (GTG = Val-BDNF; ATG = Met-BDNF).

Statistical analysis

Analysis of variance (ANOVA) was carried out to assess mean differences for continuous variables among subjects with AD, MCI and controls. Pearson's chi-square and Fisher's exact tests were carried out to assess differences in the frequency of dichotomous variables among AD, MCI and control subjects. For the genetic analysis, patients were grouped in APOE*4 carrier (homozygous and heterozygous) vs. APOE*4 non-carrier, and in Met-BDNF carrier (homozygous and heterozygous) vs. Met-BDNF non-carrier. Analysis of covariance (ANCOVA) was done to assess the effect of potential confounders on BDNF levels, such as socio-demographic and clinical variables, and APOE*4 and BNDF genes. Pearson's coefficients were determined to address the correlation between serum BDNF levels and demographic, clinical and neuropsychological variables.

ANOVA was done to ascertain mean differences in the baseline BDNF level, socio-demographic, cognitive scores according to the outcome of MCI patients. Cox regression analyses, with enter entry model, were done to examine the effect of serum BDNF levels, APOE and BDNF genes on the risk of cognitive deterioration and of conversion to AD. The probability for entry in the model was set at 0.05 and to be removed was set 0.1. All statistical analyses were carried out with the Software Package for Social Science v. 14.0 for Windows (SPPS, Chicago, IL).

Results

Baseline assessment

Patients with AD were older, less educated, with a higher frequency of the APOE*4 allele, and had worse performance on all cognitive tests. Intermediate values were observed for patients with MCI (Table I). Table II displays serum BDNF levels and the frequency of BDNF and APOE alleles according to clinical diagnoses. The presence of the Met-BDNF allele was neither associated with the risk of AD (OR = 1.0 $CI_{95\%}$ [0.4–2.5], P=0.9) nor with the risk of MCI (OR=1.5 CI95% [0.7–3.4], P=0.3). As expected, the presence of the APOE*4 allele was significantly associated with AD (OR=7.4 $CI_{95\%}$ [2.6–20.9], P<0.001), but not associated with MCI (OR=1.8 $CI_{95\%}$ [0.9–3.5], P=0.07).

Statistically significant differences in serum BDNF levels were observed across diagnostic group (AD, 581.9±379.4 pg/l; MCI, 509.2±210.5 pg/l; controls 777.5±467.8 pg/l, F=9.4, df=2, P<0.001). Such differences remained statistically significant after controlling for age, educational levels, APOE and the

Table I. Socio-demographic data and scores on cognitive tests according to diagnostic groups at baseline.

	AD (n=30)	MCI (n=71)	Controls (n=59)	P^*
Gender (female/male)[#]	19/11	51/20	50/9	0.06
Age (years)[(b)]	76.1 ± 6.6	70.5 ± 10.4	69.5 ± 5.5	<0.001
Education (years)[(a)]	7.9 ± 5.2	10.0 ± 5.1	13.8 ± 5.3	0.001
MMSE score[(b)]	19.3 ± 3.5	27.0 ± 2.0	29.1 ± 0.9	<0.001
CAMCOG (total score)[(a)]	63.7 ± 11.7	89.2 ± 6.8	98.4 ± 3.9	<0.001
RBMT (screening score)[(a)]	2.6 ± 2.7	7.9 ± 2.2	10.6 ± 1.3	<0.001
RBMT (profile score)[(a)]	6.6 ± 5.6	17.7 ± 3.8	22.2 ± 1.9	<0.001
FOME (total score)[(a)]	21.2 ± 13.2	39.4 ± 6.4	45.3 ± 2.8	<0.001
FOME (late recall score)[(b)]	3.6 ± 3.0	8.1 ± 1.6	9.5 ± 0.8	<0.001
VF (fruits category)[(a)]	9.3 ± 2.7	12.9 ± 2.8	16.6 ± 5.9	<0.001
TMT-A (s)[(b)]	128.7 ± 67.4	67.0 ± 32.5	49.2 ± 13.5	<0.001
TMT-B (s)[(a)]	244.6 ± 106.3	164.3 ± 59.1	103.2 ± 33.2	<0.001
SKT (total score)[(b)]	11.9 ± 4.3	4.0 ± 3.3	2.4 ± 1.7	<0.001

[*]ANOVA, unless otherwise specified; [#]Pearson's chi-square test; Post-hoc tests: (a) AD ≠ MCI ≠ controls; (b) AD ≠ MCI = controls; (c) AD = MCI ≠ controls. MMSE, Mini-Mental State Examination; CAMCOG, Cambridge Cognitive Test; RBMT, Rivermead Behavioral Memory Test; FOME, Fuld Object Memory Examination; VF, Verbal Fluency; SKT, Short Cognitive Test; MCI, mild cognitive impairment; TMT, Trail Making Test; AD, Alzheimer's disease.

BDNF Val66Met allele (F=6.9, df=2, P=0.001). No significant differences in BDNF levels were found according to the APOE (APOE*4 carrier, 671.0±423.8 pg/l; APOE*4 non-carrier, 629.8±378.4 pg/l; P=0.6) and BDNF (Met-BDNF carrier, 654.7±339.9 pg/l; Met-BDNF non-carrier, 650.7±432.3 pg/l, P=0.9) genotype. Also, no significant differences in BDNF levels were found according to the BDNF genotype when the diagnostic groups were analyzed separately (supplementary Table I http://informahealthcare.com/doi/abs/10.3109/15622971003797241).

We found significant negative correlations between serum BDNF levels and the performance in global cognitive (total SKT score, r=–0.2, P=0.04) and attention and executive function tests (TMT-A, r=–0.2, P=0.03; TMT-B, r=–0.2, P=0.02) in the whole sample. There were no statistically significant correlations between BDNF levels and demographic and clinical variables, including other neuropsychological tests. We further carried out correlation analyses in the distinct diagnostic groups and found that serum BDNF levels were negatively correlated with a worse performance in memory tests in AD patients (FOME total recall, r=–0.6, P=0.03; FOME late recall, r=–0.6, P=0.01) and in elderly controls (RBMT screening score, r=–0.350, P=0.02; RBMT profile score, r=–0.4, P=0.004). No significant correlations were found for patients in the MCI group.

Longitudinal assessment

Sixty-seven patients diagnosed as with MCI and 52 elderly controls at baseline had at least one re-assessment. Compliant (91.5%) and non-compliant subjects did not differ statistically with respect to clinical and biological variables at baseline (data not shown). Tables III and IV show biological, socio-demographic, clinical and cognitive data for patients with MCI and elderly controls according to the cognitive longitudinal outcome.

Given the small number of patients who actually converted to AD upon follow-up (MCI-AD, n=13), and the clinical similarities between MCI-AD and MCI-P patients, the former patients were further ana-

Table II. Serum BDNF levels, APOE and BDNF genotype distribution according to baseline diagnosis.

			BDNF genotype				APOE genotype			
	Serum BDNF (pg/l)	P^*	Met-BDNF non-carriers	Met-BDNF** carriers	OR [95% CI]¶	P	ε4 carrier*	ε4 non-carrier	OR [95% CI]¶	P
AD (n=30)	581.9 ± 379.4	<0.001	18	12 (0/12)	1.0 [0.4–2.5]	0.9	19 (2/17)	11	7.4 [2.6–20.9]	<0.001
MCI (n=71)	509.2 ± 210.5		49	22 (7/15)	1.5 [0.7–3.5]	0.3	22 (2/20)	49	1.8 [0.9–3.5]	0.07
Controls (n=59)	777.5 ± 467.8		35	24 (5/19)		–	12 (1/11)	47	–	–

[*]Uncorrected P value (ANOVA); Tukey post-hoc test for pairwise comparison: controls vs. MCI, P<0.01; controls vs. AD, P<0.01; MCI vs. AD, P=0.7.

[**]Genotype frequency (homozygous/heterozygous).

¶Odds ratio. Controls as the comparison group.

AD, Alzheimer's disease; MCI, mild cognitive impairment.

Table III. Serum BDNF levels, APOE and BDNF genotype distribution according to outcome diagnosis.

						BDNF genotype			APOE genotype		
Baseline diagnosis	Outcome	Duration of follow-up (months)	*P*	Baseline serum BDNF	*P*	Met-BDNF non-carrier	Met-BDNF carrier*	*P*	ε4 carrier*	ε4 non-carrier	*P*
MCI	MCI-AD (n=13)	20.1 ± 7.0		512.0 ± 217.4		11	2 (0/2)		8 (2/6)	5	
(n=67)	MCI-P (n=23)	18.1 ± 9.7	0.7	517.4 ± 235.8	0.9	13	10 (2/8)	0.1	12 (0/12)	11	0.001
	MCI-S (n=31)	20.2 ± 10.9		512.8 ± 198.3		23	8 (3/5)		4 (0/4)	27	
Controls	controls (n=39)	27.1 ± 15.6	0.2	724.1 ± 343.4	0.8	20	19 (4/15)	0.05	4 (0/4)	35	0.2
(n=52)	incident MCI (n=13)	21.3 ± 8.7		750.7 ± 492.7		12	1 (0/1)		3 (0/3)	10	

*Genotype frequency (homozygous/heterozygous).
MCI, mild cognitive impairment; MCI-AD, MCI converters; MCI-P, MCI progressive; MCI-S, MCI stable.

lyzed within the MCI-P group (n=36). No significant differences in baseline serum BDNF levels were observed between MCI-P and MCI-S patients (515.5±226.2 and 512.8±198.3 pg/l, P=0.9). Cox regression analysis showed that MCI patients carrying APOE*4 or Met-BDNF alleles had a significant increased risk of disease progression (APOE*4, OR=2.3 CI95% [1.1–4.8], P=0.03; Met-BDNF, OR=3.3 CI95% [1.2–7.8], P=0.02). In addition, MCI patients carrying both alleles (APOE*4 and Met-BDNF) had an even higher risk of disease progression (OR=4.3 CI95% [1.6–12.0], P=0.004). Lower serum BDNF levels and the presence of APOE*4 or Met-BDNF alleles did not significantly modify the risk of cognitive decline (i.e. incident MCI) among controls (Table V).

Discussion

This study corroborates previous findings indicative of decreased BDNF serum levels in patients with AD and is in line with the work of Yu et al. (2008) reporting reduced BDNF levels in patients with MCI as compared to cognitively unimpaired subjects. Accordingly, lower BDNF levels in patients with MCI were significantly correlated with a worse cognitive performance, mainly in tests addressing memory, attention and executive function. Despite the Val66Met polymorphisms not being associated with the cross-sectional diagnosis of MCI or AD in the current sample, the presence of the Met-BDNF allele was correlated with the progression of cognitive deficits in patients with MCI. The interaction between Met-BDNF and APOE*4 alleles further increased such risk. Recent studies demonstrated that the presence of the Met-BDNF allele and the interaction between Met-BDNF and APOE*4 alleles were associated with the progression of brain atrophy in healthy elderly controls and in patients with MCI (Nemoto et al. 2006; Hashimoto et al. 2009), in a pattern similar to that found in MCI patients who

Table IV. Socio-demographic and scores on cognitive tests according to outcome diagnosis at follow-up.

	MCI (n=67)				Controls (n=52)		
Baseline diagnosis Outcome	MCI-AD (n=13)	MCI-P (n=23)	MCI-S (n=31)	*P*	Controls (n=39)	incident MCI (n=13)	*P*
Gender (W/M)	5/8	17/6	27/4		33/6	11/2	
Education (years)	10.4 ± 5.9	9.0 ± 5.0	11.0 ± 4.9	0.3	14.5 ± 5.0	10.5 ± 5.9	0.03
Age (years)	76.5 ± 7.7	68.6 ± 15.3	69.6 ± 5.7	0.08	69.5 ± 5.4	69.7 ± 6.1	0.05
MMSE	26.7 ± 2.5	26.6 ± 1.8	27.6 ± 1.6	0.2	29.0 ± 1.0	29.5 ± 0.5	0.7
CAMCOG	87.5 ± 4.4	88.4 ± 7.9	91.2 ± 5.9	0.2	98.9 ± 3.4	96.4 ± 5.2	0.2
RBMT (screening scores)	5.83 ± 2.4	8.0 ± 1.8	8.7 ± 1.7	0.001	10.6 ± 1.4	10.8 ± 0.8	0.9
RBMT (profile scores)	14.4 ± 4.2	17.9 ± 3.4	19.3 ± 3.0	0.001	22.2 ± 1.9	22.4 ± 1.4	0.8
FOME (total scores)	36.4 ± 9.0	38.4 ± 4.9	41.6 ± 5.3	0.05	44.9 ± 2.9	45.9 ± 2.4	0.2
FOME (late recall)	7.7 ± 1.7	7.8 ± 1.5	8.5 ± 1.3	0.3	9.4 ± 0.8	9.5 ± 0.7	0.8
VF (fruits)	10.9 ± 1.7	13.6 ± 3.1	13.5 ± 2.8	0.01	17.0 ± 6.9	14.9 ± 4.0	0.3
Trail A (s)	76.3 ± 39.5	63.7 ± 17.0	63.5 ± 38.7	0.5	46.2 ± 12.1	51.2 ± 9.7	0.1
Trail B (s)	175.7 ± 60.7	177.3 ± 43.9	136.1 ± 60.3	0.05	95.9 ± 26.6	125.2 ± 40.6	0.1
SKT	5.7 ± 3.2	3.4 ± 1.8	3.6 ± 4.2	0.1	1.9 ± 1.5	3.4 ± 1.6	0.005

MMSE, Mini-Mental State Examination; CAMCOG, Cambridge Cognitive Test; RBMT, Rivermead Behavioral Memory Test; FOME, Fuld Object Memory Examination; VF, Verbal Fluency; SKT, Short Cognitive Test, MCI, Mild cognitive impairment; MCI-AD, MCI converters; MCI-P, MCI progressive; MCI-S, MCI stable.

Table V. Cox regression analysis for APOE and BDF genotype and the association with the risk of disease progression in MCI patients and elderly controls.

Baseline diagnosis	Outcome	BDNF (Met-BDNF carrier)	APOE (ε4 carrier)	APOE*BDNF
MCI	MCI-P vs. MCI-S	OR=2.3 $CI_{95\%}$ [1.1–4.8], P=0.03	OR=3.3 $CI_{95\%}$ [1.2–7.8], P=0.02	OR=4.3 $CI_{95\%}$ [1.6–12.0], P=0.004
Controls	incident MCI vs. controls	OR=0.1$CI_{95\%}$ [0.01–1.2], P=0.07	OR=1.4 $CI_{95\%}$ [0.4–4.6], P=0.6	OR=0.04 $CI_{95\%}$ [0.01–248.0], P=0.6

MCI, mild cognitive impairment.

convert to AD (Chételat et al. 2005; Davatzikos et al. 2009). Taken together, these findings suggest that the presence of the Met-BDNF allele may hasten the neurodegenerative process that ultimately leads to disease progression in the MCI-AD continuum. This association seems to be stronger in the presence of another known risk factor for AD, namely the presence of the APOE*4 allele. Several lines of evidence suggest that MCI patients who present with short-term, subtle cognitive worsening (i.e., MCI-P) share several clinical and pathological features with those who actually progress to clinical AD (converters), as opposed to MCI patients who remain stable through follow-up (Diniz et al. 2008b; Forlenza et al. 2009). Thus, the former patients may be regarded as "slow converters" to whom the follow-up length is not sufficient to reach the dementia threshold. The presence of Met-BDNF did not modify the rate of cognitive decline among controls. We speculate that other factors conferring resilience against deterioration, or simply the requirement of a longer period of time to exhibit such changes, may justify the fact that incident MCI was not more frequent among cognitively unimpaired subjects carrying the Met-BDNF allele.

Baseline serum BDNF levels were similar in patients with MCI and AD, and in MCI patients regardless their outcome. In other words, reduced baseline serum BDNF levels in patients with MCI did not predict the progression of cognitive deficits nor the conversion to AD, suggesting a possible role of serum BDNF as a state marker of the ongoing neurodegenerative process in the MCI-AD continuum. Alternatively, higher-than-expected BDNF levels in AD, secondary to the effect of the treatment of the former patients with cholinesterase inhibitors (Leyhe et al. 2008), may have attenuated the difference between these two groups. In addition, serum BDNF levels were not affected by BDNF or APOE genotypes. These findings suggest either that serum BDNF levels are under the regulation of pos-translational factors or that low serum BDNF levels is secondary to unspecific homeostatic changes rather than to specific, AD-related pathophysiological mechanisms. In fact, several studies have demonstrated that serum BDNF levels are reduced in many neurobiologically distinct conditions that are associated with cognitive deficits, such as late-life depression, bipolar disorder and Parkinson disease (Cunha et al. 2006; de Oliveira et al. 2009; Diniz et al. 2010; Scalzo et al. 2010). Thus, low serum BDNF level represent a downstream marker of unspecific disruptions of brain homeostasis.

We acknowledge the fact that the present study was conducted in a tertiary memory clinic and based on a relatively small sample of patients and controls yielding baseline and follow-up data. Thus, the present results should be interpreted with caution until replication in other settings and larger samples. A few comments must also be made on the study model itself, i.e. the extent to which serum BDNF reflects an actual brain abnormality. The cellular sources of BDNF found in the human plasma are not yet clearly defined; potential sources are the vascular endothelial and smooth muscle cells (Nakahashi et al. 2000). Since BDNF readily crosses the blood–brain barrier in both directions, a substantial proportion of the circulating BDNF is believed to originate from neurons and glial cells of the CNS (Pan et al. 1998). A recent study with non-demented elderly subjects found that CSF BDNF levels positively correlated with cognitive performance, lower levels predicting memory decline over 3-years of follow-up (Li et al. 2009).

In conclusion, the present results suggest that low serum BDNF level is a state marker of the ongoing neurodegenerative process in the prodromal stages of AD, and that the presence of the Met-BDNF allele is associated with a higher risk of cognitive deterioration, particularly in the presence of the APOE*4 allele.

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Statement of interest

The authors have neither financial disclosure nor conflict of interest concerning the information provided in this manuscript. Orestes V. Forlenza and Breno S. Diniz had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Supplementary material available online

Table showing collated results

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ORIGINAL INVESTIGATION

Serotonergic neurotransmission in early Parkinson's disease: A pilot study to assess implications for depression in this disorder

JAN C. BEUCKE[1+], IDUN UHL[2+], MICHAIL PLOTKIN[3], CHRISTINE WINTER[4,5], HANS-JÖRG ASSION[2], TANJA ENDRASS[1], HOLGER AMTHAUER[6], ANDREAS KUPSCH[7] & GEORG JUCKEL[2]

[1]*Department of Clinical Psychology, Humboldt University, Berlin, Germany,* [2]*Department of Psychiatry, Ruhr University, LWL University Hospital, Bochum, Germany,* [3]*Department of Nuclear Medicine, Campus Virchow Klinikum, Charité Universitätsmedizin, Berlin, Germany,* [4]*Department of Psychiatry, Campus Mitte, Charité Universitätsmedizin, Berlin, Germany,* [5]*Department of Psychiatry, University Hospital Carl Gustav Carus, Technical University Dresden, Germany,* [6]*Department of Radiology and Nuclear Medicine, Universitätsklinikum Magdeburg, Otto-von-Guericke Universität Magdeburg, Germany, and* [7]*Department of Neurology, Campus Virchow Klinikum, Charité Universitätsmedizin, Berlin, Germany*

Abstract

Objectives. Depression, a disease usually accompanied by a serotonergic deficit, has been observed in about 40% of patients suffering from Parkinson's disease (PD). Thus, a serotonergic dysfunction in PD can be assumed. We aimed to investigate the interaction between serotonergic (5-HT) and dopaminergic activity in early PD. We hypothesized a serotonergic as well as a dopaminergic deficit in PD patients. We also assumed a correlation between these neurotransmitters indicating a relationship between dopaminergic and serotonergic function in PD. *Methods.* Nine unmedicated PD patients before and 12 weeks after L-dopa treatment and nine healthy subjects were examined using the loudness dependence of auditory evoked potentials (LDAEP), a promising indicator of central serotonergic function. Dopaminergic transporters (DAT) were collected using ^{123}I-FP-CIT and single photon emission computer tomography (SPECT). LDAEP values were correlated with ^{123}I-FP-CIT SPECT data. *Results.* A significant difference between LDAEP of controls and patients ($P = 0.05$) suggested lower serotonergic activity in PD. Twelve weeks after initiation of L-dopa treatment this difference was lost between patients and controls ($P = 0.20$). There was a trend towards a correlation between LDAEP and DAT ($r = 0.65$; $P = 0.057$) of the unmedicated patients, suggesting a low serotonergic activity may be related to a dopamine deficit in PD. *Conclusions.* Our results support the hypothesis that serotonergic neurotransmission is decreased in untreated PD and suggest that a low serotonergic activity may be related to the dopamine pathology in PD. This could be related to the high prevalence of depression in PD.

Key words: *Parkinson's disease, LDAEP, depression, serotonin, ^{123}I-FP-CIT SPECT*

Introduction

Parkinson's disease (PD) is a neurological disease caused by degeneration of dopaminergic neurons in substantia nigra (SNc). Among the neuropsychiatric problems of PD, depression is observed in about 40% of PD patients (Schrag et al. 2007). Interestingly, symptoms of depression may precede the classical symptoms of Parkinson's disease (Nilsson et al. 2002; Leentjens et al. 2003). Three years prior to the diagnosis of PD the incidence of depression is increased, and patients who eventually develop PD in the future, have a 2.4 higher risk factor for symptoms of depression (Leentjens et al. 2003). It was tried to explain this increased risk for depression in PD with the "serotonergic hypothesis" (Mayeux 1990). Based on the findings of reduced serotonergic activity in PD, inhibition of striatal dopamine release by serotonin and the fact that reduced serotonergic tone may present a risk factor for depression it was hypothesized that reduced serotonergic tone may be a physiological adaptation to reduced dopaminergic activity (Mayeux 1990).

However, while the dopaminergic disturbances in PD have been extensively studied, less is known

Correspondence: Idun Uhl, MD, Department of Psychiatry, Ruhr-University Bochum, LWL University Hospital Bochum, Alexandrinenstr. 1, 44791 Bochum, Germany. Tel: +49 234 50770. Fax: +49 234 5077 1329. E-mail: idun.uhl@wkp-lwl.org
[+]Both authors contributed equally to this article.

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about the exact participation of the serotonergic system. There are numerous projections from the serotonergic nuclei to most cortical and subcortical areas of the human brain reflecting the involvement of serotonin in many different elementary functions, among them mood as well as motor function (Jacobs and Azmitia 1992; Sodhi and Sanders-Bush 2004; Azmitia 2007). Furthermore, neurodegeneration in PD does not only affect the dopaminergic neurons in SNc, but also other parts of the brainstem like the serotonergic raphe nuclei. Post mortem studies showed damage of serotonergic neurons of median and dorsal raphe nuclei in PD patients (Halliday et al. 1990a,b). Post mortem receptor autoradiography revealed a reduced binding of the central serotonin transporter of the striatal cortex in PD (Chinaglia et al. 1993). In a study using [^{11}C]WAY 100635 and positron emission tomography (PET) in advanced PD, 5-HT$_{1A}$-receptor binding was reduced (Doder et al. 2003). However, these findings indicate a 5-HT dysfunction in advanced PD, while the role of 5-HT in early PD remains unclear.

In addition, neuroanatomical findings suggest close interaction between the dopaminergic and the serotonergic systems (Bouthenet et al. 1987; Le and Simon 1991; Jacobs and Azmitia 1992; Peyron et al. 1995), and pharmacological and electrophysiological studies imply modulating effects of dopamine on the serotonergic function. Thus, a "facilitatory role" of dopamine on serotonergic function is suggested, since dopaminergic transmission is correlated with increased serotonergic neurotransmission (Mendlin et al. 1999).

Regarding 5-HT, the loudness dependence of auditory evoked potentials (LDAEP) represents a well established indicator for central serotonergic function. A high amplitude of the N1/P2-component indicates a low serotonergic activity, and vice versa (Hegerl and Juckel 1993; Juckel et al. 1997, 1999, 2003). Both, studies in animals (Juckel et al. 1997, 1999) and in humans (Hegerl et al. 1996; Wang et al. 1996) support this concept. A dysfunction of the central serotonergic system seems to occur in a variety of neuropsychiatric conditions and diseases like schizophrenia (Juckel et al. 2003), obsessive-compulsive disorder (OCD) (Carrillo-de-la-Pena 1992), depression (Linka et al. 2004), suicidality (Chen et al. 2005) or borderline personality disorder (Norra et al. 2003). With respect to depression the LDAEP has been used to estimate the response to serotonergic antidepressants with a high LDAEP indicating a better therapeutic outcome (Paige et al. 1994; Hegerl et al. 2001; Linka et al. 2005).

While the serotonergic and the dopaminergic systems may overlap in Parkinson's disease, the specific mechanisms are unclear. Especially the degeneration of dopaminergic neurons in PD and thus increased dopaminergic activity due to L-dopa therapy could influence the serotonergic neurotransmission (Fox et al. 2009; Nagatsu and Sawada 2009). In advanced PD, findings of 5-HT and DA dysfunction singularly coexist across studies (Taylor et al. 2009; Devos et al. 2010), but specific indicators of DA are needed in the *same* sample of patients to investigate the effect of dopaminergic pathophysiology on 5-HT in humans. In this context, it is also of particular interest, if the initialisation of L-dopa therapy affects 5-HT transmission. We used the radioligand ^{123}I-FP-CIT, specifically binding to dopamine transporters (DAT), and single photon emission computer tomography (SPECT) to collect indicators of DA degeneration and LDAEP for assessing 5-HT in unmedicated PD patients. LDAEP measurements were repeated after 12 weeks of L-dopa therapy. We hypothesized that the group of unmedicated PD patients will show a higher LDAEP, indicating lower serotonergic activity, compared to healthy controls. In terms of the interaction of DA and 5-HT, we hypothesized that LDAEP values will correlate with ^{123}I-FP-CIT SPECT data, and that the group difference in LDAEP will be absent after L-dopa therapy, thus indicating a possible influence of dopaminergic activity on central serotonergic function.

Methods and material

Patients and controls

We examined nine unmedicated patients (two females) suffering from Parkinson's disease. These patients had been recruited by the Movement Disorder Program, Clinic for Neurology, Campus Virchow Klinikum, Charité, University Medicine, Berlin. Patients were included according to the Brain Bank Criteria for Parkinson's disease (Hughes et al. 1992). The mean age was 64.33 ($\pm$8.41) years. The control group consisted of two female and seven male healthy subjects (aged 65 $\pm$ 8.3). Controls were matched according to gender and age. Thus, age of patients did not differ from age of controls ($t(16) = -0.169$, $P = 0.868$). Exclusion criteria for the study comprised: medication affecting the CNS, comorbid psychiatric disorders as assessed by the structured clinical interview for DSM-IV (SCID), drug abuse, contraindications for MRI examination.

The study was approved by the Charité ethics committee according to the Declaration of Helsinki and by the German Radioactivity Protection Authorities. All participants gave written informed consent to take part in the study.

Study design

The group of patients underwent four testing days. On the first day, ^{123}I-FP-CIT SPECT and LDAEP measurement were conducted and several questionnaires (see below) were applied. Following these procedures L-dopa treatment was initiated with increasing dosages of the drug starting with a mean dose of 150 mg L-dopa and a decarboxylase inhibitor. On the last testing day, the mean L-dopa dose of the patients was 612 (±296) mg per day. The patients were asked to answer the questionnaires 4, 8 and 12 weeks after the first appointment. During the last testing day, LDAEP measurement was repeated. Healthy controls were tested only once. LDAEP investigations and questionnaires in healthy controls followed the same procedure as in PD patients.

