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Role of brain-derived neurotrophic factor in Huntington's disease

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Abstract

Neurotrophic factors are essential contributors to the survival of peripheral and central nervous system (CNS) neurons, and demonstration of their reduced availability in diseased brains indicates that they play a role in various neurological disorders. This paper will concentrate on the role of brain-derived neurotrophic factor (BDNF) in the survival and activity of the neurons that die in Huntington's disease (HD) by reviewing the evidence indicating that it involves profound changes in BDNF levels and that attempts to restore these levels are therapeutically interesting.

BDNF is a small dimeric protein that is widely expressed in adult mammalian brain and has been shown to promote the survival of all major neuronal types affected in Alzheimer's disease (AD) and Parkinson's disease (PD). Furthermore, cortical BDNF production is required for the correct activity of the corticostriatal synapse and the survival of the GABA-ergic medium-sized spiny striatal neurons that die in HD. We will highlight the available data concerning changes in BDNF levels in HD cells, mice and human postmortem samples, describe the molecular evidence underlying this alteration, and review the data concerning the impact of the experimental manipulation of BDNF levels on HD progression. Such studies have revealed a major loss of BDNF protein in the striatum of HD patients which may contribute to the clinical manifestations of the disease. They have also opened up a molecular window into the underlying pathogenic mechanism and new therapeutic perspectives by raising the possibility that one of the mechanisms triggering the reduction in BDNF in HD may also affect the activity of many other neuronal proteins.

Keywords: Huntington's disease; Huntingtin; Brain-derived neurotrophic factor

Abbreviations: 3NP, 3-nitropropionic acid; AAV, adeno-associated viral vector; AD, Alzheimer's disease; ADP, adenosine diphosphate; AKT, protein kinase B; ALS, amyotrophic lateral sclerosis; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AP-1, activating protein-1; ARPP-21, cyclic AMP-regulated phosphoprotein, 21 kDa; ATP, adenosine triphosphate; BAC, bacterial artificial chromosome; BAF170, BRG1-associated factor 170; BAF57, BRG1-associated factor 57; Bcl-2, B-cell CLL/lymphoma 2; BDNF, brain-derived neurotrophic factor; BRAF35, BRACA-2-associated factor 35; BRG1, brahma-related gene 1 protein; cAMP, cyclic adenosyne monophosphate; CaM kinase IV, calcium/calmodulin-dependent protein kinase IV; CaRE1/2/3, calcium responsive element 1, 2 and 3; CaRF, calcium response factor; CBs, cannabinoids; CBP, CREB binding protein; C/EBPbeta, CCAAT/enhancer binding protein beta; CNS, central nervous system; CNTF, ciliary neurotrophic factor; coREST, REST corepressor; CRE, cAMP responsive element; CREB, cAMP responsive element binding protein; DARPP-32, dopamine- and cyclic AMP-regulated phosphoprotein, 32 kDa; DOPAC, 3,4-dihydrophenylacetic acid; DR, dietary restriction; ES, embryonic stem; ELISA, enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; GABA, gamma-aminobutyric acid; GDNF, glial cell line-derived neurothrophic factor; GFP, green fluorescent protein; GRIK2, glutamate receptor, ionotropic, kainate 2; GRIN2B, glutamate receptor, ionotropic, *N*-methyl-D-aspartate 2B; GSK, glycogen synthase kinase; HAP1, Huntingtin-associated protein 1; HD, Huntington's disease; Hdh, Huntington's disease gene homolog; hsc70, heat-shock protein cognate 70 kDa; HSJ1B, heat-shock protein DNAJ-containing protein 1b; KA, kainic acid; LTP, long-term potentiation; LIM, Lin-11 Isl-1 Mec-3; L-VDCC, L-type voltage-dependent Ca^{2+} channel; MAPK, mitogen-activated protein kinase; MeCP2, methyl-CpG binding protein 2; MEKK, MAPK kinase kinase; miRNA, microRNA; mSin3A, mammalian homologue of yeast Sin3A; MSN, medium-sized spiny neuron; ncRNA, noncoding RNA; NeuN, neuronal nuclei; NFKB, nuclear factor NF-kappa-B; NGF, nerve growth factor; NMDA, *N*-methyl-D-aspartic acid; NMDA-R, *N*-methyl-D-aspartate glutamate receptor; NR2B, *N*-methyl-D-aspartate receptor subtype 2B; NT3/4/5, neurotrophin-3/4/5; $p75^{NTR}$, p75 neurotrophin receptor; PD, Parkinson's disease; PI3K, phosphoinositol-3 kinase; P150, ^{Glued} 150 kDa dynein-associated polypeptide; PKA, protein kinase A; QUIN, quinolinic acid; RE1/NRSE, repressor element 1/neuron-restrictive silencer element; REST/NRSF, RE-1 silencing transcription factor/neuron-restrictive silencer factor; RILP, REST/NRSF-interacting LIM domain protein; RTT, Rett syndrome; SCG10, superior cervical ganglion-10 protein; siRNA, small interfering RNA; Sp1, specificity protein 1; SSRI, selective serotonine reuptake inhibitor; SVZ, subventricular zone; TAFII-130, TATA box binding protein (TBP)-associated factor, 130 kDa; TGase, transglutaminase; TrkB, tyrosine receptor kinase B; UCHL1, ubiquitin carboxy-terminal hydrolase L1; USF1/2, upstream stimulatory factor 1 and 2; UTR, untranslated region; Val66Met, valine-to-methionine substitution at position 66; YAC, yeast-derived artificial chromosome; X-Gal, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

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Contents

1. Introduction

Huntington's disease (HD) is a fatal, dominantly inherited, neurodegenerative disorder that usually onsets in midlife, and is characterised by psychiatric, cognitive and motor dysfunctions. It is due to an excessive repetition of the CAG trinucleotide in exon 1 of the *huntingtin* gene (Huntington's Disease Collaborative Research Group, 1993) which causes the production of a protein bearing a polyglutamine expanded tract in its N-terminus. The huntingtin mutation leads to widespread brain neurodegeneration, with cell loss mainly in the striatum and cerebral cortex (Reiner et al., 1988), although neuronal abnormalities are also found in many other brain regions (Rosas et al., 2003).

Results from various laboratories suggest that brain-derived neurotrophic factor (BDNF) is involved in the development of the human disease. BDNF was discovered in 1982 (Barde et al., 1982), as the second in a family of molecules with neurotrophic activities whose first identified member was nerve growth factor (NGF) (Levi-Montalcini and Hamburger, 1951). Although widely expressed in the adult mammalian central nervous system (CNS), BDNF is particularly abundant in the hippocampus and cerebral cortex where it is anterogradely transported to its striatal targets via the corticostriatal afferents (Hofer et al., 1990; Altar et al., 1997; Baquet et al., 2004).

Striatal neurons in the brain require BDNF for their activity and survival. Approximately 95% of striatal BDNF is actually of cortical origin, with the rest being produced in the substantia

nigra and delivered to the striatum (Altar et al., 1997; Baquet et al., 2004). BDNF expression increases during brain development, peaks after birth (Maisonpierre et al., 1990; Friedman et al., 1991; Huntley et al., 1992 Friedman et al., 1998; Schecterson and Bothwell, 1992; Kolbeck et al., 1999; Baquet et al., 2004), and does not seem to decline with age, thus suggesting that it plays an essential role in the adult CNS (Lapchak et al., 1993; Narisawa-Saito and Nawa, 1996; Katoh-Semba et al., 1997, 1998).

BDNF protein is transduced from a single, highly complex gene that carries a number of alternatively used promoters to generate a developmental, tissue-specific and stimulus-induced pattern of BDNF expression (Timmusk et al., 1993, 1994, 1995; Aid et al., 2007; Liu et al., 2006). The activity of this large regulatory region becomes progressively more complex and presumably more specialised in humans (Liu et al., 2005).

The fact that BDNF has survival promoting activity on the striatal neurons that die in HD has led to the idea that reduced endogenous trophic support may contribute to disease onset and/or progression. This hypothesis has aroused interest in BDNF and/or BDNF mimetics as potential therapeutic agents, and this has been intensified by reports of reduced BDNF levels in the cerebral cortex and striatum of people with HD (Zuccato et al., 2001), as well as in many mouse and cell models of the disease (see Cattaneo et al., 2005 for a review). Furthermore, although no underlying molecular mechanism has been proposed to explain reduced neurotrophic support in other neurological diseases such a Parkinson's disease (PD) or Alzheimer's disease (AD), it is known that the huntingtin mutation in HD reduces the transcriptional activity of the BDNF promoters, thus reducing the transcription of the BDNF gene and decreasing protein production in the cerebral cortex (Zuccato et al., 2001, 2003, 2005a). Finally, a selective defect in the transport of BDNF protein from cortex to striatum has also been proposed (Gauthier et al., 2004).