Procedures

LDAEP testing took place at the Laboratory of Clinical Neurophysiology at the Department of Psychiatry, Charité Berlin. The recordings were performed in an electrically shielded and sound-attenuated room adjacent to the recording apparatus (BrainAmp, Brain Products GmbH, Munich, Germany). Patients and controls were seated in a slightly reclined chair with a headrest. Evoked responses were recorded with 32 non-polarisable silver-silver chloride electrodes referred to FCz. Impedances remained below 5 kΩ throughout the testing. Pure sinus tones (1000 Hz, 40 ms duration with 10 ms rise and 10 ms fall time, ISI randomized between 1800 and 2200 ms) of five intensities (79, 87.5, 96, 104.5, 111 dB sound pressure level, generated by a PC stimulator) were presented binaurally in a pseudorandomised form by audiometry-headphones. Data were collected with a sampling rate of 500 Hz and an analogous bandpass filter (0.16–50 Hz). Periods of 350 ms pre-stimulus and 800 ms post-stimulus were evaluated for 70 segments of each intensity condition. The first five stimulus tones were excluded from further analysis to avoid short-term habituation effects. For artefact correction segments were excluded if the voltage amplitude had exceeded ±100 μV at any of the 32 channels at any time during the averaging period. The remaining segments were averaged separately for the five different intensities. A minimum of thirty segments per intensity was necessary for further evaluation. The peaks were determined at the Cz electrode. The lowest point between 50 and 180 ms after the stimulus was considered N1, the highest point between 150 and 270 ms P2. The peaks were localised semi-automatically, but visually controlled and corrected or excluded, if necessary. The N1/P2 amplitude was calculated as the amplitude difference between N1 and P2. The loudness dependence was calculated with the help of the median slope of the amplitudes of the different intensities, i.e. they were described as the linear regression of all possible connections between the amplitude values to the single loudness levels (Hegerl and Juckel 1993).

In order to evaluate severity of the Parkinson symptoms Unified Parkinson's Disease Rating Scale (UPDRS-III) (Fahn and Elton 1987) was used. Hamilton depression scale (HAMD) (Hamilton 1960) and Beck Depression Inventory (BDI) (Beck et al. 1961) assessed depressive symptoms before and after L-dopa treatment. These tests were carried out in their German versions before initiation of L-dopa treatment and 4, 8 and 12 weeks later.

^{123}I-FP-CIT SPECT was employed to collect data about the current DA transporter status and to support the diagnosis of idiopathic Parkinson's disease. In order to protect healthy controls from unnecessary exposure to radiation ^{123}I-FP-CIT-SPECT was not carried out in this group. SPECT imaging was performed at the Clinic of Radiology and Nuclear Medicine, Charité Berlin (Campus Virchow). The thyroid gland had been blocked using 900 mg of sodium perchlorate 30 min before the tracer was applicated intravenously. Four hours after injection, subjects were examined using a triple headed gamma camera (Multispect 3, Siemens Medical Systems, Germany) with a low-energy, high-resolution collimator. The reconstructed resolution was 13 mm full width at half-maximum. Slice thickness was fixed at 3.5 mm. For the acquisition of SPECT images a step and shoot mode with 120 projection angles over 360° was used with a matrix size of 128 × 128. The energy window was centred on 159 keV (20%). SPECT data were reconstructed with the help of a Butterworth filter (cutoff frequency 0.38 Nyquist, order 6). The first order method of Chang (Chang 1978) was employed to correct attenuation. MPI tool software (ATV Inc., Kerpen, Germany) was used for automatic coregistration of SPECT data. These data were analysed in a semi-quantitative way using irregular regions of interest (ROIs) drawn manually. These were placed on transaxial MR slices and transferred onto corresponding SPECT slices. Three ROI were located in the basal ganglia. ROI were placed in the caudate nucleus as well as in the putamen of either hemisphere. Moreover, we determined another ROI including putamen and caudate nucleus in both hemispheres to obtain data of a ROI involving the whole striatum. An area in the occipital cortex served as a reference region. The specific DAT binding was calculated as follows: DAT = specific binding in basal ganglia–non-specific binding = (basal ganglia–occipital cortex)/occipital cortex.

Statistical analysis

Statistical analysis was performed using SPSS® 12 for Windows (SPSS Inc., Chicago, IL, USA). Using the Kolmogorov–Smirnov test, data were examined for normal distribution. In order to compare LDAEP values between patients before and after therapy to healthy controls, *t*-test for independent samples was used. Pearson correlation coefficient (*r*) was performed to test for relationship between LDAEP values and ^{123}I-FP-CIT SPECT data, and for correlations between LDAEP and rating scales. In terms of clinical scales, *t*-test for paired samples was used to compare BDI and HAMD values in PD patients before and after therapy. A repeated measures analysis of variance (ANOVA), accounting for all four testings, was conducted to further address this question. Finally, effect sizes (*d*) were calculated. The significance threshold for all group comparisons and correlations was set at $P < 0.05$.

Results

In unmedicated PD patients, LDAEP at Cz was 2.41 ± 1.78 μV/7.5 dB. Twelve weeks later, the patients' LDAEP at Cz was 1.56 ± 0.85 μV/7.5 dB. Healthy subjects showed a mean LDAEP of 0.78 ± 1.52 μV/7.5 dB. This group difference between unmedicated patients and healthy controls reached significance ($t(16) = 2.088$, $P = 0.05$) (Figure 1). Correlational analysis revealed a strong trend for a positive correlation between LDAEP values and ^{123}I-FP-CIT data in unmedicated patients ($r = 0.65$, $P = 0.057$) (Figure 2). After 12 weeks of treatment, PD patients' LDAEP values did no longer differ from healthy controls ($t(16) = 1.345$, $P = 0.20$). Analysis of depression scales revealed no significant differences between patients and healthy controls (Table I). Although depressive symptoms in the patients were mild, a slight reduction of symptom severity was observed in the course of time, though not reaching significance when a one-way ANOVA with repeated measures was applied (Table I). In contrast, a significant decrease of UPDRS-III scores was observed in the course of the patients' treatment indicated by one-way repeated measures ANOVA ($F(3) = 6.4$, $P < 0.05$, $d = 0.81$). Correlation coefficients between LDAEP and the depression rating scales did not reach significance at any point of time.

Discussion

The present study aimed to test the hypothesis of decreased 5-HT function in early PD. Moreover, the relationship between dopaminergic pathophysiology in PD and 5-HT function was of interest. The LDAEP of drug-naive PD patients was collected before and after 12 weeks of L-dopa treatment and compared to healthy controls. Consistent with our hypothesis, we found a significant group difference between unmedicated PD patients and healthy controls concerning the LDAEP, suggesting low 5-HT levels in early PD. After 12 weeks of L-dopa treatment, the LDAEP of patients and healthy subjects did not differ anymore, which suggests normalization of also the serotonergic function by L-dopa therapy.

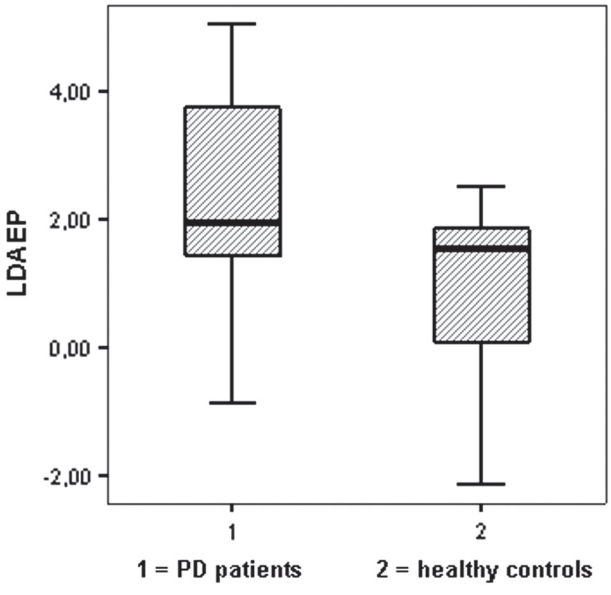

Figure 1. LDAEP at Cz before L-dopa treatment started. A significant difference between PD patients and healthy subjects was observed ($P = 0.05$).

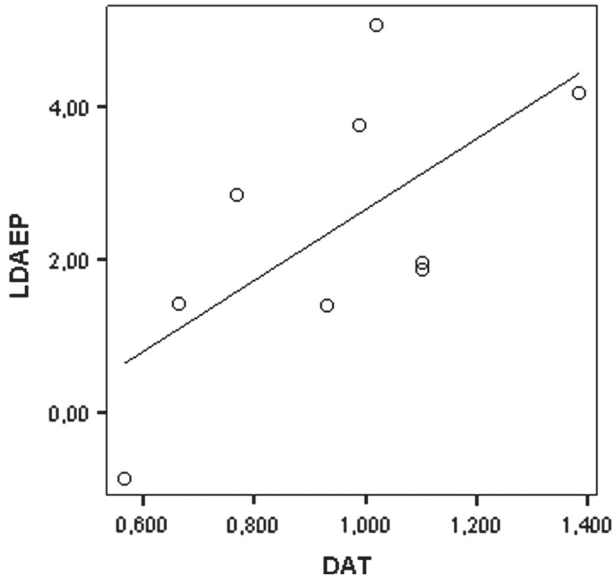

Figure 2. A positive correlation between LDAEP and FP-CIT SPECT values in the PD patients (r = 0.65, $P = 0.057$).

Table 1. Severity of depressive (HAMD, BDI) and PD (UPDRS-III) symptoms, described by mean ±SD, assessed before (t_1), during (t_2, t_3) and after (t_4) L-dopa treatment in PD patients, and once in healthy controls.

Time of examination	HAMD	BDI	UPDRS-III
t_1	4.67±4.18	4.44±3.54	24.11±8.87
t_2	1.78±1.72	3.78±2.77	22.17±9.61
t_3	2.22±1.30	3.33±3.16	19.11±6.77
t_4	1.78±1.86	3.22±2.68	19.00±5.00
P (*t*-test)	0.066	0.163	< 0.05
P (ANOVA)	0.100	0.386	< 0.05
d	0.96	0.39	0.73
healthy controls	1.56±2.30	3.56±4.04	10.33±3.09

HAMD, Hamilton Depression Rating Scale; BDI, Beck Depression Inventory; UPDRS-III, Unified Parkinson's Disease Rating Scale, motor part. *P* values from paired *t*-test and ANOVA indicate signifance; *d* effect size of improvement due to L-dopa therapy.

Thus, these results seem to support the theory of a relevant serotonergic dysfunction in Parkinson's disease which can be influenced and balanced by L-dopa treatment, and which might be related to the high incidence of depression in PD.

Our study also revealed a relationship between decreased serotonergic activity measured by LDAEP and decreased dopamine transporter level (as measured by ^{123}I-FP-CIT SPECT) which points to an interaction between the serotonergic and the dopaminergic systems with the possibility of a "facilitatory role" of dopamine (Mendlin et al. 1999). While there might also be a serotonergic dysfunction in untreated Parkinson's disease on its own, the possibility of an interaction between the dopaminergic and the serotonergic system has to be discussed. There is other evidence that the monoaminergic systems of the brain might be interacting. A decreased LDAEP was found in cats when the dopamine agonist apomorphine was applied (Juckel et al. 1997). Moreover, a current animal study by our group provided evidence for an interaction of the serotonergic and the dopaminergic systems (Winter et al. 2007). In this study, depressive-like behaviour was induced in rats by lesioning the SNc and the ventral tegmental area and could be reduced by citalopram and L-dopa treatment. In OCD patients, LDAEP correlated with the availability of serotonin transporters (SERT) in midbrain/pons and with the striatal DAT (Pogarell et al. 2004). Moreover, it was shown that the selective serotonin reuptake inhibitor citalopram caused a decreased SERT and an increased DAT availability in OCD (Pogarell et al. 2005). Furthermore, hyperechogenicity of substantia nigra and raphe hypoechogenicity in PD patients examined with transcranial sonography were associated with depression prior to the diagnosis of PD suggesting in nigrostriatal vulnerability in depressive patients (Walter et al. 2007). It was also shown that the selective serotonin reuptake inhibitor sertraline did not only improve mood in depressed PD patients but also motor symptoms of all UPDRS domains (Kulisevsky et al. 2008). This finding could also indicate interaction between serotonergic and dopaminergic function in the pathophysiology of PD. Animal studies also support the theory of an interaction between serotonergic and dopaminergic systems; L-dopa-induced dyskinesia was found to be associated to central serotonergic activity (Carta et al. 2006, Carta et al. 2007). Another animal study provided evidence of decreased 5-HT in Parkinson's disease and an increased 5-HT uptake following L-dopa treatment, suggesting a normalization of 5-HT levels similar to the results of the study presented here (Kääriäinen et al. 2008). Using a hemiparkinsonian rat model, it was found that L-dopa administration increased striatal serotonin immunoreactivity in rats with L-dopa-induced dyskinesia (LID) and that serotonin is negatively associated with LID (Gil et al. 2010). It could be concluded that, on the one hand, L-dopa therapy influences serotonin function and, on the other hand, that specific serotonergic treatment might improve adverse effects of L-dopa therapy. However, another animal study showed decreased 5-HT levels after long-term L-dopa treatment suggesting further necessity of distinguishing between different stages of PD (Borah and Mohanakumar 2007). Taylor et al. (2009) showed symptoms of depression in mice with a reduction in vesicular monoamine transporter expression (VMAT2-deficient), thus establishing a model of depression and other nonmotor symptoms in PD which also includes interaction between serotonergic and dopaminergic neurotransmission. These studies underline the hypothesis of a functional interaction between the central serotonergic and dopaminergic systems, which is further corroborated by our findings.

On the other hand, when the dopaminergic system was modulated in healthy subjects, LDAEP did not change (O'Neill et al. 2006) which might provide evidence for the specifity of LDAEP regarding the serotonergic neurotransmission. Based on this result, a serotonergic dysfunction in unmedicated PD patients might be discussed as a separated entity. This hypothesis is supported by results of other studies which showed that Lewy body degeneration in Parkinson's disease also affects serotonergic raphe nuclei (Halliday et al. 1990a,b). If possible changes of serotonergic neurotransmission in PD patients are assumed, serotonin reuptake and serotonin release reflecting synaptic availability of the neurotransmitter should be considered separately. In fact, the

present findings correspond with another study in which a significant decrease of the 5-HT_{1A}-receptor binding in PD patients compared to healthy controls was found when presynaptic conditions were examined (Halliday et al. 1990b).

An aspect that could account for contradictory results in this field of research is the fact that different methodological approaches shed light on separate mechanisms of 5-HT function. While the LDAEP is reflecting synaptical 5-HT release, the 5-HT_{1A}-receptors, inhibiting 5-HT release, and the serotonin transporters are closely associated with presynaptic conditions. A concomitant study by our group including the same patient group as in the present study could not disclose differences between midbrain serotonin transporter availability before and after L-dopa treatment using the highly specific ^{123}I-ADAM SPECT ligand (Beucke et al. in preparation). Taken together, these findings advocate for the view that synaptical 5-HT release, as measured by the LDAEP, is deficient in early PD, while the 5-HT transporter seems to be preserved.

In summary, we provide evidence for a decrease of 5-HT release in early PD, and report relationships between 5-HT function and dopaminergic function. The small sample size is one limitation of the study. Further studies attempting to further clarify the role of 5-HT in PD should include PD patients with comorbid major depression or patients at risk for PD with and without depressive symptoms. In general, depression and medication status, illness duration, and methodological differences between distinct measures of 5-HT, delineating separate mechanisms of 5-HT function, have to be considered to advance the understanding of the role of 5-HT in PD, the comorbidity with depression and successful treatment strategies.

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Statement of interest

None to declare.

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ORIGINAL INVESTIGATION

The safety and tolerability of clozapine in aged patients: A retrospective clinical file review

DANIEL W. O'CONNOR[1], CHRISTINE SIERAKOWSKI[2], LOI FEI CHIN[3] & DHIREN SINGH[1,4]

[1]*School of Psychology and Psychiatry, Monash University, Melbourne, Australia,* [2]*Caulfield Hospital, Melbourne, Australia,* [3]*Southern Health, Melbourne, Australia, and* [4]*Frankston Hospital, Melbourne, Australia*

Abstract

Objectives. A clinical file review was conducted of clozapine use in three aged psychiatry services in Melbourne, Australia, to compare its safety and tolerability with findings reported in the literature. *Methods.* The review period spanned the intervals from 2008 to the services' origins between 11 and 15 years earlier. The files of all patients treated with clozapine during this period were checked with respect to adverse effects and the reasons for ceasing treatment. *Results.* Clozapine was prescribed to 75 patients (mean age 74.2 years, range 65–89) with doses ranging from 25–800 mg daily (mean 296 mg). Treatment was stopped within the review period in 37 (49%) cases. Reasons for discontinuation included death (n=14), non-fatal adverse events (n=12), patient choice (n=8) and other factors (n=3). While none of the 14 deaths could be linked directly to treatment, orthostatic hypotension might have contributed to a single fatal cerebrovascular accident. There were three cases of "red alert" leukopenia, none of which progressed to agranulocytosis. In general, side effects were more frequent than in a previous report concerning aged patients, most probably because clozapine doses were higher. *Conclusions.* Most of the adverse events leading to treatment cessation occurred within the first month, emphasising the need for slow titration. Strict monitoring procedures ensured that there were no fatal haematological adverse events.

Key words: *Old age psychiatry, psychopharmacology, antipsychotics, clozapine, treatment*

Introduction

Clozapine, a second-generation antipsychotic, has proven efficacy in reducing both the positive and negative symptoms of otherwise treatment-resistant schizophrenia and schizoaffective disorder (Wahlbeck et al. 1999; Ciapparelli et al. 2000). It causes minimal extrapyramidal side effects, produces no tardive dyskinesia and has little effect on prolactin secretion (Miller 2000). Its use is limited, however, by agranulocytosis, an uncommon but life-threatening condition that occurs most often in the first 3 months of treatment with the risk increasing 10-fold from age 21 to 50+ years (Alvir and Liberman, 1994). Deaths have also been associated with myocarditis and cardiomyopathy (Kilian et al. 1999).

Given clozapine's potent antagonist activity at alpha-adrenergic, muscarinic and histaminergic receptors, other unwanted consequences can include sedation, orthostatic hypotension, hypersalivation, urinary retention and constipation (Stahl 2000). High rates of metabolic syndrome with weight gain, diabetes mellitus and dyslipidemia are also of concern (Lieberman 1998; Henderson et al. 2005).

These sequelae are well described but few reports have focussed on older people for whom adverse events might be commoner and more serious because of age-related pharmacokinetic changes, physical co-morbidity and drug interactions, culminating in treatment cessation. In one cohort study, patients who started clozapine after 50 years of age were 4 times more likely to stop taking it within 3 years than patients aged 17–29 years (MacGillivray et al. 2003).

A secondary analysis of clozapine's use in genuinely old patients between 1966 and 1998 identified 14 case reports and chart reviews concerning a total of 139 patients, almost all aged ≥65 years, of whom

Correspondence: Daniel O'Connor, MD, Professor, Aged Mental Health Research Unit, Kingston Centre, Warrigal Road, Cheltenham, Victoria 3192, Victoria, Australia. Tel: +61 3 9265 1700; Fax: +61 3 9265 1711. E-mail: daniel.oconnor@med.monash.edu.au

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only 15 had notable adverse effects, including three cases of leukopenia, three of orthostatic hypotension and two of hypersalivation (Barak et al. 1999). Doses were very low, however, with a mean of only 134 mg daily. In similar vein, clozapine produced no more side effects than chlorpromazine and haloperidol in two randomised trials involving 10 patients aged ≥55 years (Howanitz et al. 1999) and 42 patients aged ≥60 years, respectively (Salganik et al. 1998). By contrast, two of five patients aged ≥63 years who were prescribed clozapine 100 mg daily developed agranulocytosis (Herst and Powell 1997). These findings are too varied to permit clear conclusions.

In a recent survey of 135 British old age psychiatrists, only 45 had prescribed clozapine to their patients. Most saw it as having a useful role to play but were concerned by the few studies of safety in this population. Those who had prescribed it were more positive than those who had not (Paranthaman and Baldwin 2006). For this reason, we report here on a file review of clozapine use in three Australian aged psychiatry services with the object of adding to the existing small evidence base in older age groups.

Methods

The national clozapine database and hospital pharmacy records were used to identify all individuals prescribed clozapine by the three neighbouring publicly-funded aged psychiatry services that provide community and inpatient care to people aged ≥65 years in southeast Melbourne. The audit period spanned the intervals from 2008 to the services' origins between 11 and 15 years earlier.

Clinical files were checked by consultant psychiatrists who noted medical and nursing entries, discharge summaries, medication and observation charts, ECG and laboratory results including the full blood counts required before every prescription. All plausible adverse responses to clozapine were noted together with patients' age, gender, diagnosis, co-morbid physical conditions, length of treatment with clozapine and reasons for discontinuation where applicable. The review was approved as a quality assurance activity by the local health research ethics committee. All data were collected in a de-identified format.

In Australia, as in other countries, a nationwide monitoring scheme tracks patients' neutrophil and total white blood cell (WBC) counts at specified intervals and triggers mandatory action whenever "amber" and "red" levels are breached with the object of preventing agranulocytosis (Honigfeld et al. 1998; Novartis Pharmaceuticals 2002). An "amber" WBC of 3.0–3.5 $\times$ 10^9/l and/or a neutrophil count of 1.5–2.0 $\times$ 10^9/l prompts more frequent checks. A "red" WBC of $<3.0 \times 10^9$/l and/or a neutrophil count $<1.5 \times 10^9$/l leads to immediate cessation. Non-mandatory guidelines are now in place to minimise myocarditis and cardiomyopathy by means of ECGs and cardiac enzyme assays at baseline, day 7 and day 14, and echocardiography after 6 months.

Results

Patient details

Clozapine was prescribed to 75 people, 47 of whom were female, with a mean age of 74.2 years (range 65–89). Ages on starting treatment ranged from 54 to 88 years (mean 67.9). Clozapine doses ranged from 25–800 mg/day with a mean dose of 296 mg. Excluding four patients who were lost to follow-up after transfer to other mental health services between 1 and 7 years after starting clozapine, the mean total time on treatment was 53.4 months (range 1–168).

The reasons for prescribing clozapine included (with numbers) schizophrenia (53), schizoaffective disorder (18), Parkinson's disease with psychotic symptoms (two), bipolar disorder (one) and acquired brain injury (one). Co-morbid conditions included intellectual disability (two) and dementia (seven). All patients had failed to respond to at least two other antipsychotic agents.

Only seven patients had no documented medical problems prior to treatment. The commonest diagnoses (with numbers) were hypertension (21), asthma/chronic airways disease (19), dyslipidemia (16), ischemic heart disease, atrial fibrillation or cardiac failure (15), diabetes mellitus (14), gastro-oesophageal reflux or ulcers (11), osteoarthritis (nine), transient ischaemic attack or stroke (six), and Parkinson's disease (six). The mean number of treated medical conditions was 2.8 (range 0–9).

Data were missing for the following clinical assays (with numbers): fasting lipids (41), fasting glucose or HbA1c (38), echocardiograms (37), cardiac enzymes (23) and ECGs (22).

Discontinuations

Thirty-seven of the 75 (49%) patients had ceased treatment by the time of the review including 14 of the 28 males (50%) and 23 of the 47 females (49%). Eight patients stopped treatment within a month of starting and another eight stopped between 1 and 6 months. The remainder were scattered over the whole of the follow-up period. The reasons for stopping treatment (with numbers) included death (14), adverse events (12), patient choice (eight) and physical frailty (two).

Of the 14 patients (19%) who died while taking clozapine, none went to autopsy. Periods on medication ranged from 4 to 132 months (mean 62.4). Ages at death ranged from 62 to 88 years (mean 72). A link to treatment could not be excluded in a patient with malignancy and ischemic heart disease who died 6 years later of a cerebrovascular accident in the setting of clozapine-related orthostatic hypotension. The connection to clozapine was less clear for two patients who choked on food after treatment for 10 and 11 years, respectively, with no prior history of hypersalivation or dysphagia. The remaining deaths were judged to result from coincidental conditions including cancer, stroke, sepsis, renal failure, respiratory failure and gastrointestinal haemorrhage.

Twelve patients (16%) ceased clozapine after the adverse events listed in Table I. There were three cases of "red" level leukopenia and three of serious cardiac pathology. A patient who developed parkinsonism was also taking lithium carbonate.

Eight patients refused treatment or failed to adhere to the monitoring protocol. Two stopped clozapine because of increasing physical frailty. Reasons for discontinuation could not be established in the final case.

Other adverse effects

In addition to the adverse events listed in Table I, side effects (with numbers) that did not lead to discontinuation included sedation (28), hypersalivation (25), constipation (16), orthostatic hypotension (11), weight gain (eight), "amber" level leukopenia (four), tachycardia (three), "red" level leukopenia with successful re-challenge (two), and seizure (two). There was a single instance each of myocardial infarction, dysphagia, fever and fatigue (one). Metabolic parameters were not monitored routinely. Even so, there were five known incident cases of dyslipidemia and four of diabetes mellitus.

Discussion

This file review has several limitations. Clozapine prescriptions and WBC counts were scrupulously documented but clinical records in other respects were sometimes incomplete and difficult to interpret. For this reason, we made no attempt to rate clozapine's effectiveness, focussing instead on adverse events. More importantly, we cannot judge the relative contributions of other medications and co-morbid medical conditions to patients' reported outcomes. By way of balance, our series of patients aged ≥65 years is the largest reported to date.

Psychiatrists are naturally wary of the association of clozapine with agranulocytosis. One case of "red" alert leukopenia was accompanied by potentially life-threatening myocarditis and myocardial infarction. By contrast, two other "red" alerts resolved quickly and the patients were re-challenged without ill effects. Such a step requires consultation with an experienced haematologist. Five instances of "amber" neutrophil counts arose between 1 and 3 years after starting treatment, confirming the need for ongoing monitoring (Alvir and Liberman 1994). No deaths resulted from low WBC counts in this or earlier reports of aged patients (Oberholzer et al. 1992; Salganik et al. 1998; Barak et al. 1999; Howanitz et al. 1999) though caution is certainly warranted and protocols must be followed carefully.

The deaths of aged patients with chronic psychoses might result directly or indirectly from self-neglect, substance abuse, medical avoidance and the motoric and metabolic consequences of long-term antipsychotic medications. Co-morbidity rates in this smaller but much older sample were high and some deaths were to be expected over the lengthy audit period. Blood dyscrasias aside, apportioning responsibility to a single condition is difficult.

Of the 14 deaths reported here, none could be attributed unequivocally to clozapine though orthostatic hypotension might possibly have contributed to a death due to stroke given the recognised link between the two conditions (Eigenbrodt et al. 2000). Isolated reports exist of non-fatal choking due to clozapine-induced hypersalivation or oesophageal dysfunction (Pearlman 1994; McCarthy and Terkelsen 1994), but the two patients who died of choking had no such history in contrast to another patient whose treatment-related dysphagia improved quickly once clozapine was stopped. Other more proximal risk factors for choking in old age include neurological disorder, cognitive impairment, dry mouth and an age-related decline in oropharyngeal muscle strength and coordination (Schindler and Kelly 2002).

Orthostatic hypotension led to treatment being stopped in two cases. Rates are higher in the elderly,

Table 1. Non-fatal adverse events leading to discontinuation.

Adverse events	Months on clozapine
Orthostatic hypotension	≤1
Orthostatic hypotension	≤1
Sedation, dysphagia	≤1
Fever, tachycardia	≤1
Fever, tachycardia	≤1
Myocarditis, myocardial infarction, "red" WBC	≤1
"Red" WBC	3
Parkinsonism	4
Myocardial infarction	7
"Red" WBC	15
Sedation, ataxia	19
Cardiomyopathy	72

especially in those with diabetes mellitus, Parkinson's disease and other autonomic neuropathies, and in those taking antihypertensives and diuretics (Low 2008). The frequency of sedation, hypersalivation and constipation was broadly in keeping with earlier reports (Lieberman 1998; Miller 2000). Fever was noted in three cases and is greatly concerning as it may herald myocarditis and agranulocytosis (Lieberman 1998). We cannot comment on rates of metabolic syndrome as relevant parameters were not checked routinely.

Half (49%) of patients discontinued clozapine during the audit period, most often within a few months of starting treatment, emphasising the need for slow, graded titration. This cessation rate was higher than in three of four earlier, smaller aged series (Oberholzer et al. 1992; Herst and Powell 1997; Barak et al. 1999; Lee et al. 2007), but lower than in the CATIE study in which 75% of younger subjects stopped their assigned second-generation antipsychotic within 18 months because of a perceived lack of efficacy, side effects or personal choice (Lieberman et al. 2005). Older people are perhaps more compliant with doctors' advice; some cannot express a choice, while others have their medications dispensed by caregivers. Doctors prescribing clozapine to frail, aged people must therefore carefully balance antipsychotic efficacy with side effects, patients' preferences and their quality of life.

In conclusion, this audit covered a longer period, and included a larger number of genuinely old people, than any previous series. No deaths could be clearly attributed to clozapine though a link between orthostatic hypotension and stroke could not be excluded. Monitoring systems identified nine cases of reduced neutrophil or WBC counts but none progressed to agranulocytosis. In general, side effects were roughly twice more frequent than reported by Barak et al. (1999) in their review of 14 case series and chart reviews, most probably because mean clozapine doses were 2 times higher.

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Statement of interest

None to declare.

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ORIGINAL INVESTIGATION

Adolescent toluene exposure produces enduring social and cognitive deficits in mice: An animal model of solvent-induced psychosis

BIH-FEN LIN[2,3#], MEI-CHUN OU[1#], SHIANG-SHENG CHUNG[1,4], CHENG-YOONG PANG[2] & HWEI-HSIEN CHEN[1]

[1]*Institute of Pharmacology and Toxicology, Hualien, Taiwan,* [2]*Institute of Medical Sciences, Haulien, Taiwan,* [3]*Department of Laboratory Medicine and Biotechnology, Tzu Chi University, 701, Section 3, Chung Yang Road, Hualien, 970, Taiwan, and* [4]*Department of Pharmacy, Yuli Veterans Hospital, Hualien, Taiwan*

Abstract

Objectives. Abuse of toluene-containing volatile solvents by adolescents is a significant public health problem. The present study characterized the long-term behavioural and neurochemical consequences of toluene exposure during adolescence. *Methods*. Male NMRI mice received one injection per day of either toluene (600 mg/kg) or corn oil during postnatal days (PN) 35–37 and (750 mg/kg) during PN38–39 and PN42–46. A variety of psychiatric disorder-relevant behavioural tests were examined at PN56–P84. *Results*. The toluene-exposed mice were significantly deficient in the social interaction test, nesting behaviour, social dominance tube test, and novel objective recognition test. However, toluene exposure did not affect locomotor activity and behavioural profiles in the forced swimming test, tail suspension test, emergence test and elevated plus maze. Neurochemically, the turnover rates of dopamine in the prefrontal cortex, striatum and nucleus accumbens were reduced in toluene-treated mice. *Conclusions*. Adolescent toluene exposure leads to social deficits and cognitive impairment at adulthood as well as neurochemical dysfunction in mice, which correlate with the symptoms observed in patients suffering from solvent-induced psychosis. These findings highlight the need for understanding the effects of solvent abuse on the developing nervous system and reveal an animal model suitable for research into pathophysiology of neurological and psychiatric consequences of solvent abuse.

Key words: *Toluene abuse, adolescent, psychosis, dopamine, mice*

Introduction

Voluntary inhalation of volatile solvents found in commercial products such as glues, paint products, cleaning fluids and lighter fluids to achieve a euphoric state is prevalent worldwide, especially among juveniles and adolescent children. Recurrent solvent use is associated with serious health problems including cerebellar ataxia, Parkinsonism, encephalopathy, trigeminal neuropathy, hepatorenal syndrome, hepatotoxicity, and "sudden sniffing death" (Williams and Storck 2007). Moreover, psychiatric disorders are highly prevalent among solvent users. The solvent-induced schizophreniform psychosis is commonly observed. It is considered to be brief, lasting from a few hours to at most a few weeks beyond the intoxication. In severe cases, the patients suffering from dependence on solvents and those in a psychotic state due to chronic solvent use show psychiatric symptoms for a long time after detoxification (Byrne et al. 1991; Okudaira et al. 1996; Hernandez-Avila et al. 1998). The symptoms observed among the solvent-induced psychosis include amotivation, intoxication, emotional instability, delusions, hallucinations, disinhibition and memory impairment (Goldbloom and Chouinard 1985; Wada et al. 2005). In addition to psychotic symptoms, solvent users have a high lifetime prevalence of mood, anxiety and personality disorders (Wu and Howard 2007). It seems that solvent inhalation might be related to induction of these psychiatric disorders.

According to clinical evidence, the psychiatric sequelae of solvent abuse are serious and potentially irreversible. Given the prevalence of inhalant abuse

Correspondence: Hwei-Hsien Chen, Institute of Pharmacology and Toxicology, Tzu Chi University701, Section 3, Chung-Yang Road, Hualien, 970, Taiwan. Tel: +886 3 856 5301, ext. 7198. Fax: +886 3 856 1465. E-mail: hwei@mail.tcu.edu.tw
[#]Contributed equally to this work.

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in children and adolescents, it is important to know the long-term behavioural consequences of early toluene abuse and the possible pathophysiological mechanisms of psychiatric disorders induced by inhalants. Animal models are particularly important in this regard since they afford the opportunity to understand how solvent inhalation might lead to psychiatric disorders. Certainly, the primary difficulty in modelling solvent-induced psychiatric disorders in rodents is that they cannot self-report hallucinations, amotivation, and other related symptoms. Recently, mouse behaviours of potential relevance to signs and symptoms of schizophrenia have been developed (Powell and Miyakawa 2006). For example, increased locomotor activity or augmented locomotor response to psychotomimetic drugs, such as MK-801, ketamine or amphetamine is correlated with positive symptoms and decreased social interaction, altered social dominance in the tube test, and decreased nesting behaviour reflect social withdrawal represent the prominent negative symptoms of schizophrenia (Powell and Miyakawa 2006). In addition, several behavioural tests, such as elevated plus maze, emergence test, forced swimming test and tail suspension test, are commonly used to assess anxiety-like or depression-like behaviours in animals.

Since toluene is the chemical found in the most commonly abused inhalants, in the present study the long-lasting behavioural dysfunction after toluene exposure during adolescence in mice were examined. First, we examined schizophrenia-relevant behavioural testing in mice including spontaneous locomotor hyperactivity, enhanced locomotor hyperactivity elicited by methamphetamine, social withdrawal, and cognitive impairment. Second, we ascertained whether adolescent toluene treatment produces depression-like and/or anxiety-like behaviours. Finally, tissue monoamine contents were measured because dopaminergic and serotoninergic systems play important roles in schizophrenia.

In general, human exposed to toluene by inhalation. Continuous exposure to toluene vapours is usually used in rodent models to mimic the exposure in industrial workers. However, abusers do not continuously sniff glue for long periods, but rather prefer to titre their dose by repeatedly "huffing" very-high-exposure concentrations for only seconds to minutes. This is hard to be conducted in animal models. Actually, intraperitoneal injection of toluene to rodents, like inhalation exposure, produced biphasic locomotor activity and stereotypic behaviours, which resemble behavioural signs observed in toluene abusers (Riegel and French 1999; Chan et al. 2004) and full substitution for inhaled toluene in drug discrimination (Shelton and Slavova-Hernandez 2009). The concentration of toluene in the animal tissues at the time of testing determined the behavioural performance no matter the route of administration (Shelton and Slavova-Hernandez 2009). Furthermore, intraperitoneal injection can produce low variation of toluene concentrations in blood and this route of administration is also routinely used in animals to study drugs commonly abused by inhalation (e.g., cannabinoids and nicotine). Therefore, toluene was administered by intraperitoneal injection.

Materials and methods

Animals

Male NMRI mice (5 weeks) were supplied from the Laboratory Animal Center of Tzu Chi University (Hualien, Taiwan) and housed four to five per cage in a 12-h L:12-h D cycle with ad libitum access to water and food. All experiments were performed in accordance with the Republic of China animal protection law (Chapter III: Scientific Application of Animals) and approved by the Review Committee of the Tzu Chi University.

Forty-eight mice were treated with ascending doses of toluene (HPLC grade, 99.8%, Mallinckrodt Baker, Inc., KY, USA) (n=24) or corn oil (n=24) during postnatal day (PN) 35–46, corresponding to human adolescence. Toluene was diluted in various ratios with corn oil to achieve an injection volume of 10 ml/kg and administered intraperitoneally. Mice received one injection per day of either toluene (600 mg/kg, i.p.) or oil at PN35–37, 750 mg/kg at PN38–39, followed by a 2-day withdrawal period, and 750 mg/kg at PN42–46. The dose and duration of toluene treatment was chosen following preliminary experiments that showed this to elicit acute behavioural dysfunction such as locomotor hyperactivity and motor incoordination without subsequent animal loss.

All behavioural tests were performed in toluene-treated and control mice (n=16/group) during PN56–84. To prevent the influence of methamphetamine, those animals used for methamphetamine-induced locomotor hyperactivity (n=8/group) were not subjected to the other behavioural tests.

Toluene in blood

Blood samples were taken through heart puncture 1 and 3 h following the last injection of toluene. Toluene in blood was analyzed using a head-space gas chromatography–mass spectrometer (HP7649-HP6890-HP5973, Hewlett-Packard). The samples were thermostatted at 80°C for 20 min. Pressurization and injection times were 1.5 and 0.1 min, respectively. The temperature program for the column (HP-5MS) was as follows: 3 min at 50°C,

then 10°C/min to 60°C, then 30°C/min to 200°C, then hold for 0.5 min. The flow rate of helium carrier gas was 0.6 ml/min. Toluene concentration was determined by comparing toluene to *o*-xylene peak area ratios to a calibration curve determined from prepared standards.

Locomotor activity

The animals were placed into the test room at least 1 h before testing, moved from the home cage into an activity cage (TruScan Mouse chamber, Coulbourn Instruments Allentown, PA, USA) and spontaneous locomotor activity was measured in the activity cage (TruScan Mouse chamber, Coulbourn Instruments, Allentown, PA, USA) for 2 h. Methamphetamine-induced locomotor hyperactivity was examined by injection of methamphetamine (1 or 2 mg/kg, s.c.) or saline after the 2-h habituation period. The distance (cm) travelled was recorded for 60 min. A 70% alcohol solution was used to clean the inner surface of the apparatus between trials to remove any potentially interfering odours left by the previous mouse.

Social interaction test

This protocol was adopted for evaluation of negative schizophrenic symptom-like behaviours, which was modified from the original social interaction test in that aggressive behaviours (biting, boxing) and passive contact (sitting or lying with bodies in contact) were not included in the social interaction score (Qiao et al. 2001). The social interaction between pairs of mice was examined in an open-field box (35×35×30 cm) under normal room lighting.

Every mouse was randomly assigned to an unfamiliar partner in the same treatment group. That is a toluene-treated mouse was paired with another unfamiliar toluene-treated mouse, and a toluene-treated mouse was paired with another unfamiliar control mouse. Each pair of unfamiliar mice was placed in the apparatus for 10 min and the time that a pair spent in social interaction (sniffing and grooming the partner, following, mounting, and crawling under or over the partner) was recorded by an observer who was blind to the drug treatments.

Social dominance tube test

Toluene-treated and control mice were tested as described (Lijam et al. 1997) in a 30 cm long × 3.5 cm diameter tube. Two mice of different treatment groups were released toward each other from opposite ends of the tube. A subject was declared a "winner" when its opponent backed out of the tube. Each pairing was performed twice for a total of 32 trials.

Nest building experiment

Approximately 1 h before the dark phase, the group housed mice were transferred into individual cages. A standard piece of paper towel (23 × 23 cm) was placed in each cage overnight. The nests were assessed next morning using a scoring system described previously (Keisala et al. 2007): 0=no nest, 1=primitive flat nest (flat paper slightly elevated from the bedding), 2=more complex nest (wrapping and biting the paper), 3=complex accurate cup-shaped nest (walls and shredded paper), 4=complex hooded nest. The amount of paper damage was also assessed here, using the following scale: 0=intact paper or little damage (<5% paper destroyed), 1=some paper damage (5–20%), 2=pronounced paper damage (20–40%), 3=severe paper damage (>40%).

Novel object recognition test

The novel object recognition test was carried out as described previously (Kamei et al. 2006). The experimental apparatus consisted of a Plexiglas open-field box (35×35×30 cm high). The novel object recognition test procedure consisted of three sessions: habituation, training, and retention. The animals were videotaped in both training and retention sessions. Each mouse was individually habituated to the box, with 10 min of exploration in the absence of objects for three consecutive days (habituation session, days 1–3). During the training session, each animal was placed in the test box, and after a 5-min habituation period, two identical objects were introduced in two corners (approximately 30 cm apart from each other). Each animal was allowed to explore in the box for 5 min (day 4). An animal was considered to be exploring the object when its head was facing the object (the distance between the head and object was approximately 1 cm or less) or it was touching or sniffing the object. The time spent exploring each object was recorded by an experimenter blinded to the identity of the treatments, using stopwatches. After training, mice were immediately returned to their home cages. During the retention sessions, the animals were placed back into the same box 1 or 24 h after the training session, but one of the familiar objects used during training had been replaced with a novel object. The objects were different in shape and colour but similar in size. The animals were then allowed to explore freely for 5 min, and the time spent exploring each object was recorded as described above. A preference index in the retention session, a ratio of the amount of time spent exploring the novel object over the total time spent exploring both objects, was used to measure cognitive function. In the training session, the preference index was calculated as a ratio of the time spent

exploring the object that was replaced by the novel object in the retention session over the total exploring time. The objects used for first test (PN57–61) and second test (PN79–83) were different.

Elevated plus maze

The plus-maze was constructed of Plexiglas and consisted of two open and two closed arms (10 cm wide ×50 cm long, 50 cm walls for closed arms, 2 cm walls for open arms), intersected by a centre platform (100 cm^2), elevated 50 cm above the floor. Each animal was tested for 5 min on the maze and videotaped. The mice were placed on the central platform of the maze facing the open arm. The following indices were recorded: the total number of entries into open arms and closed arms and the total time spent in each type of arm. The percentage of time spent in the open arms was calculated for each animal and provided as the measures of anxiety. An entry was defined as the entry of all four feet into one arm and an arm exit was defined as two paws leaving the arm. Between tests, the maze was wiped clean with 70% ethanol.

Emergence test

The experimental apparatus consisted of a Plexiglas open-field box (35×35×30 cm high). The open field contained an aluminum cylinder (10 cm deep × 6.5 cm diameter) located lengthwise along one wall, with the open end 10 cm from the corner. Mice were placed into the cylinder and tested for 10 min. A trained observer blind to the treatment scored the following behaviours: the latency to leave the cylinder, defined as placement of all four paws into the open field, the number of entries into the cylinder with the initial placement counting as an entry, and the total time spent inside the cylinder.

Forced swim test

The forced swim test was conducted on two consecutive days. Mice were pre-exposed to the forced swim test for 15 min 24 h before test day to increase sensitivity (Cryan and Lucki 2000). Mice were placed in an acrylic cylinder (14 cm in height, 10 cm in diameter) filled with 10-cm high water (25±2°C). The mice were removed 15 min later, dried and placed in their home cage. Twenty-four hours after their first exposure, the animals were again placed in the swim apparatus for 5 min and behaviours were monitored. The rater of the behavioural patterns was blind with respect to the experimental conditions being scored. A time sampling technique was employed whereby the predominant behaviour in each 5-s period of the 300-s test was recorded. The predominant behaviour was assigned to one of three categories: climbing, swimming and immobility. Climbing behaviour consisted of upward directed movements of the forepaws along the side of the swim chamber. Swimming behaviour was defined as movement (usually horizontal) throughout the swim chamber, which also included crossing into another quadrant. Immobility was assigned when no additional activity was observed other than that required to keep the mouse's head above the water.

Tail suspension test

The tail suspension test was carried out as described previously (Cryan et al. 2004). Mice were individually suspended by the tail to a horizontal ring stand bar (distance from floor=30 cm) using adhesive tape (distance from tip of tail=2 cm). Typically, mice demonstrated several escape-oriented behaviours interspersed with temporally increasing bouts of immobility. A 6-min test session was used and scored by a trained observer who was unaware of the treatment. The parameter recorded was the number of seconds spent immobile.

Assay of dopamine, serotonin, and metabolites in brain by HPLC/ECD

Dopamine, serotonin and their metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA) were analyzed in the brains of mice exposed to toluene or controls (n=8). At PN85 mice were decapitated, and the brain was immediately excised and placed on ice for dissection and isolation of frontal cortex, striatum and nucleus accumbens. Tissues were weighed and stored at −80°C. Fresh-frozen brain areas were assayed for levels of DA, DOPAC, HVA, 5-HT, and 5-HIAA by using high-performance liquid chromatography with electrochemical detection. Dopamine, serotonin and their metabolites were separated on a microbore reverse-phase column (C-18, 5 mm, Unijet; BAS, West Lafayette, IN) with a mobile phase consisting of 0.1 M chloroacetic acid buffer with 0.74 mM octyl sodium sulfate, 34 nM EDTA, and 10% acetonitrile (pH 3) at a flow rate of 70 µl/min and detected by a 3-mm glass carbon electrode (Unijet; BAS) set at +0.75 V. The volume of injection was 20 µl.

Statistical analyses

Significance was determined using a Student's *t*-test. Where appropriate, for example with scaled data from the primary behavioural observations, non-parametric analyses were conducted using a Mann–Whitney test. The data obtained from the social dominance tube test were analyzed with the χ^2-test. The data for basal locomotor activity and MA-induced locomotor hyperactivity and social interaction at PN56 and PN84 were analyzed with a two-way ANOVA followed by a post hoc Student–Newman–Keuls test. $P<0.05$ was considered statistically significant.

Results

Behaviours, body weight gain, and blood toluene in the developing mice

The toluene exposure regimen used in the present studies did not produce sedation. The mice exposed to toluene showed mild ataxia but hyperactivity after the toluene injection. The toluene-treated mice were more agitated and irritable during the exposure period compared to controls. However, the emotional changes gradually disappeared after toluene withdrawal. Mice were weighed on injection day. During toluene exposure in adolescent mice (PN35–46), there was no difference in the body weight gain of toluene (6.32±0.37 g, $n=24$) and control mice (6.31±0.35 g, $n=24$). Blood toluene levels were 63.1±2.6 and 24.8 ±4.0 μg/ml, 1 and 3 h after last injection of toluene.

Locomotor activity

Locomotor activity was assessed during PN56–58. As shown in Figure 1A, Baseline locomotor activity in response to a novel environment (i.e. the locomotor chambers) was not different between toluene-treated mice and controls during the 2-h session (toluene: $F_{1,360}=0.39$, $P=0.43$; time: $F_{11,360}=0.39$, $P<0.001$). In both treatment groups, methamphetamine (1 and 2 mg/kg) induced locomotor hyperactivity ($F_{1,42}=42.6$, $P<0.001$) and there was no significant difference between groups ($F_{1,42}=0.46$, $P=0.5$) (Figure 1B).

Social interaction test

Social interaction was measured as total interaction time and non-aggressive social responses, such as sniffing, following and climbing. A significant reduction in the total duration of social interaction (Figure 2A) and

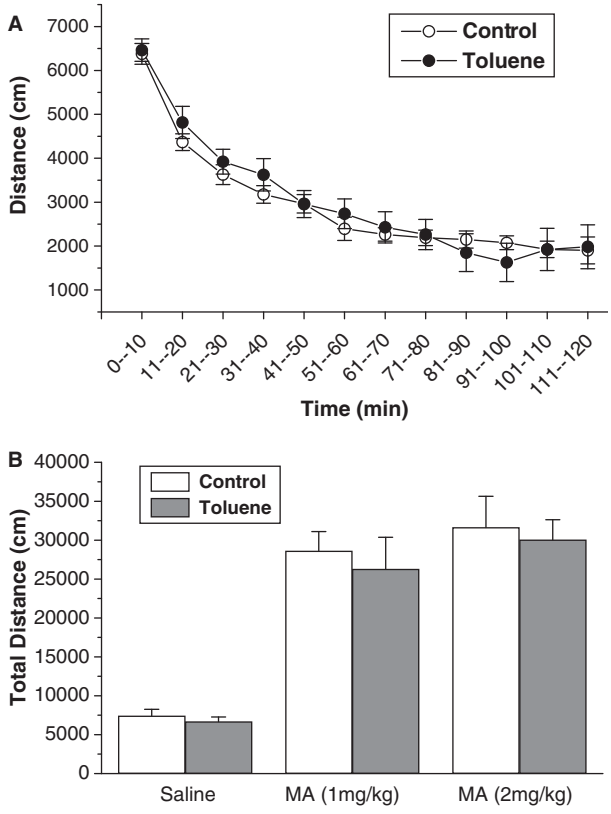

Figure 1. Effect of toluene exposure during adolescence on basal locomotor activity (A) and methamphetamine (MA)-induced locomotor hyperactivity (B). Values are mean±SEM ($n=16$ for basal activity; $n=8$ for MA-induced hyperactivity).

social responses (Figure 2B) was observed in toluene-exposed mice at PN60 and PN84. Two-way repeated measures ANOVA indicated significant main effect of toluene treatment (total duration: $F_{1,28}=57.5$, $P<0.001$; sniffing: $F_{1,28}=8.27$, $P<0.01$; following: $F_{1,28}=18.69$, $P<0.001$; climbing: $F_{1,28}=23.63$, $P<0.001$) and no significant difference between different ages ($F_{1,28}=3.19$, $P=0.08$) in the total duration of social interaction. However, the counts of social behaviours were age-dependent (sniffing: $F_{1,28}=12.82$, $P<0.01$; following: $F_{1,28}=14.31$, $P<0.001$; climbing: $F_{1,28}=11.63$, $P<0.01$).

Nest building test

Nest building was examined during PN57–58. Toluene-exposed mice showed lower nest scores and paper damage scores compared to control mice (Figure 3A).

Social dominance tube test

At PN59, social dominance tube test was conducted. In 23 of 32 trials (72%, $P<0.01$), the control mice

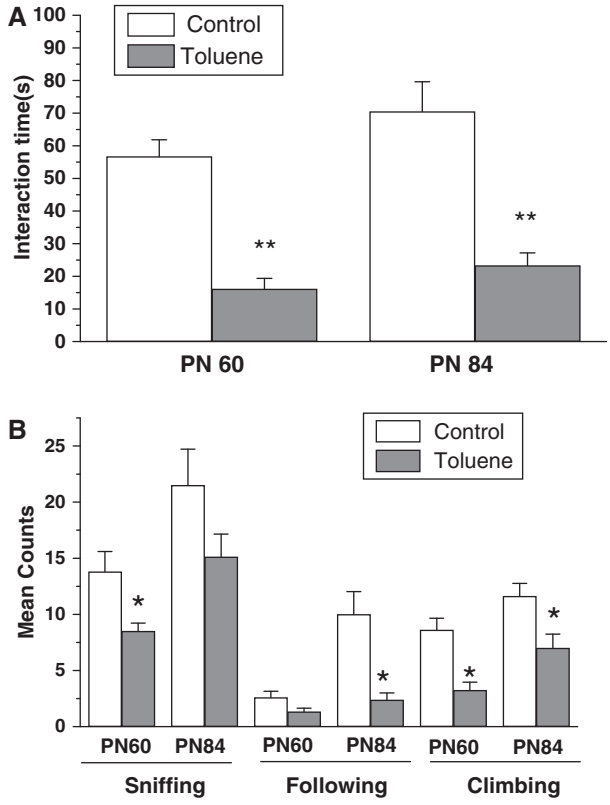

Figure 2. Effect of toluene exposure during adolescence on social behaviours in the social interaction test. The social interaction tests were performed at PN60 and PN84. The total interaction time (A) and the counts of social behaviours (B) were recorded. Values are mean±SEM (n=8). $^{*}P<0.05$, $^{**}P<0.01$ compared with control groups.

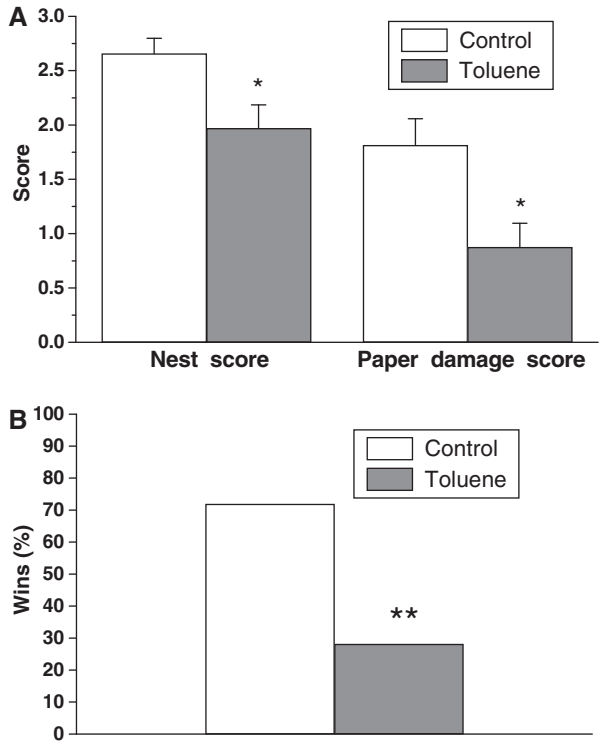

Figure 3. Effect of toluene exposure during adolescence on nesting behaviour (A) and social dominance tube test (B). The nesting score and paper damage score were analyzed by Mann–Whitney test. The percentage of dominance in 32 trials (16 toluene-exposed mice compared with control mice × 2 times) was calculated and analyzed with the χ^2-test. Values are mean±SEM (n=16). $^{*}P<0.05$ $^{**}P<0.01$ compared with control groups.

won when test against toluene-exposed mice (Figure 3B). Toluene-exposed mice were less dominant than controls in a paired social dominance test.

Tail suspension test and forced swimming test

Tail suspension test and forced swimming test were conducted at PN64 and PN65–66, respectively. There were no significant differences between toluene-exposed and control mice in forced swimming test (Figure 4A) as well as in tail suspension test (Figure 4B).

Emergence test and elevated plus maze

Emergence test and elevated plus maze were conducted at PN68 and PN69. As shown in Table I, toluene exposure did not have any effects on the emergence test or elevated plus maze.

Novel object recognition test

Novel object recognition test was performed during PN61–65 and PN79–83. All mice showed similar exploration of the left and right objects during exposure to two identical objects on the training trials with no difference between the two groups. Control mice exhibited a preference for the novel over the familiar object during two retention test sessions, 1 or 24 h after training, whereas the amount of time spent exploring the novel object relative to the familiar object was significantly reduced in toluene–exposed mice during both testing periods (Figure 5).

Brain weights and neurochemical analysis

The whole brain weights of toluene-treated mice (421.5±2.8 mg) were lower than those of control mice (447.6±3.1 mg). Neurochemical analysis showed very modest or negligible reductions in the levels of DA, 5-HT and their metabolites in toluene-exposed mice across the brain areas examined (Table II). Only the levels of DOPAC were reduced in the prefrontal cortex and striatum in the toluene-exposed mice compared with controls. The ratio of DOPAC/DA was significantly decreased in

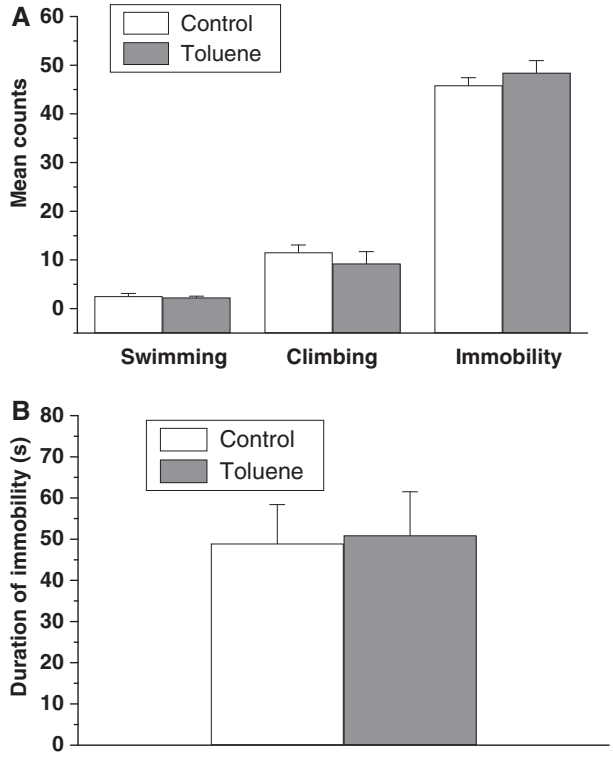

Figure 4. Effect of toluene exposure during adolescence on forced swimming test (A) and tail suspension test (B). The mean counts of swimming, climbing and immobility were recorded in the forced swimming test. The duration of immobility was measured in tail suspension test. Values are mean±SEM (n=16).

the prefrontal cortex, striatum and nucleus accumbens, whereas the ratio of (HVA + DOPAC)/DA was reduced in the striatum and nucleus accumbens.