The impact of BDNF depletion in HD mice has been tested in experiments in which the levels of endogenous BDNF have been further reduced experimentally, with the consequence of lowering the age of onset and exacerbating the motor dysfunction. The observed neuropathology correlates with morphological alterations in the brain, a finding that confirms the pivotal role of BDNF in the specific degeneration of striatal neurons (Canals et al., 2004). Early experiments in which BDNF was administered in mouse models of HD indicate that BDNF may be beneficial to striatal neurons, although delivery strategies and BDNF bioavailability need to be further improved. More recently, it has been found that administering BDNF to HD mice by means of an osmotic minipump increases the number of striatal enkephalinergic neurons, the most affected population in HD (Canals et al., 2004), thus confirming the possibility that delivering BDNF or increasing endogenous BDNF production may stop or delay the progression of the human disease.

This review discusses the evidence indicating that cortical BDNF is a critical survival factor for the striatal neurons that die in HD. It describes the available data showing the negative influence of BDNF gene inactivation on the performance of striatal neurons in control mice, considers the molecular relationship between huntingtin and BDNF, describes the mechanism by which normal but not mutant huntingtin promotes BDNF production and axonal transport, and discusses the evidence indicating a worse HD phenotype in animal models lacking one BDNF allele. We present the case that the discovery of the mechanisms by which wild-type huntingtin controls the transcription of the BDNF and other neuronal genes may be exploited in drug-screening strategies aimed at identifying compounds capable of acting as huntingtin mimetics. Finally, we summarise attempts to deliver BDNF in HD animal models and the strategies available for increasing its level in human HD.

2. BDNF, a member of the neurotrophin family

BDNF is an abundant member of the NGF family in mammalian brain. By binding specific receptors such as tyrosine receptor kinase B (TrkB) and p75 neurotrophin receptor ($p75^{NTR}$), BNDF acts in a paracrine and autocrine manner to control a variety of brain processes, including the growth, development, differentiation and maintenance of neuronal systems, neuronal plasticity, synaptic activity and neurotransmitter-mediated activities (see Chao, 2003, and Binder and Scharfman, 2004, for reviews).

Like the other main neurotrophins, NGF and neurotrophin-3, neurotrophin-4 and neurotrophin-5 (NT3, NT4 and NT5), BDNF is found in most vertebrate classes including bony fishes, amphibians, reptiles, birds and mammals (Hallbook et al., 1991). Phylogenetic analyses suggest that all neurotrophins have evolved from a common ancestor gene, which underwent an early duplication more than 460 million years ago that gave rise to BDNF/NT4/5 and NGF/NT3 ancestors; a subsequent duplication probably originated the four neurotrophins in mammals (Hallbook et al., 1998). Interestingly, phylogeny and gene mapping data of the neurotrophin and TrkB receptor gene families suggest that they both date back at least 460 million years to the early vertebrates (Hallbook, 1999; see Murer et al., 2001 for a review).

BDNF shares about 50% identity with NGF, NT3 and NT4/5. Its primary amino acid sequence reveals the presence of a signal peptide following the initiation codon (Leibrock et al., 1989; Ernfors et al., 1990; Hohn et al., 1990; Jones and Reichardt, 1990; Kaisho et al., 1990; Rosenthal et al., 1990; Maisonpierre et al., 1990; Berkemeier et al., 1991; Hallbook et al., 1991; Ip et al., 1992), and a pro-region including a N-terminal glycosylation site and a proteolytic cleavage site (Bresnahan et al., 1990; Seidah et al., 1996). The three-dimensional structure of BDNF, which is similar to that of NGF and NT3 (McDonald et al., 1991; Robinson et al., 1995), consists of three anti-parallel beta-strands connected by three beta-hairpin loops. It is held together by means of three cystine di-sulphide bonds forming a *cystine knot structure*, which is common to all neurotrophins, and determines their binding to specific receptors and consequent biological action (McDonald et al., 1991; Robinson et al., 1995).

BDNF is primarily synthesised as a pro-BDNF, a ~32 kDa precursor protein that is cleaved in the *trans*-Golgi or in secretory granules at a highly conserved dibasic amino acid cleavage site, thus generating the mature biologically active protein (~14 kDa). Novel findings indicate that the 32 kDa pro-BDNF form may also be extracellularly active at the synaptic terminals, activating intracellular pathways other than mature BDNF (Lee et al., 2001; Woo et al., 2005) and promoting apoptotic cell death (Teng et al., 2005).

2.1. Distribution in the CNS

2.1.1. Regional distribution

Since the discovery of BDNF in 1982, a number of studies have analysed its localisation and distribution in the CNS, using antibodies recognising different BDNF epitopes and giving rise to sometimes contrasting findings. This is thought to be due to antibody specificity for epitopes that are only available before complete BDNF folding or are masked by the interaction of BDNF with different intracellular proteins in different subcellular compartments. The analysis of BDNF distribution is also complicated by the fact that extracellular BDNF is internalised by neurons, antero- or retrogradely transported and rapidly secreted, thus leading to the detection of low protein levels in the cell body. It may also be present in a poor unfolded state that may prevent antibody recognition. Despite these technical problems (which can be overcome by also looking at the distribution of BDNF mRNA), it has been found that BDNF protein is widely distributed in the CNS, with higher levels in the cerebral cortex, basal forebrain, striatum, hippocampus, hypothalamus, brainstem and cerebellum (Hofer et al., 1990; Murer et al., 2001 for a review).

Baquet et al. (2004) have recently assessed BDNF expression during mouse embryonic development by means of X-gal staining in $BDNF^{lacZ/+}$ mice in which lacZ was targeted to the BDNF gene. lacZ positivity appeared in a small number of cells in the hippocampus on embryonic day 15.5 (E15.5) and was found in the piriform cortex, hippocampus, thalamus, hypothalamus and amygdaloid nucleus by E17, but in only a few cells in the neocortex and none in the striatum. Neocortical expression was apparent in deep layers V–VI of the neocortex as early as postnatal day 4 (P4) and increased with age, as previously found by Katoh-Semba et al. (1997) and Kolbeck et al. (1999).

Analyses of BDNF distribution in adult brain have revealed high levels of BDNF mRNA and protein in rodent and human cerebral cortex. BDNF protein immunoreactivity is preferentially found in neurons with a pyramidal morphology in all cortical regions examined, including the primary visual cortex and other occipital areas, the motor and somatosensory cortex, the insular cortex, and the cortex of the temporal pole (Murer et al., 1999). In humans and rat, pyramidal BDNF-immunoreactive neurons are preferentially located in layers V–VI and II–III, and seem to be more abundant in some cortical regions (frontal, parietal and temporal cortex) than others (primary motor and sensory cortices) (see Murer et al., 2001, for a review; Fusco et al., 2003).

In general, such as in the cerebral cortex, the expression of BDNF mRNA and protein is substantially similar, but there are some discrete anatomical regions in which they differ: for example, the cell localisation of immunoreactivity is different from that of mRNA distribution in some discrete regions of the hippocampus formation. Moderate BDNF immunoreactivity has been found in neuronal soma and fibres of granule cell layers (Schmidt-Kastner et al., 1996), but little or none in granule cell soma of the dentate gyrus and pyramidal neurons of the human hippocampus, despite the high level of mRNA expression (Yan et al., 1997). On the contrary, many fibres are stained in the hilus of the dentate gyrus and molecular layer, and the CA2 region shows stronger immunoreactivity (Kawamoto et al., 1996).

Some comparative studies of cellular BDNF mRNA and protein levels have revealed BDNF-labelled neurons in the striatum, but many others have reported a lack of BDNF mRNA in this brain region (Schmidt-Kastner et al., 1996; Altar et al., 1997; Baquet et al., 2004). Consistently, low levels of striatal BDNF mRNA have been demonstrated in the rodent striatum, and only become evident after a seizure (Hofer et al., 1990; Salin et al., 1995; Schmidt-Kastner et al., 1996). Nevertheless, neurons in adult rodent neostriatum contain amounts of BDNF protein that are not much less than those measured in the BDNF mRNA-rich hippocampus (Altar et al., 1997; Baquet et al., 2004). As the cortical and nigral neurons innervating the neostriatum are rich in BDNF mRNA, it has been suggested that most of the BDNF protein found in the striatum is of cortical (and more limitedly nigral) origin and then anterogradely transported (see next paragraph) (Dugich-Djordjevic et al., 1995; Altar et al., 1997; Fusco et al., 2003; Baquet et al., 2004). In humans, BDNF immunoreactive neuronal cell bodies have rarely been found in the striatum; in particular, non-homogeneous BDNF staining has been observed in the caudate nucleus, putamen and nucleus accumbens (see Murer et al., 2001 for a review).

BDNF immunoreactivity (Dugich-Djordjevic et al., 1995; Kawamoto et al., 1996, 1999; Conner et al., 1997; Yan et al., 1997; Furukawa et al., 1998) and BDNF mRNA expression have also been observed in mammalian nigral dopaminergic neurons projecting to striatal targets (Wetmore et al., 1990; Ceccatelli et al., 1991; Castren et al., 1995; Schmidt-Kastner et al., 1996; Conner et al., 1997; Pineda et al., 2005). The BDNF in nigral cells mainly comes from mesencephalic dopaminergic neurons, because specific lesions of mesencephalic dopaminergic neurons decrease nigral BDNF mRNA (Venero et al., 1994); however, they cause a very small variation in the amount of striatal BDNF (Altar et al., 1997), once again confirming that most striatal BDNF is of cortical origin.

2.1.2. Cellular distribution

A number of studies indicate that BDNF is mainly present in neurons (see Murer et al., 2001 for a review), but early works indicated that glial cells could also express BDNF under conditions of severe metabolic stress (Ceccatelli et al., 1991; Batchelor et al., 1999). Furukawa et al. (1998) have reported the presence of BDNF-immunoreactive oligodendrocytes in rat

brain white matter, and more recent studies have shown that microglia *in vitro* secrete a limited amount of BDNF (Nakajima et al., 2001). Although BDNF synthesis has been reported in glial cells, the most popular hypothesis is that most of the BDNF found in glial cells is internalised, as is suggested by the presence of receptor-bound BDNF on their plasma membrane. It has been found that glial cells express the truncated and catalytic forms of TrkB (Frisen et al., 1993; Rudge et al., 1994; Roback et al., 1995; Nakajima et al., 1998), which mediates exogenous BDNF internalisation and storage, thus indicating that astrocytes and other cells expressing the truncated form of TrkB may regulate the local bioavailability of BDNF when glial cells are activated by endogenous or exogenous stimuli (Rubio, 1997).

2.1.3. Subcellular distribution

Early data indicated the presence of BDNF in the nuclei, cell body, cytoplasm and dendrites of neurons, and suggested that pro-BDNF is processed differentially, giving rise to a mature secretory form or an alternative product that is translocated to the nucleus where it influences transcription *in vivo* (Wetmore et al., 1991, 1993; Schmidt-Kastner et al., 1996). Some later studies confirmed the presence of nuclear BDNF; however, others did not (probably because of differences in the antibodies used) (see Murer et al., 2001 for a review), and this has been excluded by experiments in which the translated product was tagged and its intracellular trafficking analysed (Moller et al., 1998; Haubensak et al., 1998; Mowla et al., 1999). Accordingly, Fusco et al. (2003) showed that nuclei were devoid of BDNF immunoreactivity by confocal microscopy, whereas a diffuse immunoreactive product was found at the perikarya and neuropil.

A recent electron microscopy study by Tongiorgi et al. (2004) has revealed that BDNF mRNA has an unequivocal somatodendritic distribution *in vivo*: in particular, it was found at distances of 70 μm from the soma, a localisation that is considered dendritic. Remarkably, it was also found in the proximity of the postsynaptic compartment of excitatory synapses, which suggests its potential availability for local protein synthesis. In addition, dendritic BDNF is increased in epileptogenesis, and may be involved in the synaptic potentiation associated with this event (Tongiorgi et al., 2004).

2.2. The different BDNF mRNA isoforms

As first shown by Timmusk et al. in 1993, the rodent BDNF gene has four 5′ exons (I–IV) associated with distinct promoters and one 3′ exon (exon V) that encodes the BDNF protein (Fig. 1) (Metsis et al., 1993; Timmusk et al., 1993; Nakayama et al., 1994; Hayes et al., 1997). Alternative use of these promoters and differential splicing generate four BDNF mRNAs with different 5′ untraslated regions fused upstream of the same coding exon. It is also known that each transcription unit uses two different polyadenylation signals at the 3′ end of exon V, thus generating eight distinct BDNF transcripts (Timmusk et al., 1993). These studies showed that BDNF

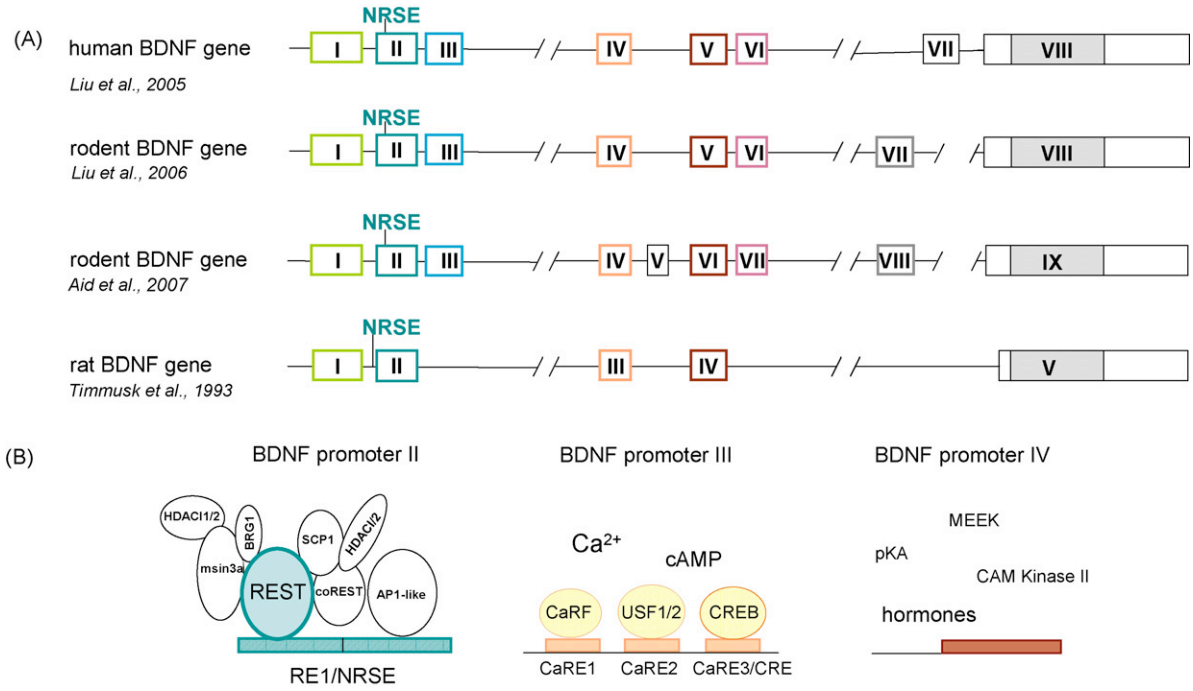

Fig. 1. The BDNF gene and its regulatory elements. (A) BDNF gene structure in humans and rodents. The BDNF coding region is indicated in grey. Homologous exons are highlighted with same colors. Note, the first description of the rat BDNF gene by Timmusk et al. (1993) is presented. Exons nomenclature in the text follows this original description. (B) Regulatory elements and proteins acting on BDNF promoters II, III and IV (BDNF exon nomenclature is according to Timmusk et al., 1993). *Left*: The RE1/NRSE silencer within exon II and the transcription factors which bind to it and activate its silencing activity. The 5′ element upper half recruits REST/NRSF protein, which, in turn, recruits different corepressors. The 3′ sequence has AP-1-like binding activity. *Centre*: Regulatory elements upstream BDNF promoter III responsive to Ca^{2+} signalling and cAMP levels. *Right*: Proteins acting on BDNF exon IV promoter.

mRNAs are transcribed in a development and stimulus-specific manner, with the transcripts containing exons I–II–III being expressed predominantly in brain, and exon IV also expressed in peripheral tissues and, particularly, lung, heart and pituitary (Timmusk et al., 1993; Bishop et al., 1994; Givalois et al., 2001).