Discussion

Given the increasing prevalence of inhalant abuse in adolescents, the need for research into the behavioural manifestations of adolescent toluene abuse-associated neural deficits is essential. Our results demonstrate that adolescent toluene exposure produces a decrease in brain weight, long-lasting social deficits and cognitive impairment, but no changes in depression-like or anxiety-like behaviours in mice. These findings are in line with psychotic symptoms in solvent abusers. In addition, the blood toluene levels are in the range obtained from toluene abusers (0.2–92 μg/ml) (Chao et al. 1993; Park et al. 1998). It appears that we have established an animal model of solvent-induced psychosis with construct and face validity.

The behavioural manifestations upon adolescent toluene exposure in mice included deficits in social interaction, nesting behaviour, social dominance and recognition memory in the novel object recognition

Table I. Effect of toluene exposure during adolescence on anxiety-like behaviours in mice.

Test	Control	Toluene
Elevated plus maze		
Closed arm entries	15.7±1.1	15. 5±1.0
Time spent in open arms (%)	40.7±4.0	32.4±3.5
Emergence test		
Latency of emergence (s)	6.5±1.1	5.1±0.6
Time spent in the cylinder (s)	64.6±10.6	70±21
Entrance number	7.5±0.8	6.9±0.8

Values are mean±SEM (n=8).

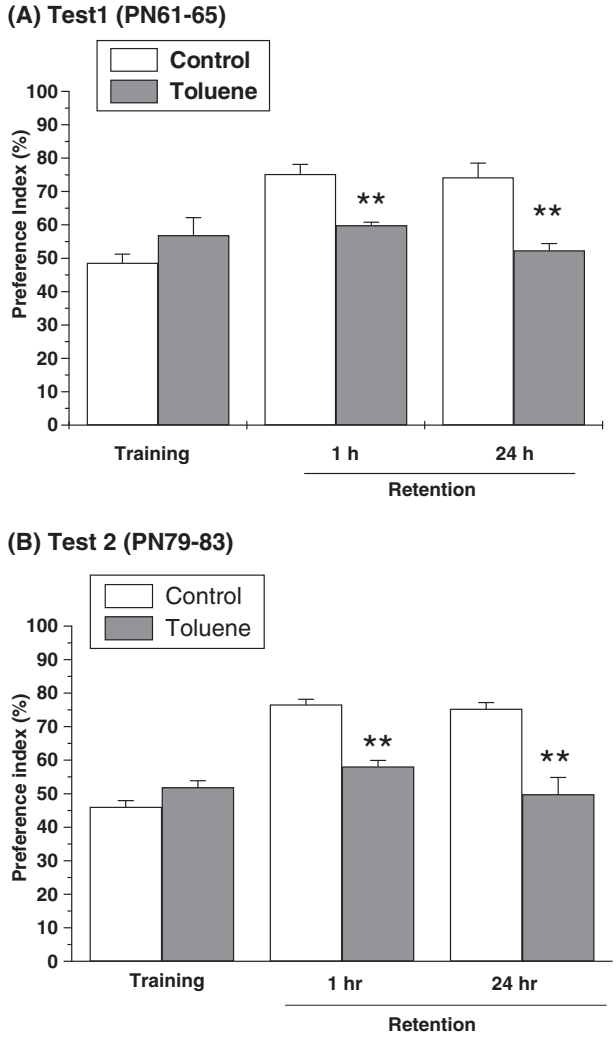

Figure 5. Effect of toluene exposure during adolescence on recognition memory. The novel object recognition tests were performed at PN61–63 (A) and PN79–83 (B). Retention session was carried out 1 and 24 h after the training, respectively. The amount of time spent exploring the objects was measured. The preference index was calculated as described in Materials and Methods. Values are mean±SEM (n=16). $^{**}P<0.01$ compared with control groups.

Table II. Tissue levels of dopamine, serotonin and their metabolites from different brain regions in control and toluene-treated mice.

Brain area		DA (pg/mg)	DOPAC	HVA	5-HT	5-HIAA	DOPAC/DA (%)	HVA/DA	(DOPAC+HVA)/DA	5-HIAA/5-HT
PFC	Control	25.46±5.50	17.48±3.08	9.17±2.85	37.80±5.79	30.59±3.61	79.7±11.2	55.8±19.2	135.5±25.6	89.84±10.35
	Toluene	19.36±6.14	7.56±2.15*	19.30±6.96	40.64±6.61	30.78±4.76	45.9±10.9*	63.5±14.2	120.0±18.1	86.62±15.69
Striatum	Control	381.96±25.76	174.02±19.61	68.14±11.14	43.84±5.87	57.18±3.83	46.4±5.1	18.6±3.5	65.0±8.2	144.5±17.21
	Toluene	416.95±27.05	119.98±12.59*	65.75±10.43	46.49±4.31	47.21±4.66	28.6±2.3*	16.2±2.8	46.2±4.4*	111.02±17.56
NAC	Control	279.76±38.03	163.43±7.75	41.00±8.55	45.66±6.45	56.29±3.54	64.0±6.8	16.2±3.4	80.2±8.3	136.8±16.62
	Toluene	294.14±38.08	133.31±18.59	40.68±11.03	61.49±6.85	46.29±3.63	44.5±3.9*	11.7±4.1	56.1±4.3*	87.56±15.89

The concentrations are in pg/mg wet weight.
DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; PFC, prefrontal cortex; NAC, nucleus accumbens.
Values are mean±SEM (n=8). *P<0.05 compared with control groups.

test. Reduced levels of social interaction, social dominance in the tube test, and nesting behaviour in mice reflect social withdrawal. The psychotic symptoms observed among the toluene abusers include amotivation, intoxication, emotional instability, delusion, hallucination, disinhibition and memory impairment (Goldbloom and Chouinard 1985; Byrne et al. 1991; Wada et al. 2005). Among them, the "amotivational syndrome" has been suggested as a characteristic feature of patients suffering from solvent-induced psychosis (Wada et al. 2005). Our treatment protocol produced long-lasting social withdrawal behaviours and cognitive dysfunction in mice, which resemble the features observed in solvent-induced psychosis. It is noted that subchronic treatment of phencyclidine, a non-competitive NMDA receptor antagonist, also induces social withdrawal behaviours (Sams-Dodd 1998; Qiao et al. 2001) and cognitive impairment in the novel object recognition task (Hashimoto et al. 2005, 2007; Shirayama et al. 2007; Fujita et al. 2008). Given that toluene can inhibit NMDA receptor-mediated current (Cruz et al. 1998), it is speculated that toluene-induced behavioural dysfunctions are related to subchronic blockade of NMDA receptors.

Although it has been reported that solvent users have a high lifetime prevalence of mood, anxiety and personality disorders (Wu and Howard 2007), in the present study mice exposed to toluene during adolescence did not show any significant effects on anxiety-like behaviours in the emergence test and the elevated plus maze. Actually, toluene exposure during brain growth spurt also had no effect on the behavioural responses in the elevated plus-maze (Liu et al. 2007). Taken together, it is suggested that toluene exposure during early or late developmental stages cannot modify anxiety-like behaviours. Since acute toluene evidently exerts anxiolytic effects (Geller et al. 1983; Bowen et al. 1996; Lopez-Rubalcava et al. 2000; Bamat et al. 2005), it is possible the solvent abusers with a high propensity to anxiety abuse toluene as a method of self-medication. Similarly, solvent abusers have been reported to have higher rates of depression (Zur and Yule 1990). It appears that the emotional dysfunction might be associated with solvent use in abusers rather than the deteriorated consequences from solvent abuse. Alternatively, toluene might not be the only drug used by solvent abusers. Our findings cannot exclude the possibility of solvents other than toluene contributing to the anxiety disorder occurring in solvent users.

Our data show that the (DOPAC+HVA)/DA ratios were reduced in the prefrontal cortex, striatum

and nucleus accumbens by adolescent toluene exposure, indicating reduced dopaminergic activity. The reduced dopamine turnover rate in these brain regions is mainly contributed by the reduction of DOPAC levels. It is of interest to determine whether the functional changes of dopamine metabolic enzymes, especially monoamine oxidase, are involved in the reduction of dopamine turnover rate after toluene exposure in the future. Alternatively, since DOPAC and HVA accumulation is primarily a reflection of extracellular metabolism of dopamine, this observation indirectly suggests that toluene exposure may produce a reduction in the extracellular dopamine concentration in these brain regions. It has been reported that repeated injections of toluene (600 mg/kg) for 7 days increased DA concentrations in the caudate nucleus and nucleus accumbens, 2 and 4 h after the last injection (Riegel et al. 2004). However, this report did not measure the long-lasting effects. Actually, long-lasting changes in dopamine turnover also occurred after neonatal toluene exposure even though the brain regions examined were different. Neonatal toluene exposure reduces dopamine levels and utilization in the olfactory tubercle and substantia nigra of the adult rat (Von Euler et al. 1989). Dysfunction of dopamine transmission is involved in psychotic behaviours in schizophrenia. Schizophrenics display increased presynaptic dopamine uptake capacity (Hietala et al. 1999; Lindstrom et al. 1999) and dopamine release (Abi-Dargham et al. 2000). Decreased activation of left ventral striatal dopamine neurons has been correlated with negative symptoms (Juckel et al. 2006). Furthermore, dopamine neurotransmission in the prefrontal cortex also plays an essential role in mediating cognitive functions. In animal studies, it is evident that prefrontal cortical dopamine D1 receptors are involved in the retrieval of object memory (Floresco and Phillips 2001; Hotte et al. 2006). The alterations in dopamine turnover in the prefrontal cortex might be involved in cognitive impairment and those in striatum and nucleus accumbens may be associated with other behavioural impairment observed in juvenile mice exposed to toluene.

A worldwide comprehensive survey has shown that the majority of toluene users start inhaling toluene when they are teenagers (Neumark et al. 1998). The two most marked neurodevelopmental changes during adolescence include a pruning of synapses (particularly in the prefrontal areas) correlated with a reduction in gray matter (Toga et al. 2006), and an increase in myelination that is correlated with an increase in the proportion of white matter (Blakemore and Choudhury 2006). In fact, the concept of "white matter dementia" in chronic toluene abuse was well substantiated worldwide by numerous studies (Filley et al. 1990; Yamanouchi et al. 1995; Fornazzari et al. 2003). We found lower brain weights in toluene-treated mice. It appears that toluene exposure during adolescence might interfere with neurodevelopment. It has been reported that rats exposed to toluene (1500 ppm for 4 h per day) for 7 days produces neurodegenerative changes. The granule cells in the dentate gyrus of the hippocampus are slightly shrunken. In the cerebellum, several Purkinje cells were shrunken and loss and the white matter was thinner than in controls (Gotohda et al. 2002). Although toluene abuse does not cause active demyelination (Aydin et al. 2003), white matter abnormality and neuronal loss might be related to adolescent toluene-induced behavioural abnormalities in adults. Moreover, organic solvents also have detrimental effects on olfactory (Gelazonia et al. 2006) and auditory systems (Lataye et al. 1999; Fuente and McPherson 2007), which may be also related to the long-lasting behavioural dysfunction, including social withdrawal and recognition memory impairment.

Although there is consistent evidence that chronic long-term toluene use during the developmental period between childhood and adolescence is likely to result in neurological deficits and cognitive impairment (Chadwick et al. 1989; Yucel et al. 2008), very little is known about the neurobiological process underlying toluene-related neuropsychological deficits. The present study demonstrated that toluene exposure during adolescence produced persistent social deficits and recognition memory impairment in mice, which in turn are believed to resemble the psychotic symptoms among patients suffering from dependence on volatile solvents and those in psychotic state due to chronic solvent use. It appears that this animal model is suitable for research into the pathophysiology of solvent-induced psychosis and development of effective treatment approaches. In order to verify the predictive validity of this model, evaluation of the effects of antipsychotics, such as haloperidol, carbamazepine (Hernandez-Avila et al. 1998) or clozapine (Shu and Tsai 2003), which are partially effective for treatment of toluene psychotic patients is needed. Additionally, these results highlight the need for further research to improve understanding of the effects of solvent abuse on the developing nervous system and the potentially enduring effects resulting from juvenile exposure.

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Statement of interest

None to declare.

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ORIGINAL INVESTIGATION

The catechol-*O*-methyl-transferase gene in tardive dyskinesia

CLEMENT C. ZAI[1], ARUN K. TIWARI[1], DANIEL J. MÜLLER[1,2], VINCENZO DE LUCA[1], TAKAHIRO SHINKAI[3], SAJID SHAIKH[1], XINGQUN NI[1], DAVID SIBONY[1], ARISTOTLE N. VOINESKOS[1], HERBERT Y. MELTZER[4], JEFFREY A. LIEBERMAN[5], STEVEN G. POTKIN[6], GARY REMINGTON[1] & JAMES L. KENNEDY[1]

[1]*Neurogenetics Section, Centre for Addiction and Mental Health, Toronto, Ontario, Canada,* [2]*Department of Psychiatry, Charité University Medicine Berlin, Campus Charité Mitte, Berlin, Germany,* [3]*Department of Psychiatry, School of Medicine, University of Occupational and Environmental Health, Yahatanishi-ku, Kitakyushu, Japan,* [4]*Psychiatric Hospital at Vanderbilt University, Nashville, TN, USA,* [5]*Department of Psychiatry, College of Physicians and Surgeons, Columbia University and the New York State Psychiatric Institute, New York City, NY, USA, and* [6]*Brain Imaging Center, University of California, Irvine, CA, USA*

Abstract

Tardive dyskinesia (TD) is a severe and potentially irreversible motor side effect linked to long-term antipsychotic exposure. Changes in dopamine neurotransmission have been implicated in the etiology of TD, and catechol-*O*-methyl-transferase (COMT) is an enzyme that metabolizes dopamine. *Objectives.* We investigated five single-nucleotide polymorphisms in addition to the functional Val158Met variant spanning the *COMT* gene for association with TD. *Methods.* We analyzed the six *COMT* single-nucleotide polymorphisms in a sample of schizophrenia/schizoaffective disorder patients ($n = 226$; 196 Caucasians and 30 African Americans). *Results.* We found a significant association between the marker rs165599 in the 3' untranslated region of *COMT* and TD (AA versus G-carrier: $OR_{AA} = 2.22$, 95% CI:1.23–4.03; $P = 0.007$). The association appeared to be originating from males. We did not find a significant association of the other five tested polymorphisms with TD in our samples. We performed a sex-stratified meta-analysis across all of the published studies ($n = 6$ plus our own data) of *COMT* and TD, and found an association between ValVal genotype and TD in females ($OR_{ValVal} = 1.63$, 95% CI: 1.09–2.45; $P = 0.019$) but not in males. *Conclusions.* Overall, our results suggest that the *COMT* gene may have a minor but consistent role in TD, although sex-stratified studies with additional markers in larger clinical samples should be performed.

Key words: *Schizophrenia, pharmacogenetics, tardive dyskinesia, COMT, meta-analysis*

Introduction

Approximately 25% of schizophrenia patients undergoing long-term treatment with typical antipsychotic medications develop TD, an involuntary and potentially irreversible involuntary movement disorder (Margolese et al. 2005; reviewed in Tarsy and Baldessarini 2006). Its presence has been linked with treatment non-adherence, stigmatization, and reduced quality of life (Marsalek 2000; Gerlach 2002). The incidence of TD has been decreasing with increasing use of atypical antipsychotic medications; however, long-term treatment of several of the atypical antipsychotic medications causes serious weight gain and metabolic abnormalities in up to 35% of patients. Recent large clinical trials have shown that typical neuroleptics are as efficacious as newer atypical antipsychotics (CATIE – Lieberman et al. 2005; CUtLASS – Jones et al. 2006). Thus clinicians may be shifting toward more use of typical neuroleptics and thus predicting those patients who are vulnerable to TD remains a clinical priority. Furthermore, the use of typicals is relatively high, due to their lower cost, in developing countries.

The complex etiopathophysiology of TD remains unclear. A number of mechanisms leading to TD have been hypothesized, including dopamine receptor hypersensitivity induced by sustained antipsychotic blockade (Tarsy and Baldessarini 1977; Klawans

Correspondence: Dr James L. Kennedy, R31 250 College Street, Toronto, Ontario, Canada M5T 1R8. Tel: 1 416 979 4987. Fax: 1 416 979 4666. E-mail: james_kennedy@camh.net

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et al. 1980; Gerlach and Casey 1988; Abilio et al. 2003). Observations of a familial pattern of TD indicate a genetic component (Müller et al. 2001, 2004). Although the dopamine D_2 receptor gene is a primary candidate, our recent meta-analysis (Zai et al. 2007a) showed only a small risk effect for the markers studied, suggesting additional genetic factors play a role in TD susceptibility.

Catechol-*O*-methyltransferase is an enzyme that degrades catecholamines including dopamine. The non-synonymous polymorphism that changes the 158th amino acid from a valine residue to methionine has been associated with lower COMT activity (Männistö and Kaakkola 1999), and this common polymorphism has been associated with numerous psychiatric disorders, including schizophrenia (Glatt et al. 2003; Winterer and Goldman 2003; Oroszi and Goldman 2004). Other *COMT* polymorphisms, rs165599 and rs737865, have also been associated with schizophrenia, but their effects appeared to be sex-specific (Shifman et al. 2002). Findings from TD studies of the Val158Met have been mixed, with a recent meta-analysis of four previous studies showing that the Val allele confers an increased risk of TD (Bakker et al. 2008). Other *COMT* polymorphisms have been largely unexamined in genetic studies of TD thus far except for a recent study by Tsai et al. (2009) in the CATIE sample, where the authors did not investigate the Val158Met polymorphism. Recent evidence pointed to the presence of additional functional *COMT* polymorphisms (Nackley et al. 2006). Nackley and coworkers found that haplotypes consisting of rs6269, rs4633, rs4818, and rs4680 (Val158Met) were associated with changes in the secondary structure of predicted messenger RNA and were correlated to the level of COMT enzyme activity. More specifically, the A-C-C-G haplotype was associated with high COMT protein level and enzyme activity, and the G-C-G-G haploytpe was associated with low COMT protein level and enzyme activity (Nackley et al. 2006). These findings prompted us to investigate the rs6269, rs4633, and rs4818 polymorphisms in addition to Val158Met for possible association with TD. Furthermore, we tested for an association between TD and two additional polymorphisms, rs165599 and rs737865, in light of their significant association with schizophrenia, and their provision of additional coverage of the *COMT* gene. Finally, we conducted an updated meta-analysis of the Val158Met polymorphism in TD.

Patients and methods

Subjects

Our sample characteristics have been described previously (Zai et al. 2007b, 2008). More specifically, subjects were recruited from four clinical sites in North America: Center for Addiction and Mental Health in Toronto, Ontario (Dr G. Remington, $N = 95$); Case Western Reserve University in Cleveland, OH (Dr HY Meltzer, $N = 69$); Hillside Hospital in Glen Oaks, NY (Dr J.A. Lieberman, $N = 50$); University of California at Irvine, CA (Dr. SG Potkin, $N = 12$). Subjects were selected based on their diagnoses for schizophrenia or schizoaffective disorder according to DSM-III-R or IV (APA 2000). Exclusion criteria include type II diabetes, head injury with loss of consciousness, and seizure disorder. We do not have detailed medication history for all patients. Patients recruited in the US (HYM, JAL, SGP) were not exposed to atypical antipsychotic medication prior to TD assessment, while the chronic patients recruited in Canada (GR) may have been on either typical or atypical antipsychotics. Nonetheless, all patients had undergone at least one year of treatment with typical antipsychotic medication before TD assessment. The rate of TD was similar between the US and Canadian samples ($P = 0.46$), and was less common in males (36%) than in females (52%) in the collective sample ($P = 0.03$). The presence of TD was assessed using the Abnormal Involuntary Movement Scale (AIMS) or the modified Hillside Simpson Dyskinesia Scale (HSDS) for 50 patients recruited from the Hillside Hospital (Guy 1976; Schooler and Kane 1982; Basile et al. 1999). Patients were identified as having TD if they have at least one moderate rating or at least two mild ratings on the first seven body items in the AIMS (Schooler and Kane 1982). Because of previous findings of higher rate of TD in African Americans than in Caucasians, we analyzed our African American and Caucasian subjects separately (Jeste 2000). Our ethnicity designation is based on self-report of probands and/or their family members regarding their grandparents' birthplace, language, and religion. In all, 196 Caucasian schizophrenia patients were studied, of which 79 were positive for the diagnosis of TD. AIMS scores were available for 162 patients. Thirty African American patients were studied, of whom 11 were positive for TD. Our Caucasian sample has over 80% power to detect an odds ratio of 2.1 ($\alpha = 0.05$, allele frequency = 0.2, additive model; Quanto v1.2.3; Gauderman and Morrison 2006).

Gene polymorphism analysis

Genomic DNA was purified from whole blood samples using a non-enzymatic method previously described (Lahiri and Nurnburger 1991). Genotyping was done after the subjects had completed the follow-up, and all laboratory staff were blind to the AIMS scores and TD status. The *COMT* polymorphisms

and their locations are shown in Figure 1. In all, we genotyped six *COMT* polymorphisms: rs737865 (5'), rs6269 (S-COMT promoter), rs4633 (His62His), rs4818 (Leu136Leu), rs4680 (Val158Met), and rs165599 (3' untranslated region). Polymerase chain reactions of 10 µl volume using 20 ng genomic DNA were performed using Assays-on-Demand (ABI) under the following conditions: 95°C for 10 min, followed by 50 cycles of 92°C 15 s, 60°C 1 min. Determination of alleles was performed using the ABI model 7500 Sequence Detection System with the Allelic Discrimination software.

Statistics

Statistical analyses were conducted using SPSS version 15.0, and Haploview version 4 (Barrett et al. 2005). Odds ratio calculations were conducted using Program RelRisk version 2.33 written by Jurg Ott. Genotype frequency distribution was tested for fitness to Hardy–Weinberg equilibrium using Haploview. The association of genotype frequencies with AIMS was assessed using ANCOVA with AIMS as the dependent variable, genotypes as the independent variables, and age and sex as factor covariates. Where the variances among genotypes differed significantly using the Levene's Test for Homogeneity of Variances, ranked AIMS scores were used. Gender differences in genotype frequencies were assessed using the χ^2 test, as were differences in allele and genotype frequencies between patients with and without TD. For contingency tables with at least one expected cell count of less than five, two-tailed Fisher's exact tests were calculated (URL: http://home.clara.net/sisa/fiveby2.htm). Haplotype analyses with TD diagnosis and linkage disequilibrium calculations were conducted using Haploview, and analyses with AIMS scores were carried out using the QTPHASE program of the UNPHASED software version 2.402 (Dudbridge, 2003).

Meta-analysis

The PubMed database of the National Centre for Biological Information (NCBI) was searched using the key terms "Tardive Dsykinesia" and "COMT" or "catecholamine-*O*-methyltransferase". Articles cited in the papers retrieved were also screened, and relevant data was extracted from these publications. We requested the genotyping information from the corresponding authors of the publications where that information was not available. We analyzed the data using the STATA Release 8 statistical software package (StataCorp. 2003. Stata Statistical Software: Release 8. College Station, TX: StataCorp LP). The odds ratios (OR) and confidence intervals of *COMT* Val158Met for TD from the individual studies were calculated using the "metan" command, with the pooled odds ratio and standard error calculated under the fixed effects and random effects (DerSimonian and Laird 1986) models. The possible effects of ethnicity, age, and sex ratio on the results from the meta-analysis were assessed by meta-regression analysis using the "metareg" command. Publication bias was estimated using the "metabias" command using the Begg's funnel plot of the log OR for each study against the standard error of log OR, and Egger's tests which is based on a weighted linear regression of standard normal deviation of OR (standardized effect) on the inverse of the standard error of OR (precision) (Egger et al. 1997).

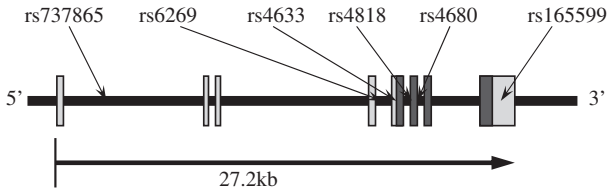

Figure 1. Schematic diagram of the *COMT* gene with the six single-nucleotide polymorphisms tested for possible association with TD in the present study. Dark filled boxes represent coding region.

Results

COMT gene in our TD sample

The genotype frequencies in our sample did not deviate significantly from Hardy–Weinberg equilibrium. Since age and sex have been associated with TD (Smith and Dunn 1979; Kane et al. 1988; Morgenstern and Glazer 1993; Woerner et al. 1998; van Os et al. 1999; Zhang et al. 2009), we included them as covariates in our analyses. The AA genotype of the marker rs165599 was associated with TD occurrence (Table I: AA versus G-carrier: $OR_{AA} = 2.22$, 95% CI:1.23–4.03; $P = 0.007$). We also found a trend for AA genotype carriers to have higher AIMS scores (Table I: AA versus G-carrier: 7.60 ± 8.423 versus 4.86 ± 6.361; $P = 0.076$). When we analyzed the sexes separately, we found the significant findings were originating from the males (AA versus G-carrier: logistic regression with age as covariate: $OR_{AA} = 3.06$, 95% CI:1.41–6.62; $P = 0.005$; ANCOVA with age as covariate: 7.74 ± 8.561 versus 3.64 ± 5.643; $P = 0.007$). The other five tested polymorphisms were not significant in the single-marker tests. The four polymorphisms, rs6269, rs4633, rs4818, and rs4680, were in linkage disequilibrium with one another (Figure 2), thus we compared the frequency distribution of haplotypes containing these four markers. We also indicated

Table I. Results from comparison of total AIMS scores among genotypes of the six *COMT* polymorphisms, as well as analysis of TD diagnoses with *COMT* alleles and genotypes.

		Caucasian				
COMT markers	Genotypes	Total AIMS score	TD (Yes/No)[1]	Alleles	Caucasian TD (Yes/No)	African American TD (Yes/No)[2]
rs737865	1/1 (G/G)	5.72±5.40	10/16	G	56/85	4/5
	1/2 (G/A)	6.33±7.35	36/53	A	100/135	14/33
	2/2 (A/A)	6.10±8.18	32/41			
	P	0.855[3]	0.488[3]	P	0.589	0.448
rs6269	1/1 (A/A)	5.68±8.007	28/40	A	96/130	11/23
	1/2 (A/G)	6.24±7.022	40/50	G	62/98	11/13
	2/2 (G/G)	6.79±8.283	11/24			
	P	0.941[3]	0.376[3]	P	0.463	0.411
rs4633	1/1 (T/T)	5.51±7.636	20/29	T	80/110	5/11
	1/2 (T/C)	6.34±7.576	40/52	C	74/116	17/27
	2/2 (C/C)	5.95±7.094	17/32			
	P	0.645[3]	0.502[3]	P	0.531	0.764
rs4818	1/1 (G/G)	6.64±8.144	11/25	G	62/99	7/6
	1/2 (G/C)	6.29±7.047	40/49	C	92/125	15/32
	2/2 (C/C)	5.54±7.770	26/38			
	P	0.911[3]	0.466[3]	P	0.447	0.197
rs4680 (V158M)	1/1 (G/G; V/V)	5.97±7.193	16/33	G	69/120	17/26
	1/2 (G/A; V/M)	6.36±7.788	39/54	A	81/110	5/12
	2/2 (A/A; M/M)	5.71±7.501	22/28			
	P	0.709[3]	0.188[3]	P	0.239	0.560
rs165599	1/1 (G/G)	5.82±5.259	8/15	G	42/87	13/25
	1/2 (G/A)	4.62±6.620	26/57	A	110/137	9/13
	2/2 (A/A)	7.60±8.423	42/4			
	P	***0.050***[3,4]	***0.031***[3]	P	***0.025***	0.782

[1]With at least one expected cell count <5; Fisher's exact test used.
[2]Fisher's exact tests were used for analyses on our African American sample.
[3]Analysis with age and sex as covariates.
[4]Variances among comparisons groups differ significantly; ranked total AIMS scores were used in place of total AIMS scores. Bolded numbers indicate $0.05 < P < 0.10$; bolded and italicized numbers indicate $P < 0.05$.

	rs737865	rs6269	rs4633	rs4818	rs4680	rs165599
rs737865		0.76 0.67–0.83	0.71 0.59–0.80	0.77 0.67–0.84	0.72 0.60–0.81	0.22 0.09–0.35
rs6269			1.00 0.96–1.00	0.99 0.96–1.00	0.97 0.91–1.00	0.30 0.15–0.43
rs4633				1.00 0.96–1.00	0.99 0.95–1.00	0.64 0.49–0.75
rs4818					0.97 0.91–1.00	0.34 0.19–0.47
rs4680						0.67 0.52–0.77
rs165599						

Figure 2. Linkage disequilibrium plot among the six *COMT* gene polymorphisms used in our entire sample. Shown are values for *D*' and 95% confidence intervals. The intensity of grey indicates the r^2 values within the polymorphism pair (white or 0%: $r^2 = 0$–20%; 20%: $r^2 = 21$–40%; 40%: $r^2 = 41$–60%; 60%: $r^2 = 61$–80%; 80%: $r^2 = 81$–100%). The LD blocks are defined by the solid spine method with 70% minimum threshold.