In another study, Timmusk et al. (1994) quantified the level of BDNF messenger RNAs transcribed by the different promoters during rat brain development. Total BDNF mRNA was determined by means of RNA protection assay, and was detectable from embryonic day (E)13, peaked on postnatal day (P)14 and remained high in all brain regions up to 30 days of age, as later demonstrated by Katoh-Semba et al. (1997) and Kolbeck et al. (1999). BDNF exon III and exon IV mRNAs are the most abundant transcripts in early embryonic development from E13 to E16; in late embryonic development (E19–E21), exon II mRNA is the major transcript. In postnatal development, exon III mRNA in most expressed and mainly accounts for the peak expression seen on P15, whereas exon II and exon III mRNAs are the most abundant in the postnatal hippocampus and cerebral cortex (Timmusk et al., 1994).

More recently, the rat and mouse BDNF gene structures and expressions have been revisited by two independent groups. Liu et al. (2006) have confirmed the initial findings by Timmusk et al. and also identified three novel BDNF exons (Fig. 1). The rodent BDNF gene therefore contains seven untranslated exons (I–VII) each linked to a separate promoter (with the exception of exon VII and VIII which share the same promoter), while the coding exon has been renamed exon VIII. In rodents, BDNF exons I, II, IV and V correspond to the previously described rat exons I, II, III and IV (Timmusk et al., 1993). In addition, intra-exon splice sites within exon II were found, which produce spliced variants called IIA, IIB and IIC. Similarly, inter-exon splice sites in exon VI, called exons VIA and VIB, and spliced with the main coding exon VIII, lead to the production of two novel transcripts: BDNFVIA, which includes exon VI and the coding exon, and the three-part BDNFVIB transcript, that also incorporates exon VII. In the light of these new findings, mouse and rat BDNF genes are now considered to encode nine distinct transcripts (Liu et al., 2006). Most of these spliced variants are detected in the hippocampus and cerebral cortex and, in line with previous results, lower levels of BDNFIV and V in the striatum (Zuccato et al., 2001; Liu et al., 2006). Interestingly, most of the BDNF splice variants were also expressed in heart and spleen (in particular, BDNFIV and BDNFV), whereas BDNFVIA expression was only observed in skeletal muscle (Liu et al., 2006). A more recent article from Timmusk's group presents several novel data which are in part complementary to the results of Liu and colleagues. According to this last description, mouse and rat BDNF gene consists of eight 5′untranslated exons (I–VIII) and one protein coding exon (IX) (Aid et al., 2007) (Fig. 1). Exon named V in this last description has not been reported by Liu et al. (2006). Transcription of each of these newly identified exons is driven by separate promoters. Thus, both mouse and rat BDNF genes produce eight different transcripts consisting of the eight 5′ exons spliced to the 3′ coding exon. In addition, a novel BDNF transcript, exon IX mRNA, consisting of only 5′ extended protein coding exon, has been detected because transcription can also initiate in the intron before the protein coding exon (Aid et al., 2007). With respect to the expression patterns of BDNF transcripts that have been identified earlier, results of this study show that mouse and rat exons I, II, III have brain-enriched expression patterns, while exons IV, V and VI are widely expressed also in non-neuronal tissues (Aid et al., 2007).

Although molecular cloning of the human BDNF gene dates back to 1991 (Maisonpierre et al., 1991), its complete structure has only recently been assembled and, as in rodents, found to consist of seven 5′ noncoding exons that are each independently spliced to the major BDNF coding exon VIII (Fig. 1) (Liu et al., 2005). In humans, independent splicing events produce a total of nine different transcripts whose number may be further increased by considering the four alternative polyadenylation sites in exon VIII. As reported by Aid et al. (2007), homology of human and rodent BDNF 5′ exons ranges from 95 to 45%, reaching 95% for exon I, 93% for exon II, 62% for exon III, 91% for exon IV, 86% for exon VI (exon V in Liu et al., 2005 description) and 45% for exon VII (exon VIA in Liu et al., 2005). Interestingly, Liu et al. (2005) have shown that protein noncoding antisense transcripts are transcribed from the human BDNF gene locus. The expression of antisense BDNF transcripts is a human- or primate-specific phenomenon, since mouse and rat BDNF genes do not produce BDNF mRNA antisense transcripts (Aid et al., 2007). This further demonstrates the complexity of the regulation of the human BDNF gene.

2.3. *Corticostriatal neurons are the main source of striatal BDNF*

The massive convergence of afferents from all areas of the cortex to the striatum indicates that the corticostriatal projection receives a number of different inputs and integrates this information in the target neurons of the striatum. The corticostriatal synapse is critical to the pathophysiology of striatal cells, as it is the site in which many major neurotransmitters and neuromodulators are released, and critical receptor systems are located. Pre- or postsynaptic defects at this level can cause severe brain dysfunctions.

The striatum and its afferents are at the centre of many pharmacological investigations. In particular, the corticostriatal afferents have been a hotspot for HD research for many years because most of the excitatory input to the striatum originates from the cerebral cortex, and the injection of excitotoxins in animals reproduces some aspects of HD (see Beal, 1994, for a review). The cortical afferents reaching the striatum intermingle with other important cellular elements (striatal targets, dopaminergic afferents and glial cells) that can influence output from the cortex (see other reviews in this issue), thus further highlighting the importance of these local interactions.

BDNF is anterogradely transported in cortical afferents to dendrites and axon terminals, and released to striatal targets in an activity-dependent manner (Blochl and Thoenen, 1995, 1996; Goodman et al., 1996; Altar et al., 1997; Altar and

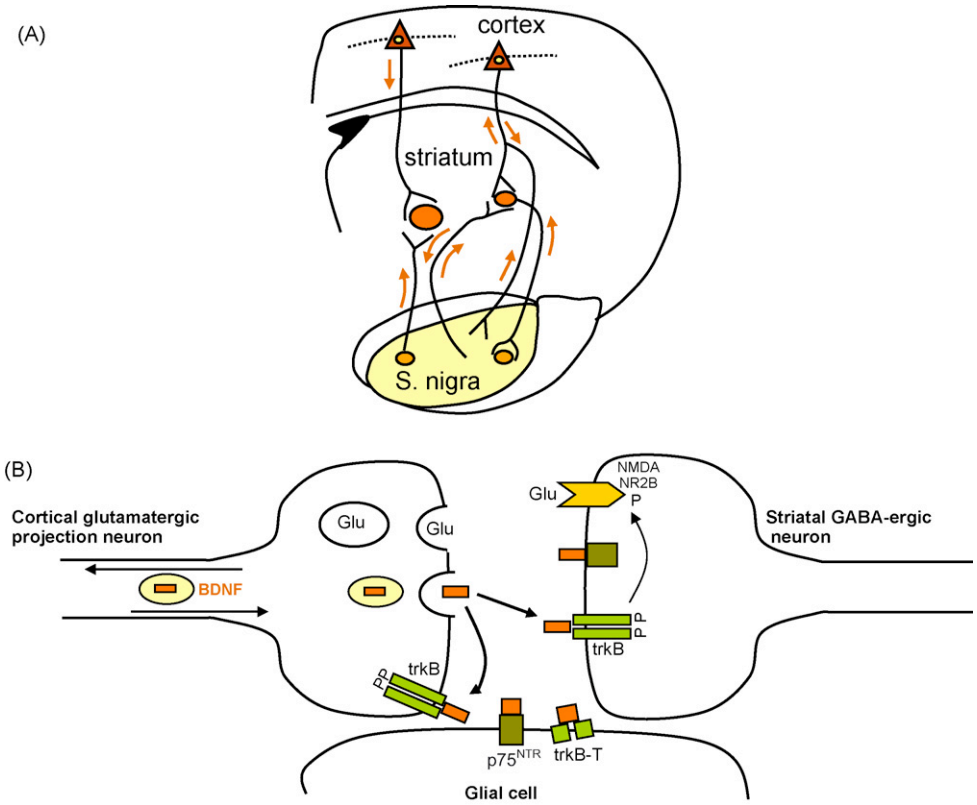

Fig. 2. BDNF and the corticostriatal synapse. (A) BDNF in basal ganglia pathways. The orange arrows indicate BDNF anterograde and retrograde transport. (B) Axodendritic connection between cortical glutamatergic projection neurons and striatal GABA-ergic neurons. BDNF release from the presynaptic terminal is evoked by depolarisation stimuli. BDNF vesicles diffuse across the synaptic cleft to activate TrkB receptors located on the postsynaptic terminal. Postsynaptic signal transduction leads to protein phosphorylation, such as the NR2B subunit of the NMDA receptor, determining enhanced synaptic transmission. BDNF activates, in an autocrine fashion, TrkB receptor on the plasma membrane at the presynaptic terminal, increasing neurotransmitter release (glutamate, Glu) by several potential mechanisms. BDNF in the synaptic cleft binds to truncated TrkB and $p75^{NTR}$ receptors on glial cells to modulate glial calcium signalling. By binding to truncated TrkB, BDNF can be stored into glial cells.

DiStefano, 1998, for a review) (Fig. 2). BDNF can also be retrogradely transported (Altar and DiStefano, 1998) from processes to the cell body via its interaction with TrkB receptors on the presynaptic nerve terminals (Heerssen et al., 2004). This induces a nuclear response that is fundamental for survival promotion and recovery from injury (Lindholm, 1994; Nikolics, 1999; Heerssen et al., 2004). In addition, BDNF released in the intracellular space can bind to pre- and postsynaptic TrkB, and participate in modulating synaptic transmission by acting in an autocrine manner to stimulate glutamate release from cortical projection neurons (Jovanovic et al., 2000; Schinder and Poo, 2000) and inhibiting GABA-ergic synaptic transmission at the postsynapse (Tanaka et al., 1997; Frerking et al., 1998; Wardle and Poo, 2003). Accordingly, a pathological increase in the frequency of GABA-ergic synaptic currents was reduced in HD mice after BDNF administration, supporting the role of BDNF in controlling synaptic transmission (Cepeda et al., 2004; Altar and DiStefano, 1998; Lu, 2003; Binder and Scharfman, 2004, for reviews).

The first evidence indicating that cortical afferents are the primary source of striatal BDNF comes from the work of Altar et al. (1997) and marks an important milestone in BDNF research. Analyses of the distribution of BDNF protein and mRNA in normal adult rat CNS were combined with lesion paradigms (including the block of axonal transport by chemical treatment or cortical ablation), and it was shown that exposure to colchicine, a drug traditionally used to disrupt microtubules, increases BDNF immunoreactivity in the cell bodies of all principal striatal afferents, including the neocortex, the pars compacta of the substantia nigra, the basolateral amygdala and the central medial thalamic nucleus (Altar et al., 1997). In line with the block of anterograde transport, BDNF immunoreactivity was markedly reduced in the striatal neuropil and increased in the white fascicles within the corpum callosum and striatum through which cortical neurons project to the striatum (Altar et al., 1997). In another experiment aimed at confirming that BDNF in the striatum was derived from extrinsic afferents, the frontoparietal cortex was ablated unilaterally (Altar et al., 1997), and it was found that BDNF protein levels determined by ELISA assays dropped by 34% in the ipsilateral striatum and by 78% in the contralateral striatum (Altar et al., 1997). Postdecortication immunocytochemical studies confirmed the dramatic loss of BDNF immunostaining in the dorsal lateral striatum and its accumulation in cortical neurons (Altar et al., 1997). The same study excluded the possibility that intrinsic striatal neurons may contribute to striatum BDNF protein levels as a quinolinic acid (QUIN) injection, which destroys striatal

neurons but spares the axon and terminals of striatal afferents, did not decrease striatal BDNF levels (Altar et al., 1997). Finally, the cortical lesions greatly depleted striatal BDNF, but nigrostriatal projection neuron lesions induced by injecting 6-hydroxydopamine into the medial forebrain bundle reduced striatal BDNF protein levels by only 14%. These early data showed that the anterograde transport of BDNF by cortical and nigral neurons accounts for the BDNF in the adult striatum (Altar et al., 1997), but more importantly, it also clearly demonstrated that striatal neurons, which are largely unable to produce their own BDNF, mainly depend on the cerebral cortex for BDNF trophic support.

Further experiments quantified GABA-ergic phenotype markers in normal and transgenic mice lacking the gene for BDNF (Altar et al., 1997). A decrease in the number of parvalbumine immunoreactive cells in the striatum of BDNF knock-out mice indicated that anterograde BDNF transport is essential for the correct phenotype development of the GABA-ergic striatal interneurons that receive synaptic inputs from the cortex.

A recent study has demonstrated the anterograde transport of cortical BDNF to the striatum in Emx-BDNF conditional knock-out mice genetically modified in such a way as to almost completely eliminate BDNF in the cerebral cortex and hippocampus (Baquet et al., 2004). Mice harbouring a floxed BDNF gene were bred with $Emx^{IREScre/+}$ to generate Emx-$BDNF^{KO}$ mice, which ELISAs showed had ~95% reduction in BDNF protein concentration in the striatum, thus further demonstrating the cortical origin of striatal BDNF (Baquet et al., 2004).

In the same study, Emx-$BDNF^{KO}$ mice were morphologically analysed postnatally in order to assess whether the absence of BDNF affected brain (and particularly striatum) volume over time. The hippocampi of the mutant mice were reduced only during early postnatal development, but the nearly complete genetic inactivation of BDNF in the cortex of the Emx-$BDNF^{KO}$ mice led to smaller cortical volumes in comparison with controls at all ages considered. In particular, on postnatal day 35, the cortex of the Emx-$BDNF^{KO}$ mice was 25% smaller than that of the wild-type, and 30% smaller on postnatal day 120.

More interestingly, striatal volume was significantly less in the Emx-$BDNF^{KO}$ than in the wild-type mice at all ages. It was 15% less on postnatal day 14, and down to 33% less on postnatal day 35, a difference that remained without further variation until postnatal day 120. Up to this age, the decreased striatal volume in Emx-$BDNF^{KO}$ mice was mainly attributed to the reduction in soma and dendrite size, and only marginally to cell death, which was evaluated by counting NeuN-positive neurons, and the reduction in NeuN-positive cells was non-significant on both postanatal days 35 and 120. BDNF of cortical origin seems to play only a minor role in determining the survival of medium-sized spiny neurons (MSNs) early in life, but a significant 35% reduction in the number of striatal cells has been observed at much later stages (1–1.5 years), thus indicating that BDNF is required for the long-term survival of striatal neurons (Baquet et al., 2004). Interestingly, BDNF seems to play a major role in dendritic morphology. Analyses of the morphology of MSNs on postnatal day 35 revealed significant dendrite thickness and a smaller cell soma size suggesting greater resistance to current flow, and so MSNs may require greater synaptic stimulation to reach the threshold for firing action potentials (Baquet et al., 2004). In addition, the number and length of dendritic spines are reduced in Emx-$BDNF^{KO}$ mice, which indicates reduced connectivity between the cortex and striatum, and the possibility that MSNs fail to receive cortically derived maturation signals other than BDNF.

Studies of the behavioural phenotype revealed that the forebrain-specific BDNF mutant mice show aspects of behavioural abnormalities seen in mouse models of HD. Interestingly, Emx-$BDNF^{KO}$ mice show a foot-clasping phenotype indicative of neurological dysfunction that becomes increasingly severe with age (Baquet et al., 2004). An earlier study by Gorski et al. (2003) indicated that Emx-$BDNF^{KO}$ mice suffered from an inability to perform a horizontal–vertical discrimination task (a test of complex learning), and it is known that complex learning is also impaired in HD (Schmidtke et al., 2002).

The release of cortical BDNF at the corticostriatal synapse is finely modulated by other neurotransmitters and neuromodulators, such as adenosine and the endocannabinoids (see other reviews in this issue by Popoli et al., and Maccarrone et al.).

Purines, such as adenosine diphosphate (ADP) and adenosine triphosphate (ATP), are crucial cellular components used as a source of energy or as part of nucleic acid. In addition to these cell functions, they are released in the extracellular space where they act as important signalling molecules that mediate biological effects through the purine receptors A_1, A_{2A}, A_{2B} and A_3 (see review by Popoli et al., this issue). A_2 receptors are particularly abundant in the striatum, and a proportion of A_{2A} receptors are located presynaptically on corticostriatal terminals, where they increase glutamate release. Recent findings indicate that activation of the presynaptic A_{2A} receptors in the hippocampus may facilitate BDNF's excitatory action on synaptic transmission (Diogenes et al., 2004).

We and others have investigated the role of A_{2A} receptors in the progression of HD, and their potential usefulness as biomarker (see Blum et al., 2003, for a review; also see the review by Popoli et al., this issue). Evidence of aberrant A_{2A} receptor function has been found in a striatal cell model of HD (Varani et al., 2001), as well as in R6/2 mice in which an early and transient alteration of A_{2A} receptor binding activity and signalling has been detected in the striatum (Tarditi et al., 2006). We have also studied a large cohort of HD and pre-HD subjects, and found that A_{2A} receptor binding activity is altered in peripheral blood cells (Varani et al., 2003).