Table II. Results from analysis of *COMT* haplotypes containing the rs6269, rs4633, rs4818, and rs4680 (Val158Met) polymorphisms related to COMT activity versus TD diagnoses for our Caucasian SCZ sample.

		Case–control		AIMS	
rs6269–rs4633–rs4818–rs4680	Haplotype defined by COMT activity (Nackley et al. 2006)	TD (Yes/No)	*P* value	Average total AIMS scores	*P* value
2211 (GCGG)	High	56/95	0.309	6.514±7.47	0.478
1122 (ATCA)	Medium	75/104	0.493	6.045±7.47	0.827
1221 (ACCG)	Low	11/16	0.910	5.083±7.47	0.456
		Global	0.656	Global	0.658

(Table II) the reported relative COMT activity defined by Nackley et al. (2006) associated with these four-marker haplotypes. Even though the high COMT-activity haplotype, as defined in Nackley et al. (2006), had the highest average total AIMS scores and the low COMT-activity haplotype had the lowest average total AIMS scores, the results from our haplotype analysis were not significant (Table II). Allelic analysis in our African American sample did not yield significant findings (Table I).

Meta-analysis

We carried out a computer search on the National Library of Medicine's PubMed online search engine database for all papers published up to November 2009 using the search terms "tardive dyskinesia" and "COMT". We also included the study by Han et al. (2005) because it was included in the Bakker et al. (2008) meta-analysis. In all, six genetic association studies were found reporting on TD and *COMT* Val158Met. The numbers of patients with and without TD and genotypes for Val158Met were available for all studies (Herken et al. 2003; Han et al. 2005; Lai et al. 2005; Srivastava et al. 2006; Kang et al. 2008), with the exception of Matsumoto et al. (2004) where only allele frequency data were available. All subjects were selected based on their diagnoses of schizophrenia or schizoaffective disorder, according to DSM-III-R or DSM-IV, using case records with or without patient interviews. The presence of TD was assessed using the AIMS, or the modified Hillside Simpson Dyskinesia Scale (HSDS) in the case of 50 patients from our study as described above. TD ratings were performed once for each patient in the majority of the studies. Including our Caucasian and African American samples, 1520 schizophrenia patients were genotyped for *COMT* Val158Met, and 553 were positive for the diagnosis of TD. Demographic information for each of the studies included in the meta-analysis is shown in Table III.

All studies were in Hardy–Weinberg equilibrium, except for the Lai et al. study ($P > 0.05$). The allelic as well as genotypic analysis, using the random effect model, did not show a significant association with TD occurrence [Figure 3a: $OR_{ValVal} = 1.38$, 95% CI:0.94–2.03; $P = 0.10$; Figure 3b: $OR_{Val} = 1.10$, 95% CI:0.87–1.40; $P = 0.43$]. No significant heterogeneity was present among the eight samples ($p > 0.05$). Sex ratio exerted a significant influence on the results observed for Val158Met ($P < 0.05$), while ethnicity, average age, and allele frequency did not ($P > 0.05$; Table III). Publication bias was

Table III. Studies included in the present meta-analysis of *COMT* Val158Met (rs4680) in TD. Only allelic information was available from the Matsumoto et al. (2004) paper, so we included this study in the allelic meta-analysis only. Results from meta-regression analysis of the variables (ethnicity, age, the number of males/females, and observed allele frequency) are shown for the meta-analyses of Val versus met and ValVal versus Met-carrier.

Study	Ethnicity	Age	M/F	Allele freq. (Met)
Herken (2003)	Turkish	32.3±9.1	93/50	0.59
Matsumoto (2004)	Japanese	54.9±9.4	106/100	0.35
Lai (2005)	Taiwanese	46.97±8.98	168/131	0.28
Han (2005)	Korean	38.69±11.21	114/0	0.31
Srivastava (2006)	N. Indian	32.28±10.9	147/125	0.46
Kang (2008)	Korean	45.22±9.55	110/99	0.26
Present study (a)	Caucasian	37.81±10.16	129/64	0.50
Present study (b)	African American	32.5±10.9	21/9	0.28
V vs. M	0.44	0.54	***0.005***	0.68
VV vs. M-carrier	0.19	0.94	***0.026***	0.40

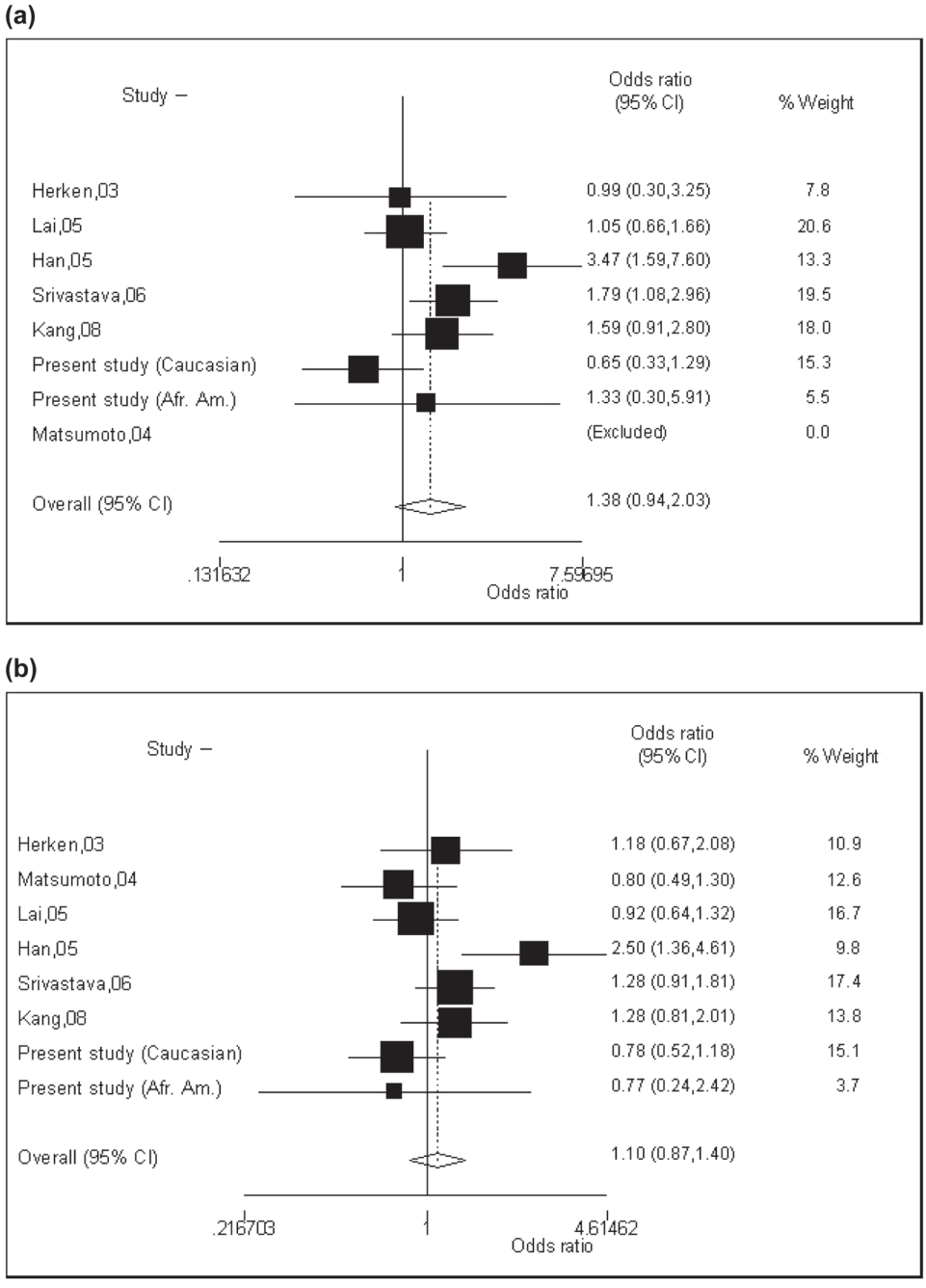

Figure 3. Meta-analysis of Val158Met in TD. (a) The upper panel shows odds ratios of ValVal genotype and (b) the lower panel shows odds ratios of Val allele.

not significant [P(Begg) > 0.05; P(Egger) > 0.05)]. Genotypic analysis under the fixed effect model revealed a significant association between the ValVal genotype and TD occurrence [$OR_{ValVal} = 1.36$, 95% CI:1.07–1.73; $P = 0.012$].

Because sex could contribute to the findings, we analyzed the males and females separately. Where the genotype information was not available from the papers, we requested the genotype counts separate for males and females from the corresponding authors of the papers. We were able to obtain genotype information from all studies except for Matsumoto et al. (2004) and Herken et al. (2003) study samples. However, we were able to calculate genotype counts from the Matsumoto et al. (2004) study for male patients, so we included this information in our meta-analysis. For males, the allelic as well as genotypic analysis, using the random effect model, did not show a significant association with TD occurrence [Figure 4a: $OR_{ValVal} = 1.31$, 95% CI:0.82–

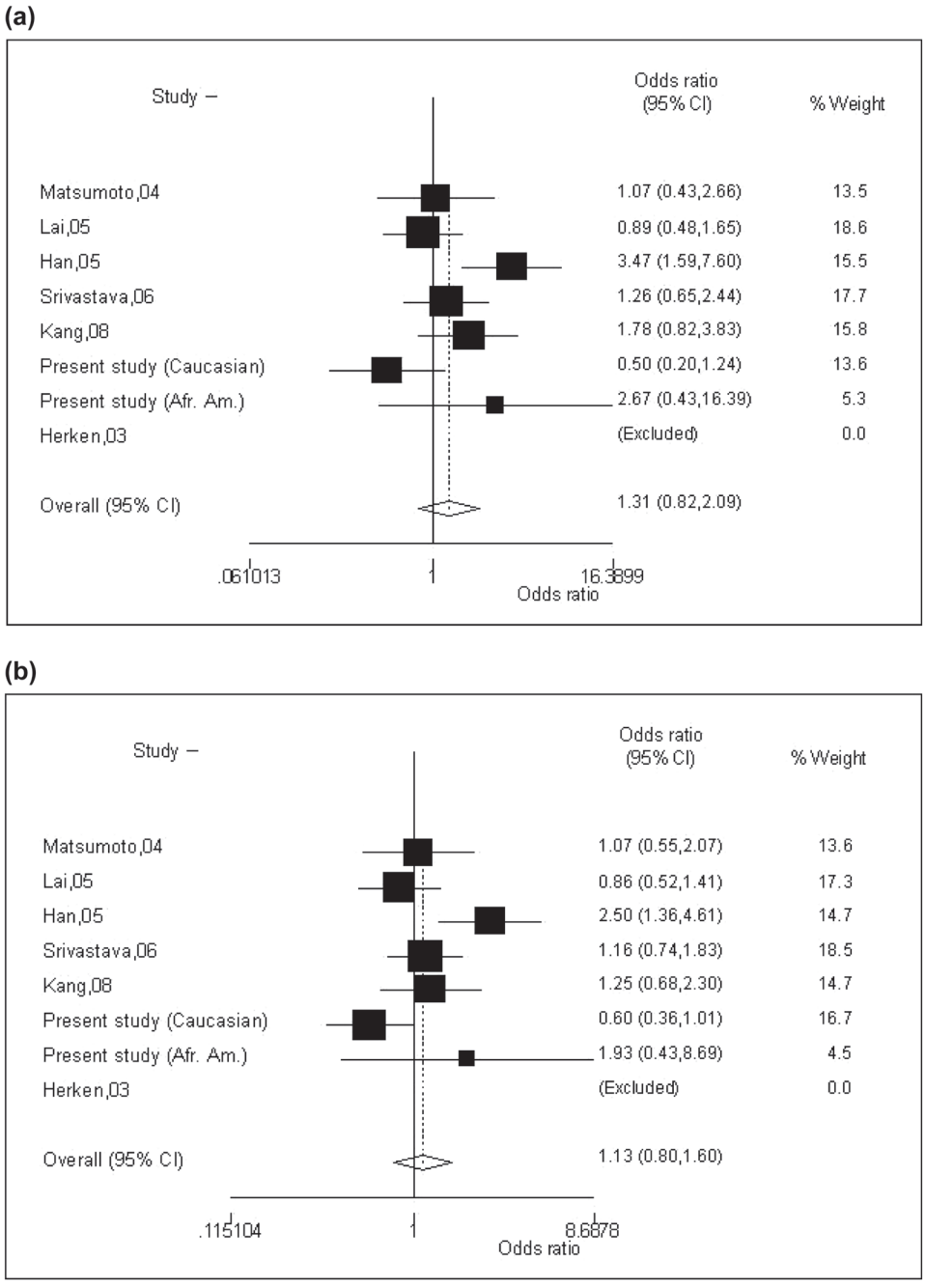

Figure 4. Meta-analysis of Val158Met in male TD patients. (a) The upper panel shows odds ratios of ValVal genotype and (b) the lower panel shows odds ratios of Val allele.

2.09; $P = 0.26$; Figure 4b: $OR_{Val} = 1.13$, 95% CI:0.80–1.60; $P = 0.50$]. Similar, non-significant results were found under the fixed effect model. For females, genotypic analysis under the random effect model revealed a significant association with TD occurrence [Figure 5a: $OR_{ValVal} = 1.63$, 95% CI: 1.09–2.45; $P = 0.019$]. Allelic analysis under the random effect model did not show a significant association with TD occurrence [Figure 5b: $OR_{Val} = 1.25$, 95% CI:0.93–1.68; $P = 0.13$]. Similar results were found under the fixed effect model.

Discussion

Our analysis of the functional Val158Met polymorphism, as well as the functional haplotypes identified by Nackley et al. (2006) did not yield significant findings in our sample. However, we found an association between the marker rs165599 in the 3' untranslated region of the *COMT* gene and TD. The results were similar when we took into consideration age and sex. When we conducted the analyses for males and females sepa-

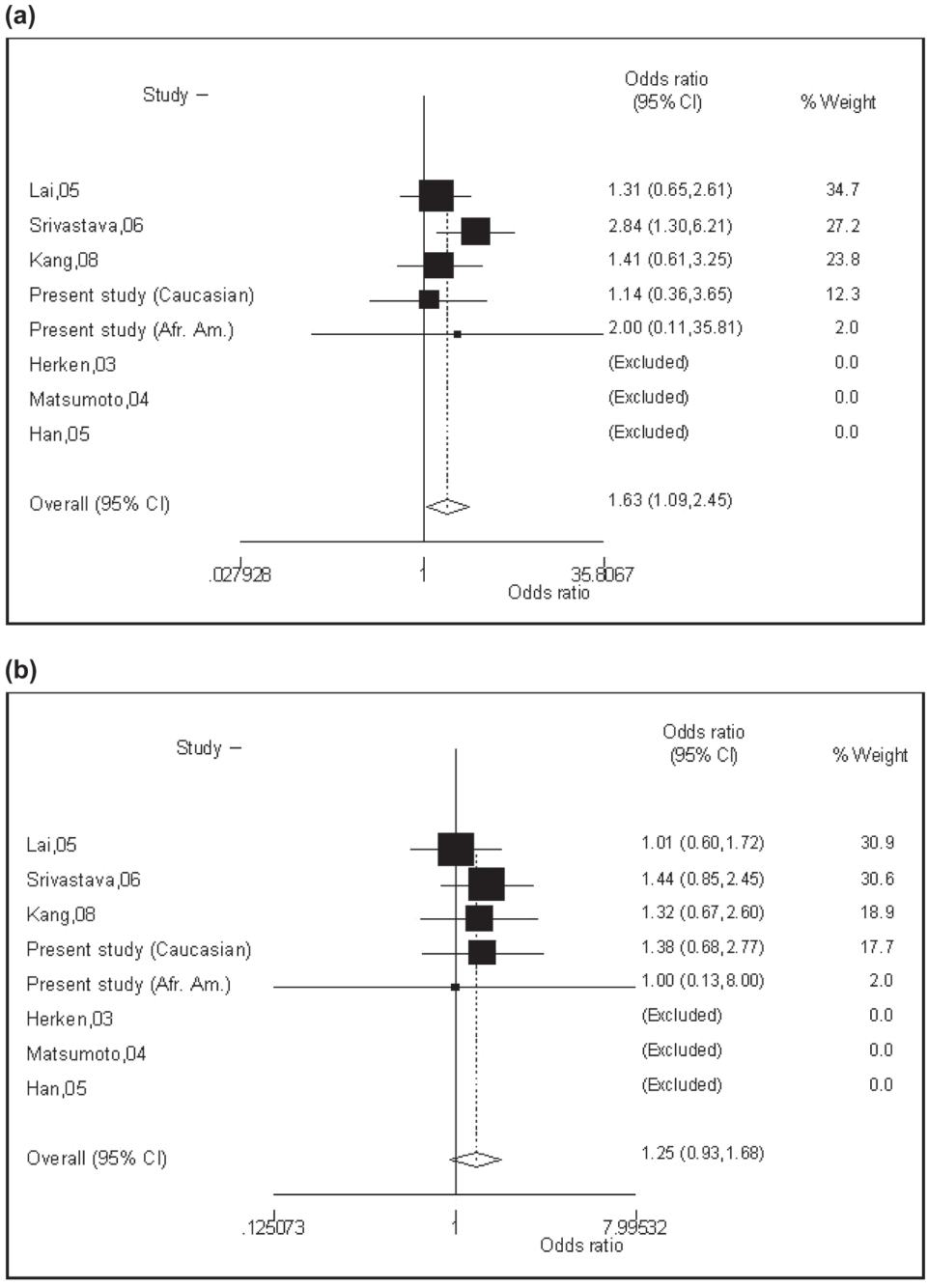

Figure 5. Meta-analysis of Val158Met in female TD patients. (a) The upper panel shows odds ratios of ValVal genotype and (b) the lower panel shows odds ratios of Val allele.

rately, the results were only significant in males. Results from the meta-analysis of Val158Met in TD indicated that the effect of Val allele or ValVal genotype was inconclusive because the results were significant only under the fixed effect model for the ValVal genotype. The risk allele for our Caucasian sample is different from that derived from the meta-analysis. Nonetheless, the risk allele is the same as that reported by Lai et al. (2005). The mixed results could be due to sampling differences among the samples used in the meta-analysis. The mixed findings could also be due to the possibility that the contribution of Val158Met in TD is weak or sex-specific. When we analyzed the sexes separately, we found the ValVal genotype to be associated with TD in the females under both random and fixed effect models, but not in the males. These sex-specific associations detected between TD and *COMT* polymorphisms mirrors the sexually dimorphic phenotypes observed in mice deficient in COMT, and the recent sex-specific findings in various psychiatric disorders (reviewed in Harrison and Tunbridge

2008), including schizophrenia (Shifman et al. 2002), attention-deficit hyperactivity disorder (Biederman et al. 2008), and obsessive-compulsive disorder (Pooley et al. 2007). The mechanism behind these sex-specific associations could be that the *COMT* gene promoter contains estrogen-response elements (ERE, Driscoll et al. 1998), and COMT is involved in estrogen metabolism. The significant polymorphisms could be markers for variants in the EREs that could influence COMT expression. Further, the *COMT* gene promoters could be differentially methylated (Sasaki et al. 2003) between males and females. Examinations into these mechanisms in TD are needed.

Smoking, drinking, and using street drugs may increase the risk for TD (Olivera et al. 1990; Menza et al. 1991; Bailey et al. 1997). We do not have detailed information on length of illness, or medication history, including treatment duration and type of medication, for our sample or for the meta-analysis. Moreover, our results with rs165599 could have been influenced by it being collected from different clinical sites. Nonetheless, the same risk allele was observed for rs165599 in the US ($OR_G = 1.52$; 95% CI: 0.82–2.79) and Canadian samples ($OR_G = 1.73$; 95% CI: 0.89–3.36). In addition, the results would not have survived correction for testing multiple markers, haplotypes and sex-stratified effects. Furthermore, our selection of polymorphisms was based on previous genetic studies of *COMT*, and these polymorphisms do not capture all the allelic variability in *COMT*. Nonetheless, replication of our findings in other TD samples including additional markers, as well as functional studies of *COMT* in TD are warranted.

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Statement of interest

AKT, DJM, VdL, TS, SS; XN, DS, ANV, GR report no competing interests. CCZ and JLK: patent application for "marker for tardive dyskinesia". HYM has received grants and/or has been a consultant to: Abbott Labs, ACADIA, Eli Lilly, Janssen, Pfizer, Wyeth, Schering Plough, SureGene, Novartis, Azur, Biovail, Lundbeck, Roche, Otsuka, Dainippon Sumitomo, Cephalon, Minster, Bristol Myers Squibb, Astra Zeneca, Glaxo-Smith-Kline, Memory, Aryx, and BiolineRx. HYM is a shareholder of ACADIA. JAL reports that he serves on the Advisory Board of Bioline, GlaxoSmithKline, Intracellular Therapies, Eli Lilly, Pierre Fabre, Psychogenics and Wyeth. He does not receive financial compensation or salary support for his participation as an advisor. He receives grant support from Allon, Forest Labs, Merck and Pfizer; and he holds a patent from Repligen. JLK has been a consultant to GSK, Sanofi-Aventis, Dainippon-Sumitomo.

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ORIGINAL INVESTIGATION

Set-shifting abilities, central coherence, and handedness in anorexia nervosa patients, their unaffected siblings and healthy controls: Exploring putative endophenotypes

ELENA TENCONI[1], PAOLO SANTONASTASO[1], DANIELA DEGORTES[1], ROMINA BOSELLO[1], FRANCESCA TITTON[1], DANIELA MAPELLI[2] & ANGELA FAVARO[1]

Department of [1]Neurosciences and [2]General Psychology, University of Padova, Padova, Italy

Abstract

Objective. There is consistent evidence that anorexia nervosa (AN) is associated with an impairment of set-shifting abilities and central coherence. No study to date investigated handedness in AN. Our aim was to study set-shifting abilities, central coherence, and handedness in subjects with lifetime AN, in a sample of unaffected sisters and in healthy controls, in order to explore their suitability as endophenotypes of AN. *Methods.* The Edinburgh Handedness Inventory and several neuropsychological tasks (Wisconsin Card Sorting Test, Trail Making Test, Rey-Osterrieth Complex Figure Test, Overlapping Figures Test, Object Assembly and Block Design) were administered to 153 subjects with lifetime AN, 28 unaffected sisters and 120 healthy controls. *Results.* AN subjects and their healthy sisters showed poorer performances on most tasks investigating set-shifting and central coherence. In addition, we did not find any differences between long-term recovered subjects, weight-restored AN patients and those in an acute phase of their illness. AN subjects were significantly more likely to be left-handed than healthy controls (OR=2.8, 95% C.I. 1.1–7.2). *Conclusions.* Set-shifting and central coherence seem to be promising cognitive endophenotypes that might help in the understanding of the pathogenetic processes involved in AN. Further studies on larger samples are needed to explore the generalizability and implications of our findings concerning handedness.

Key words: *Anorexia nervosa, endophenotype, executive functioning, neuropsychology, handedness*

Introduction

In the last few decades, cognitive functioning in anorexia nervosa (AN) patients has received increased attention in the literature. Neuropsychology is considered to be an effective method to explore the involvement of specific brain areas and functions in psychiatric disorders. Although most studies have found the presence of impairments on a broad spectrum of cognitive functions in acute AN (Szmukler et al. 1992; Green et al. 1996; Kingston et al. 1996), there are also several studies that have failed to find cognitive deficits in these patients (Touyz et al. 1986; Palazidou et al. 1990; Bradley et al. 1997) and others that surprisingly have found cognitive performance to be superior to that of healthy controls (Pieters et al. 2003, 2004). In short, the current literature is inconclusive concerning the characteristics, underlying mechanisms and reversibility of cognitive abnormalities in AN (Katzman et al. 2001; Pieters et al. 2005). The first studies suggested that cognitive deficits observed in acute AN were a consequence of starvation and may be improved once adequate nutritional status is regained (Szmukler et al. 1992; Lauer et al. 1999). However, studies concerning higher-level executive functioning failed to find an improvement after weight gain, refeeding, or other types of treatment (Green et al. 1996; Kingston et al. 1996; Tchanturia et al. 2004). This is why an impairment in specific tasks of executive functioning appears to be a good candidate endophenotype in eating disorders.

For all psychiatric disorders there is an ongoing search for intermediate cognitive phenotypes that may help clarify the relative contributions of genetic

Correspondence: Angela Favaro, MD, PhD, Clinica Psichiatrica, Dipartimento di Neuroscienze, Via Giustiniani 3, 35128 Padova, Italy. E-mail: angela.favaro@unipd.it

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and environmental risk factors. Some specific impairments of executive functions, such as inflexibility, set-shifting difficulties, and low central coherence, are considered putative endophenotypes of AN because of their stability throughout the illness (Tchanturia et al. 2004, 2005; Steinglass et al. 2006) and their heritability (Holliday et al. 2005). In addition, these impairments might play a role in the development and maintenance of AN (Bulik et al. 2007) and seem to have a negative influence on outcome (Hamsher et al. 1981; Szmukler et al. 1992; Holliday et al. 2005; Roberts et al. 2007). Notwithstanding this, few studies (Holliday et al. 2005) to date have examined the cognitive performance of unaffected sisters (or other first degree relatives) of AN patients which is considered to be an essential step in the identification of putative endophenotypes (Gottesman and Gould 2003).

Executive functions are the abilities that enable a person to engage successfully in independent, purposive and self-serving behaviour (Lezak et al. 2004). In particular, they are involved in the supervision of those cognitive processes, located primarily in the prefrontal cortex, which include restraining and delaying actions, inhibiting responses, setting goals, planning and organizing complex human behavioural output. One example of executive functioning is set-shifting ability that involves the ability to move back and forth between tasks, operations or mental sets (Miyake et al. 2000). There is consistent evidence of an impairment of set-shifting abilities in AN (Roberts et al. 2007). In addition, AN patients seem to have a better ability for the processing of details, but show worse performance on global strategies (Lopez et al. 2008a,b). This characteristic is called weak central coherence (Frith 1989) and is referred to as a cognitive style in which there is a bias towards local or analytical, detailed-focus processing of information and person is unable to integrate incoming information into meaningful context or gestalt (global integration). Global integration of perceptual stimuli involves perceptual and attentional abilities which are specifically impaired in people with lesions of the right hemisphere (temporal and frontoparietal networks) (Lezak et al. 2004). To date, the data available about central coherence in anorexia nervosa allows us to say that there is some evidence of weak global processing in anorexic patients, but there are still few data to demonstrate a superiority in local processing (Lopez et al. 2008c).