An elegant study by Huang et al. demonstrated that cannabinoids (CBs) also play a critical role at the corticostriatal synapse by regulating corticostriatal glutamate release (see review by Maccarrone et al., this issue). CBs, the principal psychoactive constituents of marijuana, have a wide range of psychotropic effects on the CNS, many of which are believed to be exerted through the activation of G-protein-coupled receptors (Pertwee, 1993). Two subtypes of CB receptors have

so far been identified: the CB_1 receptor, which is predominantly distributed in the CNS and testis (Gerard et al., 1991; Westlake et al., 1994), and the CB_2 receptor, which is restricted to the periphery (Galiegue et al., 1995). The striatum and cerebral cortex are densely populated by CB_1 receptors, and CBs inhibit glutamatergic synaptic transmission by acting on presynaptic CB_1 receptors. More importantly, it has been demonstrated that the activation of these receptors increases the amount of BDNF released from the presynaptic terminals (Marsicano et al., 2003).

Another crucial input to the striatum comes from the substantia nigra pars compacta, whose fibres represent the main striatal source of dopamine. Recent studies support the concept that dopamine can directly regulate glutamate release from corticostriatal terminals by stimulating the D2 receptors located on a subpopulation of cortical afferents (Bamford et al., 2004). However, although a number of reports indicate that BDNF can influence dopamine levels (Dluzen et al., 2002) by increasing dopamine release in the striatum, there is still no clear evidence showing that dopamine plays a role in regulating BDNF release from the corticostriatal afferents.

2.4. Cortical BDNF levels depend on striatal integrity

The data summarized so far clearly indicate that most of the BDNF found in the striatum is of cortical origin, but there is also major cross-talk between the cortical afferents and their striatal targets, and a number of studies indicate that the BDNF produced in the cerebral cortex strictly depends on striatal target integrity. Cortical BDNF mRNA is upregulated after the intrastriatal injection of toxins such as quinolinic acid (QUIN), kainic acid (KA) and 3-nitropropionic acid (3NP), which are known to damage striatal neurons (Canals et al., 1998, 2001, 2004). Intrastriatal injections of excitotoxic doses of QUIN and KA induce BDNF upregulation along the cerebral cortex, including the frontal, motor and somatosensorial areas. In particular, an increase in BDNF mRNA has been observed in cortical layers II/III, V and VI, the main neocortical and mesocortical projections to the striatum. BDNF mRNA is specifically upregulated in cortical neurons and not in glial cells, and immunohistochemical analyses have shown that the increase in BDNF protein coincides with the location of BDNF mRNA upregulation (Canals et al., 2001).

On the contrary, the administration of KCl at doses that increase synaptic activity but do not cause striatal damage does not affect cortical BDNF mRNA levels, thus suggesting that BDNF is specifically induced in the cerebral cortex by striatal damage and not neuronal activity (Canals et al., 2001). However, more recently, a study by Rite et al. (2005) has shown that BDNF mRNA levels in the cortex may also depend on neuronal activity: analyses of neuronal activation in cortical layers II/III and V indicated high levels of cFos, a marker of neuronal activation, after QUIN administration.

Pre-grafting a BDNF-secreting cell line into the striatum prevents the upregulation of BDNF expression induced by QUIN or KA, thus indicating that exogenous BDNF may be retrogradely transported to the cortex and regulate cortical BDNF expression. In addition, blocking retrograde transport by injecting colchicine leads to the upregulation of BDNF expression, which suggests that cortical neurons upregulate BDNF expression in the absence of retrograde transport or absence of striatal neurons (Canals et al., 2001).

All of these findings lead to the hypothesis that the induction of cortical BDNF after striatum damage may be a compensatory response to the loss of target neurons that provides trophic support to striatal neurons. BDNF upregulation may therefore be an adaptive mechanism against the progression of striatal and cortical neurodegeneration (Canals et al., 2001). However, it is clear from studies of genetic models of HD that prolonged exposure to toxins (and the mutant huntingtin protein can be considered a toxin) desensitises the ability of the cortex to react to target modifications, and it is known that cortical BDNF is depleted in HD cortex (see Section 4). In line with this, a study by Fusco et al. (2003) indicated that such a process could begin with the overexpression of BDNF, possibly as a local response to protect the cortex itself and its striatal target, but, in the end, there is a decrease in cortical BDNF production that was connected to the dramatic reduction in huntingtin protein levels.

We will now move on to discuss how normal huntingtin protein is linked to BDNF gene transcription and protein production in the cerebral cortex.

3. Production of BDNF is stimulated by wild-type huntingtin: physiology and mechanism

We will here review the evidence linking BDNF gene transcription to wild-type huntingtin, the protein whose mutation causes HD, as well as the data demonstrating that a well-known DNA regulatory sequence located within the BDNF promoter represents the first identified downstream molecular target of wild-type huntingtin activity. We will also review the mechanism by which wild-type huntingtin facilitates BDNF gene transcription and summarise the evidence showing that the same mechanism underlies the control of wild-type huntingtin over the transcription of other important neuronal genes.

3.1. Huntingtin and BDNF co-localise in cortical neurons

Huntingtin is an ubiquitous protein that is highly expressed in brain, particularly in the neurons of the cerebral cortex and hippocampus (Reiner et al., 1988; Fusco et al., 1999). It is expressed as early as E10 and maintained throughout brain development, increasing in parallel with the maturation of neurons in the postnatal period (Bhide et al., 1996). BDNF is found later (starting from E13) and peaks on P15/P21 (Maisonpierre et al., 1990; Hofer et al., 1990; Friedman et al., 1991; Huntley et al., 1992; Kolbeck et al., 1999; Timmusk et al., 1994; Katoh-Semba et al., 1997). Huntingtin expression increases markedly between P7 and P15 to reach its maximum level in the adult (Bhide et al., 1996).

Two studies by Fusco et al. (1999, 2003) have investigated the abundance and co-localisation of huntingtin and BDNF in

rat cerebral cortex and striatum. They found moderate BDNF immunolabelling in layers I, II and IV of the cerebral cortex (where huntingtin levels are low), but higher levels in layers III, V and VI, which are rich in huntingtin. Huntingtin and BDNF are co-localised in 99% of the pyramidal neurons of motor cortex, and, in the striatum, huntingtin is expressed in 75% of the neurons that are immunolabelled for BDNF.

At cell level, huntingtin is mainly a cytoplasmic protein associated with microtubules and perinuclear tubulovesicular membranes (Velier et al., 1998). It is associated with various organelles, including the nucleus, endoplasmic reticulum and Golgi complex (DiFiglia et al., 1995; Velier et al., 1998; Hilditch-Maguire et al., 2000; Hoffner et al., 2002; Kegel et al., 2002), and is also found in neurites and at synapses, where it associates with vesicular structures such as clathrin-coated vesicles, endosomal compartments or caveolae, and microtubules (Li et al., 2003, for a review).

The discrepancies in the results of early studies of the subcellular localisation of BDNF protein were mainly due to the use of antibodies raised against different epitopes. In a first report, Wetmore et al. (1991) reported that BDNF-immunoreactivity was not only preferentially present in the nuclei of hippocampal cells but also found in the cytoplasm and dendrites of neurons. Later studies confirmed the presence of BDNF in cytoplasm and dendrites (Kawamoto et al., 1996; Furukawa et al., 1998), but only one found it in the nuclei of neuronal cells (Furukawa et al., 1998). Similar experiments by other research groups also failed to detect BDNF immunoreactivity in the nucleus (Dugich-Djordjevic et al., 1995; Conner et al., 1997; Yan et al., 1997), as did *in vitro* studies of the intracellular trafficking of BDNF in which the translated product was tagged. Further studies indicated that BDNF is preferentially localised in the cell cytoplasm and dendrites (Moller et al., 1998; Haubensak et al., 1998; Mowla et al., 1999), and studies of cultured neurons and adult brain tissue confirmed BDNF labelling in cytoplasm, axons and dendrites, and excluded nuclear staining (see Murer et al., 2001, for a review; Fusco et al., 2003).

On the basis of the evidence that BDNF and wild-type huntingtin are both highly expressed in cortical neurons, and that cortically derived BDNF is required for the activity of the corticostriatal synapses and correct survival of striatal neurons, we proposed (and subsequently demonstrated) that wild-type huntingtin participates in controlling BDNF production in the cerebral cortex. Subsequent data suggest that wild-type huntingtin may also positively influence the delivery of BDNF from the cortex to the striatum (see Section 5).

3.2. Wild-type huntingtin increases BDNF production by stimulating gene transcription from BDNF exon II promoter

Various *in vitro* and *in vivo* data consistently show that wild-type but not mutant huntingtin facilitates cortical BDNF production by acting at the level of BDNF gene transcription (Zuccato et al., 2001). Huntingtin's ability to stimulate BDNF production was first assessed *in vitro* in a cell model of HD represented by ST14A cells derived from rat embryonic striatum via the retroviral transduction of the temperature-sensitive version of the large-T antigen (Cattaneo et al., 1994; Cattaneo and Conti, 1998), and subsequently stably transfected with human full-length wild-type or mutant huntingtin (Rigamonti et al., 2000). ELISAs of the different stable ST14A transfectants showed increased BDNF production in the cells overexpressing wild-type huntingtin in comparison with the mutant clones, which had a lower BDNF content than the mock-transfected ST14A cells.

A second series of experiments showed that the increased BDNF production in wild-type huntingtin cells was due to enhanced BDNF gene transcription. Timmusk et al. (1993) found that the rat BDNF gene contained four 5′ exons (I–IV) linked to individual promoters, which are activated in a time- and stimulus-dependent manner to produce four different transcripts (mRNA I–IV) and then spliced to the fifth 3′ exon (V) to produce the BDNF protein (see Fig. 1). We used polymerase chain reaction (PCR) experiments and gene reporter assays to show that cells bearing extra wild-type huntingtin produce a specific increase in transcription from BDNF promoter II, whereas transcription from BDNF promoter I, III and IV was unaffected (Zuccato et al., 2001).

A third series of experiments confirmed these data *in vivo* using mice bearing an additional yeast-derived artificial chromosome (YAC) that includes the full-length wild-type huntingtin gene (YAC18 mice), and therefore overexpressing the wild-type protein (Hodgson et al., 1999). It was found that lysates from the cerebral cortex of 9-month-old YAC18 mice contained 48% more BDNF protein than those from their littermates and, consistently, there was 50% increase in BDNF protein levels in the striatum and hippocampus (Zuccato et al., 2001). We also found increased *in vivo* transcription from BDNF exon II promoter, which accounted for the increased amount of BDNF protein in the cerebral cortex of the mice overexpressing wild-type huntingtin (Zuccato et al., 2001) and further confirmed the pro-stimulatory action of the protein on BDNF promoter II. Transcription from promoter II is severely affected in HD mice (Zuccato et al., 2001; Hermel et al., 2004).

Extra copies of wild-type (but not mutant) huntingtin increase BDNF production *in vitro* and *in vivo*, but brain tissue and cells depleted of endogenous huntingtin are characterised by reduced BDNF levels, thus suggesting that the reduced BDNF transcription observed in HD may depend on the level/activity of the wild-type protein (Zuccato et al., 2003; Zuccato et al., unpublished manuscript). In line with data showing increased BDNF mRNA II levels in wild-type huntingtin overexpression systems, reduced BDNF mRNA II has been found in the cerebral cortex of heterozygous huntingtin knock-out mice (Zuccato et al., 2003), and we have more recently found that neuronal inactivation of huntingtin in conditional homozygous knock-out mice (Dragatsis et al., 2000) leads to a statistically significant reduction in BDNF mRNA levels. In this system, the reduced BDNF mRNA II level is responsible for the lower amount of total BDNF mRNA found in the absence of endogenous huntingtin and the fact that transcription from BDNF promoter III and IV is unaffected indicates that the reduced activity of wild-type huntingtin in

HD specifically affects transcription from BDNF exon II promoter (Zuccato et al., unpublished manuscript).

As a similar dysfunction is seen in HD samples (see Section 4), we believe that the reduced BDNF levels in HD may be at least partially due to decreased normal huntingtin activity (Cattaneo et al., 2005). Although there is a component of loss of huntingtin function in BDNF levels in HD, we will show below that the cortical BDNF deficit in patients is also due to the enhanced toxicity of the mutant protein further reducing transcription from BDNF III and IV promoters.

3.3. Wild-type huntingtin influences the activity of the RE1/NRSE silencer

The regulation of BDNF exon II promoter is well documented and known to depend mainly on the activity of a repressor element 1/neuron-restrictive silencer element (RE1/NRSE) localised in the proximal region of the exon, and the activity of RE1/NRSE primarily depends on RE1 silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF) (Timmusk et al., 1999). Our recent findings link wild-type huntingtin to the RE1/NRSE silencer and show that the wild-type but not the mutant protein activates BDNF gene transcription by inhibiting the RE1/NRSE within BDNF promoter II. Furthermore, recent bioinformatic data indicate that RE1/NRSE is not only present in the BDNF gene but also found in a large number of neuronal genes, thus suggesting the possibility that wild-type huntingtin may be more broadly involved in regulating RE1/NRSE-dependent neuronal gene transcription.

3.3.1. RE1/NRSE neuronal genes and REST/NRSF transcriptional repressor

RE1/NRSE was initially defined as a 23 bp DNA sequence capable of repressing the transcription of a number of neuronally expressed genes in non-neuronal tissues. It was discovered independently by two laboratories, and found to downregulate the expression of SCG10 and type II sodium channel genes in non-neuronal cells, with little effect on their expression in neuronal cells (Mori et al., 1992; Maue et al., 1990). When positioned upstream of a minimal promoter, RE1/NRSE represses transcription in an orientation- and distance-independent manner, thus satisfying the criteria for a silencer element. RE1/NRSEs were subsequently found in a number of genes that are fundamental for the maintenance and terminal differentiation of neurons (Schoenherr et al., 1996), and more recent findings from Buckley's group indicate that there are more than 1300 RE1/NRSE sites in the human and murine genomes (Bruce et al., 2004; http://bioinformatics.leeds.ac.uk/RE1db_mkII/), most of which encode for ion channels, neurotransmitters, growth factors and hormones, factors involved in axonal guidance and vesicle trafficking, and molecules involved in maintaining the cytoskeleton and extracellular matrix (Bruce et al., 2004).

The RE1/NRSE binding protein (REST/NRSF) was discovered in 1995, and described as a C2H2 zinc-finger protein related to members of the Gli-Kruppel family of transcriptional repressors (Chong et al., 1995; Schoenherr et al., 1996). It contains an eight zinc-finger cluster at the N-terminal end, which is required for binding to RE1/NRSE. There are differently spliced isoforms of REST/NRSF (Palm et al., 1998), one of which (REST4) acts as a dominant-negative regulator by competing with REST/NRSF for DNA binding in neurons (Shimojo et al., 1999). Furthermore, REST/NRSF and REST4 interact with the REST/NRSF interacting LIM domain protein (RILP), a protein of the LIM domain (named from the Lin-11, Isl-1 and Mec-3 genes) for nuclear translocation (Shimojo and Hersh, 2003; Shimojo, 2006; Shimojo and Hersh, 2006).

In the nucleus, REST/NRSF interacts with RE1/NRSE sites and recruits co-repressors, including the mammalian homologue of yeast Sin3A (mSin3A) (Huang et al., 1999; Roopra et al., 2000) and REST co-repressor (CoREST) (Andres et al., 1999; Ballas et al., 2001), which in turn recruit multiple co-factors and transcriptional regulators, including brahma-related gene 1 protein (BRG1), BRG1-associated factor 57 (BAF57) and BRG1-associated factor 170 (BAF170) (Battaglioli et al., 2002), and BRACA-2-associated factor 35 (BRAF35) (Hakimi et al., 2002).

Early *in situ* hybridisation studies showed that REST/NRSF is expressed in most non-neuronal tissues throughout embryonic development and into adulthood whereas, in the developing nervous system, it is transiently expressed by neuroepithelial cells, but seems to be extinguished upon terminal differentiation (Chong et al., 1995; Schoenherr and Anderson, 1995). All of these early observations were consistent with a role of REST/NRSF as a global silencer of neuronal gene transcription in non-neuronal cells (Chong et al., 1995; Schoenherr and Anderson, 1995), and seemed to exclude the possibility of its presence and activity in mature neuronal stages. However, later findings argued strongly that the principal role of RE1/NRSE in genes is to modulate gene expression in neurons rather than silence expression in non-neuronal cells. Evidence for a role of the RE1/NRSE in neurons came from three independent transgenic mouse studies that used different RE1/NRSE-containing promoters fused upstream reporter genes (Bessis et al., 1997; Kallunki et al., 1998; Timmusk et al., 1999), none of these studies found that RE1/NRSE mutation led to widespread reporter gene expression in non-neuronal areas as would have been expected if the role of REST/NRSF was to silence gene transcription in non-neuronal cells. Similarly, transgenic mice lacking functional REST/NRSF do not show the upregulation of neuronal genes in non-neuronal tissues, but the fact that they die E11.5 precludes any analysis of the mutation's effect on neurogenesis or neuronal gene expression (Chen et al., 1998). In an attempt to clarify the role of RE1/NRSE in regulating neuronal gene expression, REST/NRSF-dominant negative retroviral constructs were used to infect chick embryos, and some neuronal genes controlled by the silencer showed de-repression in subsets of infected neurons, once again indicating that the role of REST extends beyond silencing gene expression in non-neuronal cells (Chen et al., 1998).