Although handedness has been considered as a putative endophenotype in other psychiatric disorders (Savitz et al. 2007) and seems to be consistently associated with schizophrenia and other neurodevelopmental disorders (Dragovic et al. 2005; Ramadhani et al. 2006), no study to date has explored the distribution of handedness in AN. Handedness is in part due to genetic factors, and in part can be conditioned by early environmental insults (prenatal or perinatal). Recent studies have hypothesized that eating disorders may have a neurodevelopmental origin (Connan et al. 2003; Strober et al. 2007) and their risk seems to be increased by the occurrence of perinatal complication (Favaro et al. 2006). For this reason, we included neuropsychological characteristics and handedness among the putative endophenotypes of AN.

The aim of the present investigation was to study handedness and performance on some specific neuropsychological tasks which explore set-shifting abilities and central coherence in women with lifetime AN, their unaffected sisters, and a control group, in order to explore their suitability as putative endophenotypes.

Methods

Subjects

The subjects of the study were 153 female patients with a lifetime diagnosis of anorexia nervosa (AN) referred to the Eating Disorders Outpatient Unit of Padua Hospital. According to the diagnostic status at the time of neuropsychological assessment the subjects were classified into three groups: (1) acute AN according to DSM-IV criteria (APA 1994) in 60 cases (39%), (2) weight-restored AN in 63 cases (41%), and (3) no eating disorder in the other 30 cases (20%). The second group consisted in subjects with previous AN, who were weight-restored, but who were still symptomatic at the time of neuropsychological assessment (binge eating or purging, hyperactivity, fasting). This sample included 14 subjects with bulimia nervosa, 10 subjects with EDNOS with binge eating or purging behaviour, and 39 subjects with EDNOS without binge eating or purging behaviour. The third group was composed of subjects with complete recovery from AN (at least 3 years of normal weight, regular menses, no eating symptoms, and good social and interpersonal outcome). An adapted version of the Morgan–Russell criteria have been used for definition of recovery (Morgan and Hayward 1988). The age of the AN sample ranged from 14 to 47 years with a mean of 26.2 years (SD=6.9) and the mean level of education was 14.2 years (SD=2.8). The mean age of onset for anorexia nervosa was 17.8 years (SD=4.1). The mean body mass index was 16.2 (SD=1.5; range 9.9–17.5) in the acute AN group, 20.5 (SD=3.1; range 18.0–37.0) in the weight-restored group, and 20.6 (SD=1.5; range 19.0–24.4) in the remitted group.

Criteria for participation in the study were: a lifetime diagnosis of anorexia nervosa according to the DSM-IV criteria (APA 1994); more than 14 years of age; written informed consent from patients and, in the case of patients younger than 18, from one parent. Exclusion criteria were: traumatic brain injury, lifetime presence of any neurologic or systemic illness independent from the eating disorder; lifetime presence of Axis I comorbidity (except for depressive and anxious disorders), presence of alcohol or substance abuse, psychoactive medication, except for the use of antidepressants.

After permission was obtained from the patient, we invited the sisters of the patients to participate in the study. We were able to contact 56 out of 72 sisters. Seven sisters were excluded because of the lifetime presence of an eating disorder, another six because their age was below 14, and in 15 cases either the patient or the sister refused. The final sample consisted of 28 healthy sisters, with a mean age of 27.5 years (SD = 8.7), a mean BMI of 22.0 (SD = 2.2) and mean level of education of 13.8 years (SD = 3.4).

The healthy control group consisted of 120 subjects with no history of eating disorder, with a mean age at assessment of 27.4 (SD = 4.5), a mean BMI of 21.8 (SD = 3.0) and mean level of education of 16.4 years (SD = 2.3). Criteria of exclusion were: BMI below 18, presence of a first degree relative with a lifetime eating disorder, traumatic brain injury, presence of any neurologic, psychiatric or systemic illness; presence of alcohol or substance abuse; psychoactive medication. All subjects gave informed written consent for the use of data in an anonymous form and institutional approval for the study was obtained.

Clinical and cognitive assessment

In all subjects (patients, their sisters, and controls), clinical interviews were performed using the eating disorders section of the Structured Clinical Interview for DSM-IV (First et al. 1995) and a semistructured interview to gather socio-demographic and clinical variables, such as family psychiatric morbidity, history of weight, and history of treatments. In addition, they were asked to complete the Hopkins Symptoms Checklist (Derogatis et al., 1974), the State Trait Anxiety Inventory (STAI; Spielberger et al. 1970), and the Tridimensional Personality Questionnaire (TPQ; Cloninger 1987).

Handedness was assessed through the Edinburgh Handedness Inventory (Oldfield 1971), which yields scores ranging from –100, denoting consistent left-handedness, to +100, denoting consistent right-handedness. As in previous studies (Christman et al. 2007), we considered mixed-handedness a score between –80 to +80. One AN subject and three controls did not complete this measure.

A neuropsychological test battery was used to assess some executive functions.

Set-shifting abilities: (1) the *Wisconsin Card Sorting Test* (WCST; Bergh 1948) investigates the ability of abstraction and cognitive flexibility. Particularly, the execution of the WCST presupposes the involvement of manifold cognitive operations, such as the process of initial abstraction, the appeal to functional strategies of problem solving, the ability to modify the strategy when the situation requires a change of rule, the ability to learn and to memorize functional rules (Laiacona et al. 2000). In addition to the indices usually considered in previous studies (number of completed categories, number of perseverative errors and responses, number of non perseverative errors), we used a measure of global efficiency, called the global score (Global score = [no. of trials – (no. of achieved categories × 10)]; Laiacona et al. 2000). The global index, the number of perseverative responses, and the number of non perseverative errors were adjusted for age, sex and educational level as reported in Laiacona et al. (2000). (2) the *Trail Making Test* (TMT; Reitan 1958) to measure attention and mental tracking. The first part of the task (TMT-A) evaluates selective attention and visuospatial ability, whereas the second (TMT-B) is a set-switching task that appraises selective and alternate attention.

Global versus local processing style (central coherence): (1) The *Rey–Osterrieth Complex Figure Test* (ROFT; Osterrieth 1944) appraises different cognitive functions such as perception, visuospatial ability, planning and visuospatial memory. The subject must copy and recall, after an interval of 3 minutes, a complex geometric figure. We used the standard scoring system developed by L. Taylor (Lezak et al. 2004) to assess the drawing accuracy of the Rey figure. The performance score is valued from the accuracy and placement of the reproduction of the 18 details of the figure. Using the Rey Figure Test, it is possible to calculate a central coherence index (ICC), that results from the order of construction index (number of global and local elements drawn in the initial stage of copying the figure) and the style index (the degree of continuity in the drawing process; Booth's method described by Lopez et al. (2008a)). A global approach during the copying task (high index of central coherence) seems to improve the performance on the recall task (Lopez et al. 2008a). In the present study, the copy and recall accuracy scores were adjusted for sex, age and educational level, as reported in Caffarra et al. (2002). (2) The *Unsegmented/Segmented Block*

Design and *Object Assembly* subtests of the revised version of the Wechsler Adult Intelligence Scale (WAIS-R; Wechsler 1981) were used as measures of visuospatial ability, problem solving and central coherence. In the Block Design test, subjects are asked to reproduce complex geometric figures by putting together some cubes. The difference between the time spent in the segmented and unsegmented trials is called benefit from segmentation. It has been hypothesized that subjects with a weak central coherence would gain less advantage from segmentation. In the Object Assembly test the subjects must recompose some jigsaw-type puzzle pieces into a whole figure (mannequin, head, hand, elephant), with no final example given to the subject. A shorter time suggests a better ability to create an integrated global representation from its parts. (3) The *Overlapping Figures Test* (Della Sala et al. 1995) appraises the ability to discriminate figures from the background (visual interference), spatial exploration, and denomination. An entangled sketch composed of many overlapping and segmented figures of animals, fruits, persons, numbers, and various other objects is given to the participant who must recognize and denominate the greatest number of figures in 4 minutes of time. Excessive local processing or weak global processing is associated with impaired performance.

Each subject was assessed individually in a quiet and well-illuminated room by a trained neuropsychologist and the examination took about 90 minutes.

Statistical analysis

All variables were tested for normality. Only the performance indices of the WCST were not normally distributed. We used a natural log transformation for normalization of these variables. After normalization, we used parametric tests to compare independent groups. An exception was the number of completed categories at the WCST which is an ordinal variable and required nonparametric statistical tests. When adjusted scores were not available, an analysis of variance with educational level and age as covariates (ANCOVA) was used to examine groups differences when significant differences in age or education were present between groups. Analyses for paired subjects were performed to compare AN patients and their healthy sisters. These procedures were implemented with Statistical Product and Service Solutions software (SPSS, Inc, Chicago, IL).

Results

Neuropsychological tasks: AN subjects versus controls

Table I shows the scores obtained by AN subjects and control subjects on the neuropsychological assessment. As regards the WCST, AN subjects had an overall worse performance in comparison to controls, reporting a higher global score, a lower number of categories, and a higher number of both perseverative and non perseverative errors (Table I).

Concerning the other neuropsychological tests, AN subjects reported a poorer performance on the Trail Making Test part B, the accuracy of ROFT recall, the central coherence index, the Object Assembly test, the Block Design Test Unsegmented/Segmented and the Overlapping Figures Test. Regarding the Block Design Test, we found no difference between groups across the two conditions of the tasks (benefit score). The inclusion of the Edinburgh score (handedness) among the covariates did not change any of the findings.

In AN, no significant correlation was found between neuropsychological performance and age of onset, BMI at the time of assessment, and lowest lifetime BMI. No differences have been found between subjects with a restricting phenotype and those with binge eating or purging behaviour. State anxiety (STAI) showed a moderate correlation with Trail Making Test part A ($r=0.23$; $P=0.007$), Overlapping Figure Test ($r=-0.37$; $P=0.001$), and Object Assembly test ($r=-0.36$; $P=0.008$). The inclusion of anxiety scores as a covariate in the comparison between AN patients and controls did not change the findings reported in Table I. No significant correlation has been found between neuropsychological performance and SCL depression/SCL obsessionality. Exploring correlations with temperamental variables, we found moderate correlations between the Reward Dependence subscale and ROFT Central Coherence Index ($r=-0.23$; $P=0.007$), ROFT Order Index Copy ($r=-0.26$; $P=0.002$), and ROFT Style Index Copy ($r=-0.17$; $P=0.045$).

No difference was found between medicated ($n=32$) and unmedicated ($n=120$) AN subjects, with the exception of ROFT Accuracy Copy (28.9 ± 4.2 vs. 30.4 ± 3.2; $t=2.18$; $P<0.04$) and the segmented Block Design (41.8 ± 9.4 vs. 48.2 ± 4.2; $t=2.68$; $P<0.02$). The exclusion of medicated subjects from the case–control comparison did not change the results of Table I, with the exception of the segmented Block Design (no significant difference between cases and controls).

Neuropsychological tasks: disease status

We divided the clinical sample according to the diagnostic status at the time of assessment (acute AN, weight-recovered but still symptomatic AN, long-term fully recovered AN) in order to compare the three groups (Table II). The three groups differ as regards education (13.7 ± 2.7; 14.0 ± 2.6; 15.3 ± 2.9;

Table I. Neuropsychological performance in anorexia nervosa patients and healthy controls.

	Raw scores			
WCST[2]	Anorexia nervosa Mean (SD) n=152	Controls Mean (SD) n=117	[1] $F(1,268)$	Effect size
Global score	48.6 (34.2)	32.3 (25.5)	33.37**	0.54
Number of categories[3]	5.2 (1.5)	5.7 (0.9)	–3.64**	0.40
Perseverative errors	14.5 (10.9)	10.5 (8.6)	5.12*	0.41
Non-perseverative errors	15.4 (12.8)	9.7 (8.1)	27.32**	0.53
Perseverative responses	16.2 (13.3)	11.7 (10.5)	28.31**	0.38
	n=152	n=120	$F(1,271)$	
Trail Making Test A	30.7 (11.0)	28.0 (8.1)	2.67	0.28
Trail Making Test B	67.5 (25.3)	55.4 (14.6)	13.26**	0.59
Rey Figure	n=152	n=118	$F(1,264)$	
Copy accuracy	30.1 (3.5)	30.9 (3.1)	1.53	0.24
Copy order	1.94 (0.72)	2.26 (0.70)	6.14*	0.45
Copy style	1.23 (0.46)	1.41 (0.36)	6.09*	0.44
Central coherence Index	1.20 (0.42)	1.38 (0.35)	6.14*	0.47
Recall accuracy	18.6 (5.4)	20.6 (5.0)	7.18**	0.38
	n=98	n=114	$F(1,211)$	
Overlapping Figures Test	35.2 (6.6)	39.2 (6.4)	11.55**	0.61
	n=59	n=84	$F(1,142)$	
Block design unsegm.	34.8 (8.6)	40.5 (6.1)	8.86**	0.76
Block design segm.	46.2 (6.8)	48.4 (3.4)	4.57*	0.41
Benefit from segm.	11.4 (9.3)	8.0 (7.0)	0.88	0.41
Object assembly	27.7 (6.1)	32.7 (4.3)	21.40**	0.95

[1]An analysis of variance was used. Educational level was included as a covariate when adjusted scores were not available (see Methods).
[2]Data about WCST were analysed after logarithmic transformation.
[3]Ordinal variable (Mann–Whitney *U*-test, z not adjusted for educational level).
*$P<0.05$; **$P<0.005$.

$F(2,150)=3.65$; $P=0.028$) and age (25.7 ± 7.7; 24.5 ± 6.1; 30.8 ± 4.6; $F(2,150)=9.58$; $P<0.001$). For this reason, both variables have been used as covariate in Table II. The comparison did not reveal any significant differences between the three groups on any of the neuropsychological measures, with the exception of the accuracy score of the ROFT copy on which the recovered group performed significantly worse.

Neuropsychological tasks: healthy sisters

Table III shows the scores reported by those AN subjects whose sisters participated in the study, the scores reported by their healthy sisters, and those of controls. The performance of healthy sisters is generally in an intermediate position between the other two groups. The performance of AN subjects and their healthy sisters did not differ significantly on any of the neuropsychological tasks, with the exception of the ROFT Central Coherence Index and Copy Order Index. AN patients and their sisters did not differ in a significant way as regards age and education ($P>0.3$). On the contrary, healthy sisters of AN patients differed from controls as regards education ($t=4.87$; $P<0.001$), but not age ($P>0.9$). Compared with controls, the healthy sisters of AN patients reported a poorer performance as regards the number of categories, the global score, the number of perseverative responses and non perseverative errors of the WCST, the Trail Making Test part B, the Overlapping Figures Test, Block Design and Object Assembly. There was no difference between sisters and controls on the ROFT scores, and benefit from segmentation at the Block Design Task.

Handedness

Subjects with AN (13 vs. 5%; $\chi^2=4.88$; df=1; $P<0.03$; OR=2.8, 95% C.I. 1.1–7.2) were significantly more left-handed than controls. A similar percentage of left-handedness was present in the healthy sisters of AN patients (14 vs. 5%; $\chi^2=2.95$; df=1; $P<0.09$; OR=3.1, 95% C.I. 0.8–11.8), but the difference was not statistically significant. No differences emerged as regards the rate of mixed-handedness or

Table II. Neuropsychological performance according to disease status.

WCST[2]	Acute anorexia nervosa Mean (SD) $n=60$	Weight recovered eating disorder Mean (SD) $n=63$	Full remission Mean (SD) $n=29$	[1] $F(2,151)$
Global score	47.4 (34.4)	48.5 (35.2)	51.1 (32.7)	0.17
Number of categories[3]	5.2 (1.6)	5.1 (1.5)	5.2 (1.4)	0.40
Perseverative errors	14.6 (11.1)	14.5 (11.1)	14.4 (10.2)	0.01
Non perseverative errors	15.7 (13.0)	14.8 (13.6)	15.8 (10.6)	0.50
Perseverative responses	16.1 (13.4)	16.1 (13.4)	16.9 (13.6)	0.06
TMT	$n=59$	$n=63$	$n=30$	$F(2, 151)$
Trail Making Test A	30.1 (9.8)	30.7 (10.2)	31.9 (14.5)	0.20
Trail Making Test B	73.3 (27.6)	64.9 (20.9)	61.8 (27.6)	2.48
ROFT	$n=59$	$n=63$	$n=30$	$F(2, 151)$
Copy accuracy	31.2 (3.0)	29.7 (3.6)	28.9 (3.7)	5.05*
Copy order index	1.97 (0.76)	1.92 (0.71)	1.93 (0.68)	0.19
Copy style index	1.24 (0.60)	1.19 (0.44)	1.28 (0.44)	0.30
Central coherence index	1.22 (0.45)	1.18 (0.40)	1.22 (0.40)	0.21
Rey Figure recall	19.1 (5.5)	18.1 (5.6)	18.5 (5.1)	1.16
	$n=29$	$n=41$	$n=28$	$F(2, 97)$
Overlapping Figures Test	33.9 (6.5)	35.4 (6.0)	36.1 (7.7)	1.14
	$n=21$	$n=30$	$n=6$	$F(2, 56)$
Block design unsegmented	35.1 (8.9)	35.2 (7.0)	31.7 (15.1)	0.09
Block design segmented	47.2 (4.2)	45.6 (8.3)	46.2 (6.5)	0.50
Benefit from segmentation	12.1 (8.5)	10.2 (9.6)	14.5 (10.8)	0.31
Object assembly	27.4 (6.4)	27.8 (5.1)	28.3 (10.4)	0.13

[1]An analysis of variance was used. Educational level and age were included as covariates when adjusted scores were not available (see Methods).
[2]Data about WCST were analysed after logarithmic transformation.
[3]Ordinal variable (Kruskal–Wallis test, χ^2 not adjusted for age and educational level).
*$P<0.05$; **$P<0.005$.

in the degree of lateralization. In both AN and controls, handedness or degree of lateralization did not correlate with cognitive performance.

Discussion

Physical, biological or psychological traits may represent endophenotypes or partial phenotypes of a specific disorder when they are measurable, heritable, associated with the illness, independent of the clinical state, and more frequent in first-degree relatives of affected subjects than in the general population. Of a series of putative AN endophenotypes, the present study provides data about three characteristics: association with the illness, independency of the clinical state, and frequency in a sample of unaffected sisters. The traits considered by our study are set-shifting abilities, central coherence, and handedness.

Set-shifting abilities

Our study represents the largest dataset using the WCST in anorexia nervosa (Roberts et al. 2007). Analysing the performance of AN patients on this test, we found a significant impairment both in perseverative and nonperseverative errors. While the first type of errors are indicative of an impairment of set-shifting abilities, the presence of a high rate of both types of errors can be caused by low motivation or an impairment of abstraction, working memory, learning abilities, problem solving, or poor inhibition (Lezak et al. 2004). Both perseverative and nonperseverative errors significantly distinguished healthy sisters of AN patients and controls and did not show differences according to disease status. So, our data indicate that AN patients and their sisters seem to share not only an impairment of set-shifting abilities, but also a more global impairment of executive functions (Laiacona et al. 2000; Lezak et al. 2004). The performance on the TMT confirmed the impairment of flexibility and set-shifting abilities in AN patients and their sisters, but also showed that AN patients were not impaired in their attentive functions, in visual tracking and motor speed (Lezak et al. 2004). The literature provides evidence that set-shifting is a promising cognitive endophenotype for AN. An

Table III. Neuropsychological performance in AN subjects, their healthy siblings and controls.

WCST[3]	Anorexia nervosa Mean (SD) *n*=28	Healthy sisters Mean (SD) *n*=28	Controls Mean (SD) *n*=117	Sisters vs. AN[1] *t*	Sisters vs. controls[2] *F* (1, 144)
Global score	48.5 (39.3)	51.1 (34.5)	32.3 (25.5)	–0.36	14.30**
Number of categories[4]	4.9 (1.8)	5.2 (1.1)	5.7 (0.9)	–0.68	–3.52*
Perseverative errors	15.0 (12.2)	16.3 (10.7)	10.5 (8.6)	–0.42	4.85*
Non perseverative errors	16.2 (14.6)	14.9 (11.1)	9.7 (8.1)	0.24	10.48**
Perseverative responses	16.8 (14.8)	18.2 (12.7)	11.7 (10.5)	–0.53	15.90**
TMT	*n*=28	*n*=28	*n*=12		*F*(1, 147)
Trail Making Test A	31.1 (12.9)	30.5 (9.6)	028.0 (8.1)	0.24	0.75
Trail Making Test B	71.3 (28.2)	63.5 (15.8)	55.4 (14.6)	1.56	4.10*
ROFT	*n*=28	*n*=28	*n*=118		*F*(1, 145)
Copy accuracy	29.8 (3.0)	29.7 (3.9)	30.9 (3.1)	0.16	1.27
Copy order index	1.73 (0.66)	2.19 (0.61)	2.26 (0.70)	–3.71**	0.32
Copy style index	1.13 (0.51)	1.27 (0.37)	1.41 (0.36)	–1.21	0.50
Central coherence index	1.09 (0.43)	1.30 (0.34)	1.38 (0.35)	–2.40*	0.03
Rey Figure recall	17.8 (5.4)	18.7 (4.6)	20.6 (5.0)	–1.02	1.37
	n=17	*n*=19	*n*=114		*F*(1, 132)
Overlapping Figures Test	35.5 (7.4)	33.5 (8.2)	39.2 (6.4)	0.68	5.41*
	n=11	*n*=13	*n*=84		*F*(1, 96)
Block design unsegm.	35.3 (8.9)	30.8 (8.0)	40.5 (6.1)	2.03	13.13**
Block design segm.	47.3 (5.2)	45.1 (7.0)	48.4 (3.4)	–0.05	12.97**
Benefit from segm.	12.0 (8.4)	14.3 (11.2)	8.0 (7.0)	–1.86	0.71
Object assembly	27.4 (5.8)	26.0 (6.4)	32.7 (4.3)	0.79	13.26**

[1]*t*-test for dependent samples.
[2]An analysis of variance was used. Educational level was included as a covariate when adjusted scores were not available (see Methods).
[3]Data about WCST were analysed after logarithmic transformation.
[4]Ordinal variable (Mann–Whitney *U*-test and Wilcoxon test for paired samples).
*$P<0.05$; **$P<0.005$.

impairment of set-shifting abilities seems to be heritable (Anokhin et al. 2003; Friedman et al. 2006), to be consistently associated with AN (Tchanturia et al. 2005; Roberts et al. 2007), to be still present after weight recovery (Green et al. 1996; Kingston et al. 1996; Steinlass et al. 2006) and in long-term recovered subjects (Tchanturia et al. 2004), and more frequent in healthy siblings of AN patients than in healthy controls (Holliday et al. 2005). Our study confirmed that poor set-shifting abilities and inflexibility have the characteristics required for being considered among the putative endophenotypes of AN. However, our data also suggest that a wider impairment of executive functioning seems to be present in both AN patients and their healthy sisters, and to be independent of disease status.

Central coherence

There is less literature regarding central coherence as a possible endophenotype for AN. In a recent review, Lopez et al. (2008b) confirmed the presence of a weak central coherence in AN patients. However, they also concluded that studies are consistent in finding an impairment in global processing associated with eating disorders, but there is still little evidence about the presence of superior local processing in these patients (Lopez et al. 2008b). A weakness in central coherence is postulated to reflect an impairment of those functions that are normally responsible for integrating individual pieces of perceptual information to ascertain a global meaning (Frith 1989). Abilities in perception, visuo-spatial organization, abstraction, perceptual flexibility, planning and integration are all involved, in different measures, in the performance of the tests that we used in our study. However, as previously described (Lopez et al. 2008c), the performance on some of these tasks seems to benefit more from global processing (ROFT, Object Assembly, Overlapping Figures) while others benefit more from local processing (Segmented/Unsegmented Block Design). In our study, the performance of AN patients on all the tests investigating central coherence provided support for the presence of weak central coherence in AN, similarly to what has been found in subjects with autism spectrum disorders (Losh et al. 2009). However, AN patients did not report any difference in the benefit

from segmentation on the Block Design test in comparison to control subjects. So, they seem to differentiate from patients with autism spectrum disorders, because their performance is indicative of poor global processing, but not of superior local processing. In addition, while in autism spectrum disorder both social sensitivity and central coherence are impaired (Frith 1989; Soderstrom et al. 2002), in AN patients we found a significant correlation between a weak central coherence and high reward dependence. To our knowledge, this is the first study exploring the relationship between temperament and cognitive functioning in AN. The correlation between these two variables might be due to the fact that some of the brain regions involved in social cognition (orbitofrontal cortex and temporal lobes) are the same involved in central coherence and visuo-spatial abilities. However, while in autism spectrum disorders there is a severe impairment of both central coherence and social functioning (Frith, 1989), in our AN sample an impairment of central coherence seemed to be associated with high sentimentality and dependence. A further exploration of this point in future studies is important, because some authors have found an impairment of social cognition in acute AN (Oldershaw et al. 2009) and severe AN is often associated with social isolation.

Confirming previous studies (Lopez et al. 2008c), a weak central coherence is present in AN patients independently of disease status. Our study is, on the contrary, the first to explore central coherence in healthy sisters of AN patients. Sisters seemed to share with AN patients the presence of weak central coherence and in particular the presence of poor global processing, as shown by performance on the Object Assembly, Block Design and Overlapping Figure tests. The only exception was the performance on the ROFT, that seemed to significantly differentiate AN patients and their sisters as regards the central coherence index and the style used to copy the complex figure. We are uncertain as to why this difference emerged only on the ROFT and not on the other tests. Compared to the other tests, ROFT seems to involve more abstraction abilities (Lezak et al. 2004); in particular, it requires more integration of strategic organizational processes, spatial skills, and nonverbal memory for successful performance. This kind of nonverbal memory might to be more susceptible than other spatial processing tasks (such as Object Assembly, Block Design and Overlapping Figures Test) to executive impairment because of its more abstract nature and greater reliance on organizational capacity. However, the complexity of these tasks makes it difficult to formulate reliable hypotheses about the component of the performance which can explain this difference. When a trait is associated with the illness, independent from the disease status, but not shared with first degree relatives, the possible hypotheses are: (1) the trait is a consequence of the illness (scar hypothesis); (2) the trait is linked to individual environmental risk factors; or (3) the trait is linked to non-shared genetic factors. Although the independence of the central coherence index from disease status makes the first hypothesis improbable, our data did not allow us to choose one of these hypotheses to explain our findings. In any case, this finding is the only that contradicts the hypothesis that weak central coherence represents a good candidate as a putative endophenotype of AN. For this reason, this finding suggests the need for replication in other samples of first degree relatives.

Handedness

Our study is the first, to our knowledge, to explore the characteristics of hand lateralization in AN. Hand preference is believed to be determined in part by genetic factors, that account for about 25% of variance (Medland et al. 2009) and in part by prenatal or very early environmental factors (Ramadhani et al. 2006; Vuoksimaa et al. 2009). Most studies report a higher prevalence of left-handedness in males (rates around 9–10%) than in females (6–8%) (Vuoksimaa et al. 2009). The rate of left-handedness in our sample of AN subjects is significantly higher than the rate of healthy controls and seems higher than the rates reported in female healthy subjects described in other studies (Vuoksimaa et al. 2009). If replicated in larger samples, our finding might have important implications for the understanding of the pathogenesis of AN. It would confirm a neurodevelopmental hypothesis for AN (Connan et al. 2003; Favaro et al. 2006; Strober et al., 2007). An alteration of neurodevelopmental trajectories could be the cause for both the higher frequency of left-handedness and an abnormal development or lateralization of brain functions that could be involved in the development of the illness (Smeets and Kosslyn 2001; Uher et al. 2005). In the literature, there seems to be a tendency for right-handers to perform better than left-handers on visuospatial tasks (Lezak et al. 2004). This is in line with the hypothesis of a dysfunction in lateralized somatosensory functions that are implicated in the visual representation of body image (Smeets and Kosslyn 2001; Grunwald et al. 2001). In a nonclinical sample, Christman et al. (2006) found that strong degrees of handedness, but not type of handedness, are associated with deficits in accurate representation of body image. According to these two hypotheses, we would expect a greater impairment on visuo-spatial tasks (Rey–Osterreth Figure Test, Overlapping Figures Test, Object Assembly, Block Design) according to

type or degree of handedness in anorexia nervosa and control subjects. However, we found no correlation between these tasks and handedness. Studies about brain lesions found that lesions of the left hemisphere (parietal and temporal areas) are associated to deficits in the detail processing, whereas right lesions are associated to an impairment in the global visuo-spatial processing (Lezak et al. 2004). It is possible that abnormal lateralization of brain functions in AN patients is at the origin of both the observed excess of left-handers and weak central coherence.

The excess of left-handers in unaffected sisters of AN patients confirmed that there may be some overlap between the genetic factors that increase the risk for developing AN and those that influence hand lateralization. This means that left-handedness could be included among the putative endophenotypes of AN.

Strengths and limitations

The present study has several strengths, as well as important limitations that should be taken into consideration. It is the first study, to our knowledge, to provide data about handedness in a sample of subjects with lifetime AN and one of the few to explore executive functioning in a large sample of AN patients and in a sample of healthy sisters of AN cases. However, some caution is required and replication in other samples is needed because AN subjects referred for outpatient treatment cannot be considered to be completely representative of all cases existing in the general population and the sample of healthy sisters was not large. In this study, we did not assess psychiatric comorbidity using a Structured Diagnostic Interview. For this reason, we cannot be conclusive about whether our findings are specific to AN or are due to the presence of a comorbid disorder, such as depression (Austin et al. 2001) or obsessive-compulsive disorder (Savage et al. 1999; Henry 2006). However, the lack of significant correlations between obsessive-compulsive and depression symptoms, as measured by the Hopkins Symptoms Checklist, and cognitive performance made this hypothesis unlikely. We found, on the contrary, that state anxiety correlated with the performance of Trail Making Test A, Object Assembly, and Overlapping Figures Test. It is well known that anxiety symptoms can impair cognitive functioning, because of an impaired efficiency of attentional control (Eysenck et al. 2007). The inclusion of anxiety scores among the covariates of analysis of variance did not change any of the findings of the present study.

Although all sisters were assessed after the mean age of onset for anorexia nervosa (Favaro et al. 2009), we cannot rule out that some sisters might develop an eating disorder after our assessment. In addition, given the relative low frequency of left-handers, our study did not have the power to analyse the relationship between handedness and cognitive functioning reliably.

Conclusions

In conclusion, the present study lends support to the hypothesis that impaired set-shifting and low central coherence might be considered good candidate as endophenotypes of AN. Moreover, it explored and provided the first evidence concerning the possibility that handedness could be included among the putative endophenotypes of this illness. Our findings need to be replicated in other samples and the nature of the cognitive impairment measured by the single tasks to be clarified. These findings not only have important implications for future studies addressing the etiopathogenesis of AN, but also could have some clinical utility. Poor abstract thought, visuo-spatial impairment and relative difficulty in integrative processing may not only represent important predisposing and/or maintenance factors to AN, but they may also interfere with treatment course and outcome of illness. In particular, a reduced ability in problem solving, and poor conceptual thinking may explain in part the low consciousness of illness, the neglect of long-term secondary health problems and the lack of full engagement in therapeutic work.

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Statement of interest

None.

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ORIGINAL INVESTIGATION

Evidence of complex involvement of serotonergic genes with restrictive and binge purge subtypes of anorexia nervosa

KIRSTY KIEZEBRINK[1], EVLEEN T. MANN[2], SARAH R. BUJAC[3], MICHAEL J. STUBBINS[4], DAVID A. CAMPBELL[5] & JOHN E. BLUNDELL[1]

[1]*Institute of Psychological Sciences, University of Leeds, Leeds, UK,* [2]*Yorkshire Centre for Eating Disorders, Seacroft Hospital, Leeds,Yorkshire, UK,* [3]*AstraZeneca, Research and Development Genetics, Macclesfield, Cheshire, UK,* [4]*Discovery & Pipeline Genetics, GlaxoSmithKline, New Frontiers Science Park North, Harlow, Essex, UK, and* [5]*SDG Life Sciences, A Unit of IMS, London, UK*

Abstract

Objectives. There is mixed evidence of association of serotoninergic genes with anorexia nervosa (AN), but substantial evidence for the involvement of serotonergic mechanisms in appetite control. This study was designed to investigate possible associations between the two subtypes of AN (Restricting-RAN, and Binge-purging–BPAN) and polymorphisms within five genes encoding for proteins involved in the serotoninergic system. *Methods.* In order to carry out this investigation we have conducted a case–control association study on 226 females meeting the criteria for AN, and 678 matched healthy females. *Results.* Our data show a significant association between polymorphisms with the gene encoding HTR2A with both AN subtypes, an association between polymorphisms within the genes encoding HTR1D and HTR1B with RAN, and an association between polymorphisms within the gene encoding HTR2C with BPAN. No associations were found for any polymorphisms of the serotonin transporter gene. This outcome indicates a substantial and complex inter-relationship between serotoninergic genes and AN. *Conclusions.* Given these data we hypothesis that the expression or control of expression of several genes of the serotoninergic system, and interactions between these genes, could exert considerable influence over the specific symptomatology of the subtypes of AN.

Key words: *Eating disorder, anorexia nervosa, genetics, serotonin, serotonin recpetor*

Introduction

Anorexia nervosa (AN) is a severely debilitating disorder that affects primarily women (Hebebrand et al. 1996; Elfhag and Linne 2005) and has the highest mortality rate of any of the psychiatric disorders (Sullivan 1995; Steinhausen 2002). Occurring predominantly during adolescence (Halmi et al. 1979) the disease is characterised by a pathological obsession for thinness through the control of eating behaviour. Evidence from family and twin studies have suggested that both genetic and environmental components contribute to the development of AN (Vandereycken and Pierloot 1981; Garfinkel and Garner 1982; Lilenfeld et al. 1998; Fairburn et al. 1999; Sokol et al. 2009). The genetic component has been estimated through meta analysis of twin studies as contributing up to 76% of the susceptibility to AN (Treasure and Holland 1990; Klump and Culbert 2007). Although no large pedigree studies showing Mendelian inheritance have been reported to date, and despite the existence of a large number of published genetic studies, the genetic component of this complex disorder is still far from being understood. This is likely to be due in part to the relatively low prevalence rate of this disorder. A further difficulty could be the existence of separate sub-types of this disorder (American Psychiatric Association 1994), which are likely to have somewhat different genetic profiles.

The serotonin theory of satiety was proposed more than 30 years ago (Blundell 1977), and subsequent

Correspondence: Dr K Kiezebrink, School of Contemporary Sciences, University of Abertay Dundee, Dundee DD1 1HG, UK. Tel: +44 1382 308205. Fax: +44 1382 30800. E-mail:k.kiezebrink@abertay.ac.uk

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research by many authors has confirmed the main tenets of this approach (Blundell and Halford 1998; Halford and Blundell 2000; Halford et al. 2004). In general, the release or blockade of re-uptake of serotonin causes an inhibition of eating, and drugs such as fenfluramine, fluoxetine and sibutramine have all been shown to suppress food intake and cause weight loss in obese people (Van der Ploeg 2000; Halford 2006). Fluoxetine has further more been shown to result in reduce rates of relapse after inpatient weight gain in anorexia nervosa (Kaye et al. 2001). Considerable research on the serotonin receptor subtypes has implicated the HTR1B and HTR2C subtypes in the suppression of appetite (Park et al. 1999). Animal studies have shown that the hypophagia induced by the serotonergic releasing drug fenfluramine can be antagonised by a highly selective HTR1B antagonist (Simansky and Nicklous 2002) injected directly into the parabrachial nucleus, and by pre-treatment with the selective HTR2C antagonist. The hypophagia induced by the sertraline can also be blocked by serotonin antagonists acting on HTR1B and HTR2C receptor (Lucki et al. 1988). In addition the HTR1B receptor agonist significantly suppressed food intake and modified satiety (Halford and Blundell 1996), and a similar effect was shown by the selective HTR2C receptor agonist (Smith et al. 2006). These animal studies were complemented by studies in humans indicating that the HTR1B/1D receptor agonist (Sumatriptan) decreases food intake in healthy women (Boules et al. 2000). The preferential HTR2C receptor agonist mCPP induces weight loss over a 2-week period in obese subjects (Sargent et al. 1997). Taken together, these studies strongly suggest the involvement of HTR1B and HTR2C receptors in the inhibition of food intake. Transgenic animal studies have shown that the HTR1B and HTR2C knock-outs cause over-consumption and weight gain (Clifton et al. 2003). In keeping with this body of evidence are specific findings indicating a disturbance of serotonin metabolism in eating disorders (Kaye 1997; Kaye et al. 2005).

More broadly, in the eating disorders field the evidence for serotonin dysregulation has been intensively reviewed (Brewerton 1995) and several studies have shown an alteration of serotonin neurotransmission in AN and bulimia nervosa (BN) even after the restoration of body weight and or recovery (Kaye et al. 1998; Frank et al. 2002). Furthermore it has been reported that elevated cerebrospinal fluid concentrations of 5-hydroxyindoleactic acid (5-HIAA) occur both during and after recovery from AN and BN (Kaye et al. 1991). Studies have gone on to show that in individuals recovered from AN and BN exposed to serotonin challenges show altered behavioural responses (Frank et al. 2001, Kaye et al. 2003, Smith et al. 1999). The serotonergic pathway has been shown to be involved in feeding, satiety, fasting, mood, anxiety, impulsivity, addiction, body image, perception and gender (Brewerton 1995; Steiner et al. 1997). Given the relationship of these behaviours to phenotypic expression it has been argued that there is clear evidence for dysregulation of serotonin systems in AN (Jimerson et al. 1990). The reduced dietary intake of tryptophan is unlikely to play a major role in altered serotonin metabolism as tryptophan can be provided through tissue catabolism (Favaro et al. 2000). Over 20 years ago dysregulation implied an inappropriate release or reuptake of serotonin around the synapse, or aberrant 5HIAA levels in the CSF. However with the discovery of multiple subtypes (between 15 and 19 depending on the criteria adopted) of the serotonin receptor clustering within seven separate families, the notion of serotonin dysregulation has become more complex and can take many forms. The term dysregulation can now be applied to unusual combinations of different subtypes of serotonin receptors with serotonin playing a role in complex behaviours through an interaction with multiple receptors. Our approach to the understanding of the subtypes of AN is based on this receptor model of dysregulation.

Several studies have investigated possible associations between AN and polymorphisms within serotoninergic genes. There are multiple reports on the serotonin transporter (SLC6A4) gene and AN (Gorwood et al. 2003). Specifically research has focused on a functional polymorphism in the 5' regulatory promoter region which consists of two common alleles which vary due to a 44-base pair insertion (long L-allele) or deletion (short S-allele). In the S-allele form functional studies have revealed a reduction in serotonin transporter gene expression and uptake. There has been mixed evidence for the association of this polymorphism and anorexia nervosa, an initial meta-analysis of four studies revealed that there is a moderated yet significant association with the S-allele and anorexia nervosa (Gorwood 2004). A more recent meta-analysis of eight studies further supported the Gorwood study and showed a strong association with the S form of SLC6A4 and AN (Lee and Lin 2009). A number of studies have examined the role of polymorphisms within the gene encoding HTR2A receptors and genetic susceptibility to AN. The HTR2A promoter polymorphism –14385G/A has been shown to be associated with susceptibility to AN, although this finding is not consistent across all studies. A meta-analysis of 14 studies suggests that when all data are combined there is statistically significant evidence of an association (Gorwood et al. 2003). One of the possible explanations for the diversity of these findings is that many studies have classified patients with the generic

diagnosis of AN whereas others have investigated the specific sub-types. Since it is likely that different allelic profiles and physiological processes underlie each specific sub-type, the use of phenotypic sub-types is likely to be more robust.

A whole genome linkage study has produced the first report of an association of the HTR1D receptor locus with RAN (Bergen et al. 2003). We have confirmed this association (Brown et al. 2007). Of the four studies that have investigated the gene encoding the HTR2C receptor, 2 have reported positive associations (Hinney et al. 1997; Nacmias et al. 1999; Karwautz et al. 2001; Hu et al. 2003). Despite the strong evidence for the involvement of HTR1B receptors in the inhibition of appetite, there appear to be no studies on the link between AN and the gene encoding the HTR1B receptor (although it may be associated with low weight in BN (Levitan et al. 2001).

Given these data we have hypothesised that polymorphisms within genes encoding major components of the serotonergic system play an important role in defining genetic susceptibility to AN. Moreover, it is possible that different patterns of serotonin receptor genes could distinguish the restricting from the binge-purging subtypes of AN. In the current study we have performed a systematic analysis of polymorphisms within the genes encoding for SLC6A4, HTR1B, HTR1D, HTR2A and HTR2C in a single cohort of well characterised individuals with DSM-IV sub-typed AN.

Methods and materials

Subjects

A total of 226 female Caucasian patients, registered at Yorkshire Centre for Eating Disorders (Leeds, UK) between 1998 and 2002. All patients recruited to this study had at some point been admitted to the centre for inpatient treatment however at the date of completing the study the majority were outpatients ($n=176$). For each patient recruited, three female British Caucasian individuals were used as controls (total control population $n=678$). The control group was selected from a large database of healthy individuals in the general population collected for comparison purposes in genetic studies. From this healthy population we selected women who were matched with the cases for month and year of birth; therefore there was a good match for age and gender between the cases and controls. In addition the controls did not contain any individual with any clinical condition. Patients were diagnosed according to DSM-IV criteria for eating disorders by a consultant psychiatrist during clinical interview before being approached to take part in this study. Diagnosis was confirmed through the administration of the structured interview for Anorexia and Bulimia (Fichter et al. 1991; Fichter and Quadflieg 2001). Patients were classified into two groups identified as restricting AN (RAN) or binge-purge AN (BPAN). For this study patients had to meet the diagnostic criteria for current diagnosis (i.e. previous 12 months). We further extended this to ensure that no diagnostic crossover had occurred within the last 36 months, but it should however be noted that in those cases where the patient had less than 3 years diagnosis of AN this would not be possible and as such it is likely that they may undergo diagnostic crossover within the next few years. Based on this analysis of the 226 AN patients 122 were characterised as RAN and 104 were characterised as BPAN. After complete description of the study to each subject, written informed consent was obtained. The study was approved by the Leeds United Hospital Trust Ethical Review Committee (submission number 01/085).

Group definitions

Participants were dichotomised according to DSM-IV subtype classification. Reports of current age, current weight, lowest and highest weight and age of onset were also recorded. From these data BMIs were calculated (BMI = weight (kg)/height $(m)^2$) see Table I.

Statistics

Departure from Hardy–Weinberg Equilibrium (HWE) in the control population was assessed for each polymorphism using a chi-square test. An exact HWE permutation test was performed if the HWE chi-square P value was <0.05 and if at least one genotype cell had an expected count <5 (Zaykin et al. 1995). Polymorphisms with a HWE P value (chi-square or exact) <0.005 in control subjects were excluded from analysis.

Table I. Mean scores and standard deviation of group descriptive (mean BMI for participants divided by AN subtype along with age on completion of study and length of time since diagnosis with eating disorders (ED)).

	Current BMI (kg/m^2)	Lowest BMI (kg/m^2)	Highest BMI (kg/m^2)	Length of ED (years)	Current age (years)
Restrict (RAN)($n=122$)	16.45 (±2.55)	13.53 (±2.42)	22.38 (±2.88)	10.9 (±8.06)	28.6 (±7.32)
Binge/purge (BPAN)($n=104$)	18.1 (±3.49)	14.5 (±2.63)	24.11 (±4.11)	8.00 (±6.74)	27.5 (±6.40)

For each SNP, testing for association between alleles/genotypes and disease status was carried out using the fast Fisher's exact test (FET) procedure. The fast FET computes exact p-values for contingency tables using the network algorithm developed by Mehta and Patel (1983).

For each SNP two parameters are calculated: (1) an odds ratio (95% CI) for the "at risk allele" (the allele that appears more frequently in cases than controls); (2) an odds ratio for the "genotype" (determined by identifying the genotype that has the largest chi-square value when compared against the other two genotypes. For example, if a SNP has genotypes AA, Aa and aa, three chi-square association tests are performed: (a) AA vs Aa+aa, (b) Aa vs AA+aa and (c) aa vs AA+Aa. If test (a) yields the highest chi-square value, then an odds ratio is calculated for the AA genotype versus the Aa+aa genotypes combined). In some cases the genotype may result in an increased risk and in some cases a decreased risk. Where the odds ratio is below 1 this represents a decreased risk and when the odds ratio is above 1 this represents an increased risk. The allele data are always presented as the allele which increases the risk.

Odds ratios (OR) were constructed for the "at risk allele" and "genotype" according to the formula $OR = (n_{11} * n_{22})/(n_{12} * n_{21})$, where n_{11} = cases with "at risk allele"/"genotype", n_{21} = cases without "at risk allele"/"genotype", n_{12} = controls with "at risk allele"/"genotype", n_{22} = controls without "at risk allele"/"genotype". In order to avoid division or multiplication by 0, 0.5 was added to each cell in the contingency table; 95% confidence intervals for the ORs were calculated as follows: lower limit = $OR^* \exp(-z\sqrt{v})$, upper limit = $OR^* \exp(z\sqrt{v})$, where z = 97.5th percentile of the standard normal distribution and v = S-allele$[1/(n_{11})] + [1/(n_{12})] + [1/(n_{21})] + [1/(n_{22})]$.

Power of the sample

The current study examines a population of 226 AN patients and 678 healthy control individuals. This single cohort represents one of the largest AN case-control populations studied to date and given a minor allele frequency of 0.1 and an expected effect size of between 1.6 and 2.6 this study has power of between 77 and 99% at the $P = 0.05$ level.

Association analysis was performed for each of the described SNPs comparing cases versus controls; RAN versus controls; BPAN versus controls; and, RAN versus BPAN.

Multiple testing issues

The current analysis forms part of a much larger study of 42 candidate genes for AN (data to be published elsewhere). In total we tested 176 SNPs for association with AN and its subtypes. Due to the large size of the overall study and the number of statistical tests performed, we would expect to see a number of associations, with $P < 0.05$, simply by chance. However, given the complex nature of the disorder, coupled with the nature of a candidate gene study, it has been impossible to put an exact figure on how many false positives may have been generated. In order to reduce the number of false positives, a semi-conservative Bonferroni-type correction has been applied to the data presented here, by correcting for the number of genes analysed in the whole study. After applying this correction factor, a P value of less than 0.00119 would be evidence of a strong significant association, a P value of between 0.01 and 0.00120 would be evidence of a possible association.

Results

We identified three polymorphisms within the gene for SLC6A4, three polymorphisms within the gene for HTR1B, four polymorphisms within the gene for HTR1D, 10 polymorphisms within the gene for HTR2A and 15 polymorphisms within the gene for HTR2C which had minor allele frequencies of greater than 0.10. The SNP identification number (HGVBASE at http://hgvbase.cgb.ki.se), the NCBI-34 mapping position, the region of the associated gene where the polymorphisms maps and the observed allele frequency in the control population are available online from http://informahealthcare.com/doi/abs/10.3109/15622975.2010.484550.

Hardy–Weinberg equilibrium and pairwise linkage disequilibrium

One SNP was found to be out of HWE in the control population (rs2020934 in the gene encoding SLC6A4, HWE $P = 4.67 \times 10^{-9}$) and was excluded from further analysis; The strength of pairwise linkage disequilibrium (LD) varied across the 10 polymorphisms within the gene for HTR2A (Figure 1). Complete linkage disequilibrium across each of the loci for HTR1B, HTR1D (Brown et al. 2007) and HTR2C was observed in this control population (data not shown). There was no evidence of LD between the two remaining polymorphisms for SLC6A4.

Association of polymorphisms within the genes encoding, SLC6A4, HTR1B, HTR1D, HTR2A and HTR2C

SLC6A4. No evidence of association with DSM-IV™ sub-grouped AN or with AN as a whole, was found in this population with either of the polymorphisms (rs1872924 and rs3794809) identified in

Gap (bp)		6431	4576	9034	8605	1758	6903	9848	8094	3447
	rs3803189	rs977003	rs3742278	rs643627	rs1928042	rs2770293	rs582854	rs985934	rs2025296	rs2070040
rs3803189		0.63	0.45	0.41	0.09	0.04	0.05	0.06	0.06	0.07
rs977003			0.49	0.33	0.08	0.20	0.21	0.15	0.04	0.19
rs3742278				0.48	0.30	0.12	0.11	0.08	0.43	0.01
rs643627					0.05	0.08	0.15	0.03	0.25	0.08
rs1928042						0.54	0.53	0.30	0.19	0.57
rs2770293							1.00	0.42	0.24	0.74
rs582854								0.43	0.24	0.75
rs985934									0.55	0.38
rs2025296										0.46
rs2070040										

Figure 1. Modular *D*' values for polymorphisms within the gene encoding HTR2A. There appears no discernable pattern of linkage disequilibrium (LD) across the gene for HTR2A with only polymorphisms rs582854 and rs2770293 appearing to be in strong LD.

the serotonin transporter gene (SLC6A4) (Table of analysis available online from http://informahealthcare.com/doi/abs/10.3109/15622975.2010.484550).

HRT1B. Both genotype (OR=0.53, 95%CI 0.37–0.78; $P=0.0019$) and allelic (OR = 1.33, 95% CI 1.07–1.66; $P=0.0103$) frequencies at the polymorphism rs1213371 of HTR1B show significant association in a comparison between cases and controls (Table IV). There was no association with either rs1738538 or rs1145835 (Table showing additional data of non-association available online from http://informahealthcare.com/doi/abs/10.3109/15622975.2010.484550).

HTR2A. Of the 10 SNPs analysed across the HTR2A gene, two were associated with AN. Polymorphism rs3742278 was found to be significantly associated at both the genotypic (OR = 0.60, 95%CI 0.43–0.84; $P=0.0003$) and allelic (OR=1.65, 95%CI 1.23-2.22; $P=0.0012$) levels when comparing all cases with controls. This possible association appears to be specific to the BPAN with significant associations in both the genotypic (OR=0.47, 95%CI 0.29–0.75; $P=0.0015$) (however this fails to meet the stringent correction for significance at the $P=0.00119$ level) and allelic (OR=2.04, 95%CI 1.37–3.03; $P=0.0006$) comparisons with BPAN and controls which remains significant upon applying the correction for significance (Figure 2) (Table with additional data of non-association available online from http://informahealthcare.com/doi/abs/10.3109/15622975.2010.484550).

Polymorphism rs985934 was also significantly different in the control population from the case when comparing genotypic frequencies (OR=1.79, 95%CI 1.32–2.44; $P=0.0007$). This association appears to be driven by an association of RAN compared with controls in the genotypic level (OR=2.11, 95%CI 1.38–3.21; $P=0.0021$) (however this fails to meet the stringent correction for significance at the $P=0.00119$ level) (Figure 3).

HTR2C. Of the 16 SNPs investigated within the gene for HTR2C, 10 were found to be significantly associated with BPAN when comparing genotypic frequencies with the control population however upon correction for multiple testing only one of these remained significant this was rs2428720 ($OR=0.36$, 95%CI 0.20–0.65; $P=0.000276$) (Table with full data on all non-associated SNPS available online from http://informahealthcare.com/doi/abs/10.3109/15622975.2010.484550).

Discussion

The outcome of this investigation has provided evidence for a substantial, and complex, interlinking of serotoninergic gene polymorphisms and genetic predisposition to subtypes of AN. The findings of the association analysis with HTR1D have previously been published (Brown et al. 2007). Significant associations have been demonstrated with genes for four separate serotoninergic receptors: genetic polymorphisms of the HTR1B and HTR1D genes appear to differentiate the RAN from control subjects; HTR2C alleles appear to differentiate BPAN from controls; and there is allelic variation in the HTR2A gene both within and between BPAN and RAN.

A considerable number of previous studies have used the candidate gene approach to ascertain the relationship between polymorphisms in the gene encoding HTR2A and anorexia nervosa. Most of

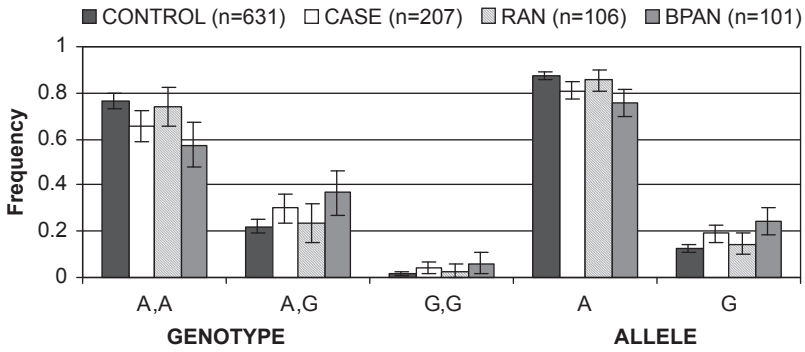

Figure 2. Genotypic and allelic frequency and 95% CI for HTR2A polymorphism rs3742278. A significant difference was found in the allelic frequency between cases and controls ($P=0.0011$) there was a trend towards significant difference in the genotypic test, however this failed to stand up to the correction factor ($P=0.0032$). When comparing BPAN and controls the frequency of the G allele was significantly higher in the BPAN than in the controls ($P=0.0006$) This association may to be a reflection of the extremely low frequency of the G,G genotype. However there were no significant differences when comparing the RAN with controls or with BPAN on either genotypic or allelic frequencies.

these studies have reported no association but in the positive studies where DSM-1V classification had been applied, an association was reported to relate to the restricting subtype. In the present study, taking the anorexia nervosa group as a whole, a case–control comparison demonstrated a strong association ($P=0.0007$) with a polymorphism within the HTR2A gene. Moreover, whilst there was significant allelic variation between RAN and controls, there was also an association shown for BPAN and cases in comparison with controls. Consequently we have concluded that considerable allelic variation in polymorphisms of the HTR2A gene exists across the whole spectrum of patients with AN. However, the occurrence of differences between restricting and binge-purging anorexia nervosa suggests that specific features of this gene not only differ between all anorexia nervosa patients and controls but also differ between RAN and BPAN.

Of the 10 polymorphisms used to investigate the HTR2A gene, eight did not demonstrate statistically significant associations. Polymorphism rs3742278 showed significant genotype and allelic variation between BPAN and controls (and between cases and controls), polymorphism rs985934 demonstrated highly significant genotypic association between cases and controls. Therefore, depending upon the specific polymorphism chosen in any candidate gene study, an association could be found with RAN, BPAN, both subtypes, or neither. This could account,

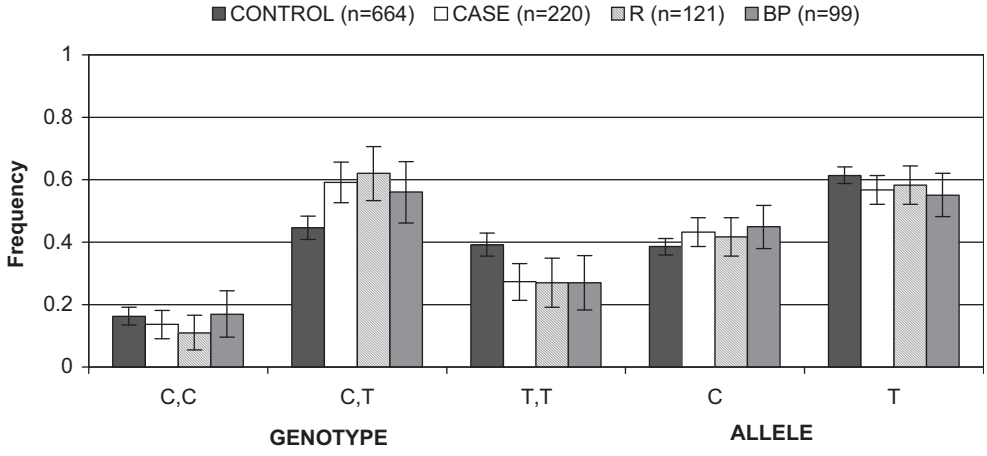

Figure 3. Genotypic and allelic frequency and 95% CI for HTR2A polymorphism rs985934. A significant difference was found in the genotypic frequency between cases and controls ($P=0.0007$). The frequency of the C,T genotype was increased in anorexia nervosa patients compared with the controls and this finding is complemented by a decreased frequency of T,T genotype in the anorexia nervosa population compared with the controls. In the comparison between RAN and controls there was a significant association ($P=0.0021$) in the genotypic analysis. There was no significant difference when comparing RAN with control by alleles ($P=0.495$). There was no significant differences either when comparing BPAN with control (genotypic $P=0.086$, allelic $P=0.079$) there was also no significant differences between RAN and BPAN (genotypic $P=0.355$, allelic $P=0.500$).

at least in part, for the positive and negative findings of association previously reported in the literature (Klump and Gobrogge 2005). We are aware that diagnostic stability (or rigidity as it is sometimes called) is an issue and that diagnostic crossover is a feature in this disorder. An attempt to ensure this diagnostic stability was made by requiring subtype criteria to have been met for the previous 36 months. However there were some cases in which the eating disorder had not been diagnosed for the full 36 months and in these cases there would still be the possibility of diagnostic crossover prior to recruitment into the study. There would also be the possibility in those cases with diagnosis of longer than 36 months that diagnostic crossover had previously occurred. It is extremely difficult to eliminate the possibility of cross-over within the lifetime of the patient, but we feel that we have certainly eliminated the occurrence of cross-over within several years of the inception of this study.

A full genome linkage analysis study (Bergen et al. 2003) has, for the first time, implicated the HTR1D gene in AN. A region of human chromosome 1, incorporating this gene, was linked with genetic susceptibility to restricting, but not binge-purging, anorexia nervosa. We have recently provided a replication of these findings (Brown et al. 2007). Of the four polymorphisms within this gene included in the analysis, one (rs856510) showed significant genotypic and allelic variation between RAN and controls, and a separate polymorphism (rs674386) also showed significant allelic variation between RAN and controls. These findings suggest that further analysis of the relationship between this gene and AN is warranted.

There is considerable evidence to implicate the HTR1B and HTR2C receptors in the control of appetite, and specifically with an inhibition of eating in animals and humans (Blundell and Halford 1998). There is therefore considerable circumstantial evidence for considering that variation in the genes for these two receptors could be associated with severe forms of human appetite disorders. There appears to have been no previous reports in the literature of association between AN and the HTR1B receptor gene. Of the three polymorphisms included in the current genotyping analysis, one (rs1213371) showed a significantly different frequency of occurrence in AN versus controls, and this was largely due to an association with the RAN subgroup. This polymorphism showed significant genotypic and allelic variation with the RAN subgroup. The association between polymorphisms within the gene for the HTR1B receptor (exclusively with the restricting subtype) is consistent with the known functional activity of this receptor in the appetite control systems. The HTR1B receptor agonist CP-94,253 inhibits food intake and intensifies satiety in rats (Halford and Blundell 1996), whilst in humans sumatriptan, which has a high affinity for the HTR1B (and HTR1D) receptor, has been shown to reduce intake at a meal (Boeler et al. 1997). Given this evidence, it is plausible to envisage that some adjustment to the structure of the HTR1B gene, if this caused a change in levels or sensitivity of HTR1B receptors, could promote a tendency to food restriction.

In contrast to the association of the HTR1B genes and RAN, a quite different pattern was observed for the HTR2C gene. Here, of the 16 polymorphisms investigated, 10 were found to be significantly and exclusively associated with the BPAN subtype. Moreover, there was also variation of the HTR2C genotypic and or allelic frequencies between the RAN and BPAN groups. Clearly, one reason for the significant associations with so many polymorphisms is because these polymorphisms were in LD indicating that a sizeable block of this gene is inherited as a complete unit. There is considerable evidence from animal studies that agonism at the HTR2C receptor is a sufficient condition for inhibition of food intake (Halford et al. 2004). Indeed, agonists at HTR2C receptors are currently in development as anti-obesity agents (Halford et al. 2004). This account is plausible since the HTR2C receptor agonist has been shown to decrease appetite and body weight in obese subjects (Smith et al. 2006).

In addition the transgenic HTR2C knock-out mouse displays hyperphagic behaviour and is obese (Tecott et al. 1995). A further interesting feature is that moderate food deprivation causes HTR2C receptor super-sensitivity (Cowen et al. 1996). Although three studies have found no association of the Cys-23-Ser alteration of the HTR2C receptor gene and either obese or underweight children (Hinney et al. 1997), anorexia nervosa patients (Nacmias et al. 1999), or sib-pair with anorexia nervosa (Karwautz et al. 2001) two other studies have reported an association between the Cys-23-Ser alteration of the HTR2C gene with low weight in teenage girls (Westberg et al. 2002) and minimum body mass index in AN (Hu et al. 2003). The results of the present study support an association but also go further to link the HTR2C receptor gene with BPAN. In the light of the experimental evidence concerning the HTR2C receptor and appetite control, an association between polymorphisms within this gene and AN is entirely plausible. The reason why this gene should be associated exclusively with BPAN is not immediately clear. However there exists the interesting possibility that the powerful restriction of food intake in RAN and BPAN patients may

be facilitated, at least in part, by two distinct serotoninergic receptor subtypes; the HTR1B in RAN and the HTR2C in BPAN. However, this suggestion should be treated with caution. The complex relationship between serotonin genes and the expression of appetite, and the reciprocal influence of dieting (and nutrition) on the sensitivity of serotonin receptors (as discussed by Hu et al. 2003) means that several possible neurochemical scenarios could be envisaged to mediate between polymorphisms of a gene for a specific receptor and the processes underlying the variability in symptomatology in AN patients. An area for further investigation is the link between tryptophan and serotonin and the possible mediating effects of tryptophan in eating disorders (Russo et al. 2007).

The outcome of this investigation supports the view that a complex variation in the genes from four different serotoninergic receptors (HTR1B, HTR1D, HTR2A and HTR2C) influences genetic susceptibility across the spectrum of symptomatology in AN. The investigation was driven by a strong hypothesis concerning the relationship between receptor subtypes in the serotoninergic systems and the clinical expression of sub-types of AN. We have decided to publish the results for all receptor sub-types together, rather than singly in order to provide the fullest picture of the inter-relationships. The study had considerable power and the associations (where significant) high probabilities. Some of the associations are complex. For example with the HTR2A gene different polymorphisms show significant associations when comparisons are made between the whole group of AN cases, RAN or BPAN. This is intriguing and suggests that there is a controlling element in this gene relevant to the phenotypic expression of anorexia nervosa. For this, and the other associations seen for HTR1D, HTR1B and HTR2C genes further interpretation would be pure supposition. Taken as a whole these findings suggest an important relationship between serotonin receptor activity and clinical symptoms in AN.

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Statement of interest

Dr Kiezebrink reports having received research funding from MRC and GlaxoSmithKline (GSK) in the form of a PhD case studentship. Dr Kiezebrink also reports receiving a travel award from Carnegie Trust with respect of this work. Ms Bujac and Dr Stubbins both report having been in the employ of GSK at the time of the study completion, Ms Bujac is now in the employ of AstraZeneca. Dr Mann, Dr Campbell and Professor Blundell all reported no biomedical financial interests or potential conflicts of interest.

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Supplementary material available online

Table I – Details of SNPs used in genotyping of SLC6A4, HTR1B, HTR1D, HTR2A and HTR2.
Table II – Results of case–control and subtype association analysis with SLC6A4.
Table III – Results of case:control and subtype association analysis with HTR1B.
Table IV – Results of case:control and subtype association analysis with HTR2A.
Table V – Results of case:control and subtype association analysis with HTR2C.

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BRIEF REPORT

Depressive symptoms and 10-year risk for cardiovascular morbidity and mortality

HANNU KOPONEN[1], JARI JOKELAINEN[2,3], SIRKKA KEINÄNEN-KIUKAANNIEMI[2,3] & MAUNO VANHALA[4,5]

[1]*Department of Psychiatry, Kuopio University Hospital and University of Eastern Finland, Kuopio, Finland,* [2]*Institute of Health Sciences, University of Oulu, Oulu, Finland and* [3]*Unit of General Practice, Oulu University Hospital, Oulu, Finland,* [4]*Unit of Family Practice, Central Hospital of Middle Finland, Jyväskylä, Finland, and* [5]*School of Public Health and Clinical Nutrition, Department of Family Medicine, University of Eastern Finland, Kuopio, Finland*

Abstract

Objectives. Depression is associated with increased physical morbidity and overall mortality. As less is known about how much depression increases the 10-year risk for fatal and nonfatal cardiovascular (CV) events, we evaluated the cross-sectional risk with two well-characterized risk functions measuring CV mortality and total CV event risk. *Methods.* The prevalence of increased depressive symptoms was measured with the Beck Depression Inventory (BDI), and the SCORE and Framingham risk functions were calculated in a middle-aged population-based sample ($N=923$). For metabolic syndrome (MetS), the modified National Cholesterol Education Program – Adult Treatment Panel III criteria were employed. *Results.* Depressive symptoms were associated with increased CV mortality and morbidity risk in men: OR for SCORE 2.9; 95%CI 1.4–5.7 and OR for Framingham function 2.2 (95%CI 1.1–4.2). In women, the corresponding figures were 1.4 (95%CI 0.3–6.9) and 1.3 (95%CI 0.7–2.6). The BDI scores showed significant correlations with SCORE ($r=0.18$ for men, $P < 0.001$; and $r=0.14$ for women, $P=0.002$), and Framingham function (for men $r=0.16$, $P < 0.001$; and for women $r=0.13$, $P=0.005$). *Conclusions.* Our results suggest that screening and effective treatment of depression are important in the primary and secondary prevention of cardiovascular events, especially in males.

Key words: *Beck Depression Inventory, cardiovascular event, Framingham risk function, mortality, SCORE risk function*

Acronyms: BDI, Beck Depression Inventory; CV, Cardiovascular; MetS, metabolic syndrome; NCEP-ATPIII, National Cholesterol Education Program – Adult Treatment Panel III; OR, odds ratio; SCORE, Systematic Coronary Risk Evaluation; SD, standard deviation

Introduction

Many studies have reported an association between depression or depressive symptoms and physical morbidity or overall mortality (Cuijpers and Smit 2002). A major part of this risk relates to fatal and nonfatal cardiovascular (CV) events (Stewart et al. 2003; Wulsin and Singal 2003; Barth et al. 2004; Gump et al. 2005), although there have also been studies showing no association (Everson-Rose et al. 2004; Hildrum et al. 2009). It is not clear what mechanisms convey this increased cardiovascular morbidity and mortality rate, but patients' lifestyle, hypercortisolemia, increased platelet aggregation, suppressed immune system functioning, altered heart rate variability, unbalanced dietary habits and side-effects of antidepressive medications have been suggested (Bonnet et al. 2005; Dimsdale 2008; Ovaskainen et al. 2009). Depression is also a risk factor for the development of metabolic syndrome (MetS; Vaccarino et al. 2008; Vanhala et al. 2008), and part of the increased risk for cardiovascular diseases may be explained by a higher prevalence of MetS in depressed patients (McNeill et al. 2005; Lorenzo et al. 2007, Vaccarino et al. 2008).

Correspondence: Hannu Koponen, Professor, Department of Psychiatry, Kuopio University, Hospital and University of Eastern Finland, PO Box 1777, FIN-70211 Kuopio, Finland. Tel: +358 40 550 5996. Fax: +358 17 172966. E-mail: hannujuhani.koponen@uku.fi

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Depression is, however, also more common in existing cardiovascular diseases, and it is also possible that other factors (such as socioeconomic status or health behaviours) are responsible for both outcomes (Thorndike and Rigotti 2009).

As MetS and depression are interrelated (Koponen et al. 2008; Vanhala et al. 2009), knowledge of the unique contribution of depression to the CV risk would be important for preventive purposes. However, there are no specific data on the depression-related CV event risk evaluated by established risk functions (e.g., Framingham or Systematic Coronary Risk Evaluation function [SCORE]; Wilson et al. 1988; Conroy et al. 2003). In this study we evaluated the 10-year risk for fatal and nonfatal cardiovascular events in cases with or without depressive symptoms in a large population-based study sample. Secondly, the effect of MetS on the depression-related cardiovascular event risk was evaluated.

Methods and materials

Subjects

The study was conducted in the Pieksämäki area of South-Savo, Finland. All inhabitants born in the years 1942, 1947, 1952, 1957 and 1962 in Pieksämäki ($N=1294$) were invited for a comprehensive health check-up during 1998 (Vanhala et al. 2002). The study was carried out in accordance with the latest version of the Declaration of Helsinki. All participants ($N=923$, i.e. 71.3%) provided written informed consent, and the study protocol was approved by the Ethics Committee of Kuopio University Hospital and the University of Kuopio. Normally, in epidemiological studies the participation rate has ranged between 50 and 70% in Finland. No data was collected from the non-participants due to the lack of consent.

Procedures

At the 1998 study visit all participants completed a standard questionnaire containing questions about their use of medications. In addition, data were collected on smoking habits, alcohol use (number of drinks per week) and physical activity (number of exercise sessions of over 30 min per week). Depressive symptoms were evaluated with the Beck Depression Inventory (BDI), in which a score of 10 points was used as a cut-off point (Beck et al. 1961). Blood samples for the determination of glucose and lipid levels were drawn between 08:00 and 11:00 after 12 h of fasting. The physical examination included weight, height, waist circumference and blood pressure taken on the same study visit.

The 10-year risk for fatal cardiovascular events was estimated using the SCORE function (Conroy et al. 2003), including coronary death, sudden death, stroke, aneurysm and heart failure. The Framingham function (Wilson et al. 1998) was used to estimate the overall risk for any fatal or nonfatal CV event (including the previously mentioned fatal events and any type of angina, myocardial infarction, coronary ischemia, congestive heart failure, intermittent claudication and peripheral arterial ischemia). In the SCORE function, the cardiovascular mortality risk is calculated from the values for age, gender, total cholesterol, HDL cholesterol, systolic blood pressure and smoking habit. With the Framingham function, risk is calculated from the same values as the SCORE function, but with the addition of the presence of diabetes. Originally, the reported predictive values for SCORE have ranged from 0.71 to 0.84 (Conroy et al. 2003), and in the review of Tzoulaki and coworkers similar figures are reported for Framingham risk score (median 0.74; Tzoulaki et al. 2009). In this study, the participants with appropriate data for calculating risk functions ($N=909$) were classified according to the probability of presenting a "high/very high" risk for cardiovascular mortality (SCORE $\geq$ 3%) and for a fatal or non-fatal cardiovascular event risk (Framingham function$>$10%) within 10 years (Wilson et al. 1998; Conroy et al. 2003).

In the evaluation of MetS, we used the modified NCEP-ATPIII criteria with the 100 mg/dl blood glucose cut-off point (Grundy et al. 2005). Originally, the NCEP defined metabolic syndrome as having three or more of the following criteria: (1) fasting serum glucose of 110 mg/dl or higher, (2) serum triglycerides of 150 mg/dl or higher, (3) serum HDL cholesterol less than 40 mg/dl in men or less than 50 mg/dl in women, (4) blood pressure of 130/85 mmHg or higher, and waist circumference greater than 102 cm in men or greater than 88 cm in women (Expert Panel 2001).

Statistical methods

Depression caseness was modelled using logistic regression, to examine associations of depression with SCORE and Framingham risk functions. Separate models were created for males and females In multivariate analyses, participants with diabetes were excluded, and the following possible confounding factors were adjusted: age, education, marital status, smoking, physical activity, alcohol consumption, and use of antidepressive medication and hormone replacement therapy; and adding MetS, to determine whether the latter mediated the association between depressive symptoms and outcome. Above specified possible confounding factors were selected

because these factors has shown to be associated with both depression and CVD-risk (Tzoulaki et al. 2009). The mediation analyses were conducted using a SAS-macro as described by Jasti et al. (2008). All statistical testing was performed at a 5% level of significance using SAS for Windows version 9.1.

Results

Females and males with depressive symptoms were older and used more often antidepressants as compared to nondepressed males and females. In addition, depressed males consumed alcohol and were single or divorced more often than nondepressed males (Table I). The prevalence of depressive symptoms in the whole study population was 14%, and the prevance of MetS 30.1% (Table II).

In the whole study population, 15.9% ($N=144$) and 27.2% ($N=209$) of all cases showed a high/very high 10-year CV event risk according to the SCORE (> 3%) and Framingham (>10%) function, respectively. When subdivided according to the gender, 33.8% of males and 1.8% of females had SCORE values>3%; the corresponding figures for Framingham >10% were 44.4% (males) and 13.6% (females). The risk scores were significantly higher in males with depressive symptoms, who also were more frequent smokers. In the logistic regression analysis, depressive symptoms were associated with high cardiovascular mortality and morbidity risk in men (SCORE OR 2.9; 95%CI 1.4–5.7 and Framingham function OR 2.2; 95%CI 1.1–4.2). In women, the OR was 1.4 (95%CI 0.3–6.9) for SCORE and 1.3 (95%CI 0.7–2.6) for the Framingham function.

The relationship between depressive symptoms and CVD risk as measured by SCORE was not mediated by the presence of MetS, the amount of mediation was only 2.6% for males ($P=0.8987$; Sobel test), and 0.5% ($P=0.8645$) for females. For Framingham the mediation for males was 5.4% ($P=0.7653$) and females 12.2% ($P=0.9848$). Out of 44 males with depressive symptoms, 14 had MetS but only 10 of them had SCORE>3%. This co-occurrence of depressive symptoms, MetS and high SCORE risk>3% in males is also described in Figure 1. The BDI scores showed significant correlations with SCORE ($r=0.18$ for men, $P<0.001$; and $r=0.14$ for women, $P=0.002$), and Framingham function (for men $r=0.16$, $P<0.001$; and for women $r=0.13$, $P=0.005$).

Discussion

The novel finding in our population-based study containing different middle-aged subgroups and both sexes was that men with depressive symptoms demonstrated a 2.2–2.9-fold higher 10-year cardiovascular morbidity and mortality risk as evaluated by established risk functions, i.e. Framingham function and SCORE. The finding was significant even after adjusting for multiple sociodemographic factors, in which the differences were nonsignificant or only modest. Our observation is in agreement with the results of Bremmer et al. (2006), Surtees et al. (2008), and Kamphuis et al. (2009) showing twofold mortality in patients with major depression at baseline in older study cohorts. In our study, the difference between risk scores in the female group with and without depressive symptoms was insignificant. This is in line with previous observations that women develop coronary heart disease at an older age, and that the observed cardiovascular event rates in middle-aged women are lower (Wang et al. 2007). The more frequent history of divorce and

Table I. Study population characteristics.

	Women			Men		
Characteristic	Depressed ($n=84$, 19.8%)	Non depressed ($n=424$)	*P* value	Depressed ($n=44$; 12.3%)	Non depressed ($n=357$)	*P* value
Age, mean (SD)	47.7 (6.4)	46.2 (6.2)	0.037	49.1 (6.1)	46.3 (6.3)	0.005
Current use of antidepressant	8.3%	2.6%	0.009	6.8%	1.4%	0.015
Basic education,%			0.105			0.145
elementary school	16.7%	9.0%		15.9%	12.0%	
middle school	46.4%	52.1%		72.7%	63.6%	
matriculation	39.9%	36.9%		11.4%	24.4%	
Marital status,%			0.089			0.043
Single	9.5%	8.0%		18.2%	9.8%	
Married/cohabit	71.4%	81.1%		70.4%	85.2%	
Divorced/ widowed	19.1%	10.9%		11.4%	5.0%	
Physically active,%	27.4%	29.2%	0.744	31.8%	31.1%	0.922
Alcohol use%	10.7%	7.8%	0.376	45.5%	30.5%	0.046
Low fruits and vegetables intake	13.1%	10.1%	0.422	36.4%	26.6%	0.173
High dietary fat intake	38.1%	46.5%	0.159	43.2%	42.6%	0.939

Table II. Metabolic characteristics in various study subgroups.

	Women			Men		
Characteristic	Depressed (n=84; 19.8%)	Non depressed (n=424)	P value	Depressed (n=44; 12.3%)	Non depressed (n=357)	P value
SCORE, mean (SD)	0.8 (1.0)	0.6 (0.7)	0.100	5 (4.6)	2.8 (2.8)	<0.001
≥3%	2.4%	1.7%	0.647	56.8%	30.1%	<0.001
Framingham score	4 (5.7)	3.4 (5.3)	0.332	6.2 (2.4)	4.9 (2.5)	0.001
≥10%, 10-years absolute risk for total CHD	16.7%	13.0%	0.376	63.6%	42.0%	0.014
Current smokers,%	23.8%	21.8%	0.678	54.6%	31.4%	0.002
Waist circumference, mean (SD)	85.6 (14.4)	83.0 (11.8)	0.079	95.9 (10.2)	93.3 (10.2)	0.108
BMI, mean (SD)	26.7 (5.8)	26.3 (4.8)	0.462	27.0 (4.1)	26.6 (3.6)	0.429
Weight (kg), mean (SD)	71.5 (14.9)	69.8 (13.1)	0.289	83.8 (13.2)	83.4 (13.2)	0.835
Systolic BP, mean (SD)	132.1 (18.7)	132.8 (18)	0.744	140.7 (22.8)	137.7 (16.8)	0.284
Diastolic BP, mean (SD)	80.7 (9.9)	79.8 (9.4)	0.405	84.5 (10.3)	84.0 (10.2)	0.741
Hypertension (sys ≥ 135 and/or dia≥85)	53.6%	55.0%	0.816	65.9%	69.2%	0.658
Antihypertensive medication	11.9%	8.3%	0.2821	13.6%	9.8%	0.4285
Triglycerides, mean (SD)	1.3 (0.6)	1.2 (0.6)	0.220	1.9 (2.4)	1.6 (1.0)	0.448
HDL, mean (SD)	1.5 (0.3)	1.5 (0.3)	0.612	1.3 (0.3)	1.3 (0.3)	0.825
Medication for dyslipidemia	2.4%	0.9%	0.2652	2.3%	3.1%	0.7665
Glucose, mean (SD)	5.7 (0.6)	5.6 (0.6)	0.581	6.1 (0.7)	6.0 (0.9)	0.150
MetS, according NCEP -criteria	27.4%	28.3%	0.864	31.8%	32.8%	0.899

alcohol consumption in males with increased depressive symptoms may represent the negative effects of depression. The use of antidepressants was more common in males with depressive symptoms. However, the number of cases using antidepressants is low in every subgroup and their exclusion from the analyses does not change the results.

In our study population the relationship between depressive symptoms and CVD risk was not mediated by the presence of MetS. In a previous study of Vaccarino et al. (2008), both MetS and depression were independent predictors of cardiovascular disease

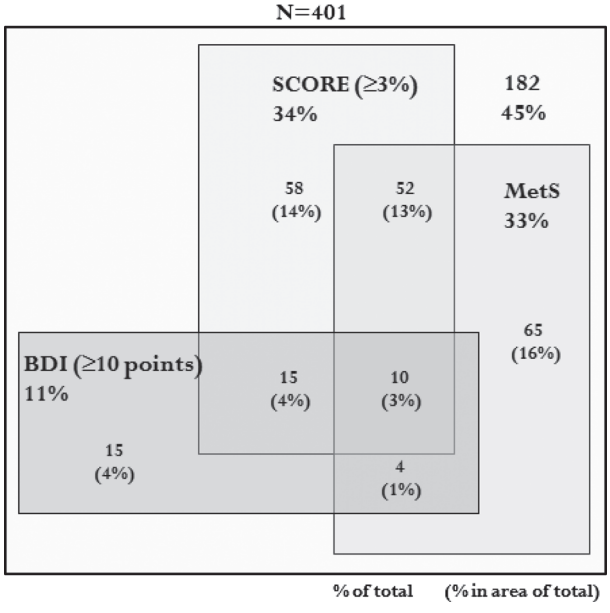

Figure 1. Mediation of depressive symptoms, MetS and≥3% SCORE risk in males (areas not proportional).

(CVD) in a pure female population, and MetS explained only 20% of the association between depression and CVD. In our study the amount of mediation was only 2.6% for males, and 0.5% for females in SCORE and 5.4% (males) and 12.2% (females) for Framingham. Although the differences may be due to differences in study populations the magnitude of mediation may be substantially lower than previously expected. In other recent studies, in a large meta-analysis of eleven European prospective cohort studies, MetS was associated with increased cardiovascular mortality in both women (OR 2.8) and men (OR 2.3; Hu et al. 2004), and the same has also been true in other studies (e.g. Malik et al. 2004; Hunt et al. 2007).

Previous studies have demonstrated that low-grade inflammation and involvement of the cytokine system may be common phenomena underlying MetS, cardiovascular morbidity and depressive symptoms (Alesci et al. 2005; Ferketich et al. 2005; Capuron et al. 2008). Previously, we have reported an elevation of IL-1RA levels and the IL-1RA/ IL-1 beta ratio in males with depressive symptoms (Ovaskainen et al. 2009), which further supports the role of cytokines. However, inflammatory biomarkers explain only a part of the association between depression and the CV event incidence (Vaccarino et al. 2007), which suggest that other mechanisms, such as insulin resistance, may also be involved (Ramasubbu 2002).

There are several limitations in this study. The study setting was cross-sectional, and the identification of depressive symptoms was based on a self-report scale, not a diagnostic interview. Although not initially constructed as a diagnostic scale, the BDI with a cut-off

score of 10 points has been shown to be a useful instrument for detecting depressive disorders, and has been demonstrated to predict the cardiovascular outcomes (Steer et al. 1999; Lasa et al. 2000; Timonen et al. 2005; Vaccarino et al. 2008). Secondly, our genetically homogenous study population may hamper the generalizability of the results. Thirdly, the study was carried out more than 10 years ago and primary and secondary prevention of cardiovascular diseases has been changed limiting the generalizability of our results. The modest correlations between BDI scores and SCORE and Framingham function need also further confirmation.

Our results suggest that depression and MetS are independent risk factors of cardiovascular diseases. Secondly, screening and effective treatment of depression are important in the primary and secondary prevention of cardiovascular events, especially in males.

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Statement of interest

None.

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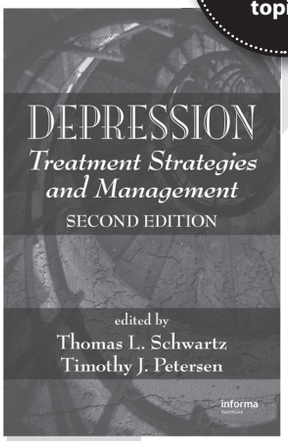

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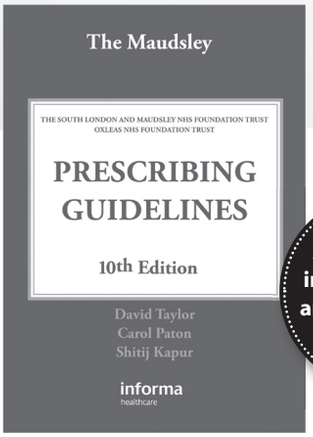

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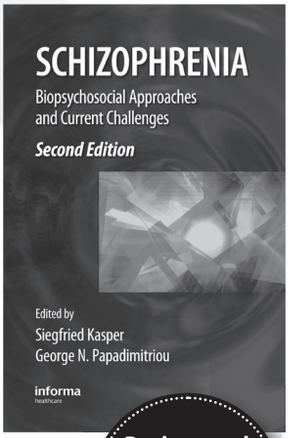

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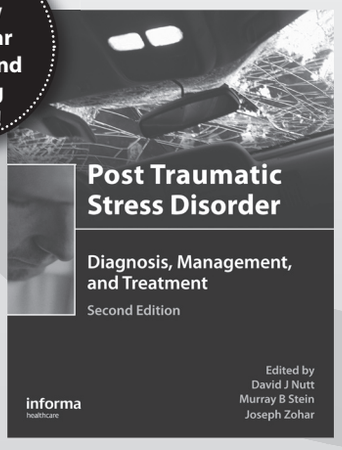

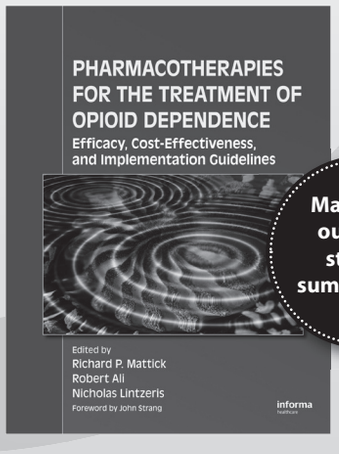

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