Further support for a role of REST/NRSF in neuronal function is provided by a number of studies reporting detectable

levels of REST/NRSF in the adult nervous system, although its expression is more limited than in non-neuronal cells or the developing nervous system. In 1998, Palm et al. reported the presence of low levels of REST/NRSF in adult brain and, 1 year later, Shimojo et al. (1999) suggested that, despite the low level of REST/NRSF expression in neurons, neuronal gene transcription was allowed by REST4-mediated regulation of REST/NRSF levels: i.e. the extent of transcriptional repression might be determined by the relative levels of REST/NRSF and REST4, rather than those of REST/NRSF alone. More recent findings have confirmed that REST/NRSF mRNA and protein can be expressed in many adult neurons, particularly those of the hippocampus, in which both REST/NRSF and its target genes are modulated in response to ischemic or epileptic insults (Palm et al., 1998; Calderone et al., 2003; Kuwabara et al., 2004). By means of Western blot analyses, we and others found that REST/NRSF is expressed in differentiated primary cortical neurons as well as in mouse and human cerebral cortex (Zuccato et al., 2003; Wood et al., 2003), and Wood et al. (2003) also showed that, in neurons, REST/NRSF can be recruited to the RE1/NRSE of transcriptionally active genes and act to repress gene transcription.

Another set of studies identified REST/NRSF as crucial for defining neuronal identity (Belyaev et al., 2004; Bruce et al., 2004). By means of different mechanisms, it is sufficient to drive the neuronal differentiation of adult neural stem cells (Kuwabara et al., 2004; Su et al., 2004) and even myoblasts (Watanabe et al., 2004). In particular, Kuwabara et al. have shown that, at the onset of neuronal differentiation, 21–25 bp noncoding double-strand (ds) RNAs (whose defined sequence is a RE1/NRSE) play a critical role in mediating RE1/NRSE-regulated gene transcription. The RE1/NRSEdsRNAs interact directly with the RE1/NRSE dsDNA-REST/NRSF machinery within the genome, and convert REST/NRSF from a repressor to an activator by dismissing co-repressors and recruiting co-activators (Kuwabara et al., 2004). Alternatively, they proposed that the RE1/NRSEdsRNA could bind a monomeric form of REST/NRSF, thus altering REST/NRSF function (possibly by inducing a conformational change) and causing changes in its activity or its binding to the proteins driving its association with the RE1/NRSEdsDNA (Kuwabara et al., 2004). In both cases, as the REST/NRSF complex can no longer associate with the proteins of the repressor complex after interacting with RE1/NRSEdsRNA, increased gene transcription can be expected. It is still not known whether REST/NRSF is capable of binding RE1/NRSEdsRNAs alone or must be part of a larger protein complex (Kuwabara et al., 2004).

In a more recent paper, Ballas et al. proposed a different mechanism of action for REST/NRSF during neuronal differentiation. There are high levels of REST/NRSF in the nuclei of embryonic stem (ES) cells but, as these cells turn into restricted neural progenitors, the protein is post-translationally downregulated by the proteosomal pathway and degraded to levels that are just sufficient to maintain neuronal gene chromatin in an inactive state (albeit poised for expression). As the progenitors differentiate into cortical neurons, REST/NRSF and its co-repressors dissociate from the RE1/NRSE sites and trigger the activation of neuronal genes (Ballas et al., 2005).

Unlike Ballas et al. (2005), Sun et al. found that REST/NRSF is expressed in hippocampal stem cells and adult neurons. This difference probably indicates the diversity of REST/NRSF actions in different populations of CNS progenitors and neurons, but may also reflect differences in developmental stage as most target genes are silent or expressed at low levels in ES and neural stem cells, but expressed at much higher levels in differentiated cells (Sun et al., 2005). These data indicate that the REST/NRSF regulon may be specific to each developmental stage, and support the notion that REST/NRSF plays distinct roles in regulating gene expression during the transition from ES to neural stem cells, and in mature neurons.

Increasing the complexity of the RE1/NRSE–REST/NRSF pathway is the recent discovery of a family of mouse microRNA (miRNA) genes containing RE1/NRSE sites controlled by REST/NRSF (Conaco et al., 2006). miRNA are a group of small noncoding RNA (ncRNA) molecules that are distinct from but related to small interfering RNAs (siRNAs), and have been identified in a variety of organisms (for reviews, see He and Hannon, 2004; Bartel, 2004). These small 20–22 nucleotide (nt) RNAs are transcribed as part of molecules that are several kilobases (kb) in length and are nuclearly processed into hairpin RNAs of 70–100 nt by the double-stranded RNA-specific ribonuclease Drosha (Cullen, 2004). The hairpin RNAs are transported via an exportin 5-dependent mechanism to the cytoplasm, where they are digested by a second, double-stranded specific ribonuclease called Dicer. In animals, single-stranded microRNA binds specific mRNA through sequences that are significantly, but not completely, complementary to the target mRNA, mainly to the 3′ untranslated region (3′UTR). By means of a mechanism that is not fully understood, the bound mRNA remains untranslated or it is degraded, thus leading to reduced levels of the corresponding protein. In their recent study, Conaco et al. found that REST/NRSF in non-neuronal cells binds to a consensus RE1/NRSE site in the miR-124a gene encoding for a brain-specific miRNA, whose target is represented by the mRNA of non-neuronal genes. The repression of miR-124a results in the persistence of hundreds of non-neuronal transcripts, thus favouring the non-neuronal phenotype. In neurons, where the levels of REST/NRSF are lower, the miR-124a gene is transcribed, thus leading to the downregulation of non-neuronal transcripts in favour of a neuronal phenotype (Conaco et al., 2006).

All of this evidence indicates that the role of REST/NRSF in controlling the transcription of RE1/NRSE target genes may involve a number of different complex mechanisms. Furthermore, the data now available suggest that the activity of the REST/NRSF regulon may be specific to each developmental stage and support the notion that REST/NRSF plays distinct roles in regulating gene expression during the transition from immature to mature neurons.

3.3.2. Wild-type huntingtin inhibits RE1/NRSE silencing activity: an effect that goes beyond BDNF itself

The RE1/NRSE in promoter II of the BDNF gene is a 53 bp inverted repeat. The 5′ element (upper half) has 89% identity with a general RE1/NRSE consensus, whereas the 3′ element (lower half) has only 57% identity, thus suggesting it is an atypical RE1/NRSE. The 5′ sequence recruits REST/NRSF protein, whereas the 3′ sequence has AP1-like binding activity (Timmusk et al., 1999). We have found that wild-type huntingtin retains REST/NRSF in the cytoplasm, thus reducing RE1/NRSE activation and allowing BDNF gene transcription (Zuccato et al., 2003). Subsequent data indicate that wild-type huntingtin and REST/NRSF co-localise (Zuccato et al., 2005b), but mutated huntingtin causes the pathological entry of REST/NRSF into the nucleus where it can bind to the RE1/NRSE site and lead to BDNF repression (Zuccato et al., 2003).

More recent data indicate that wild-type huntingtin plays a broader role in regulating neuronal gene transcription because ST14A cells and YAC18 mice expressing increased wild-type huntingtin levels have higher levels of the mRNAs transcribed from many other RE1/NRSE-containing neuronal genes (Zuccato et al., 2003; Zuccato et al., unpublished manuscript). In particular, the levels of synapsin-1, cholinergic receptor, nicotinic beta-polypetide 2 and dynamin 1 mRNA are increased in the cerebral cortex of YAC18 mice, thus indicating that huntingtin may act as a general facilitator of neuronal gene transcription in the nervous system (Zuccato et al., 2003). Further evidence in favour of a role of wild-type huntingtin in controlling RE1/NRSE-controlled neuronal gene transcription comes from recent chromatin immunoprecipation data showing that REST/NRSF occupancy is significantly lower in cells and mice expressing wild-type huntingtin than in HD models. In order to prove that the increased REST/NRSF binding at RE1/NRSE loci in HD strictly depends on the reduced activity of normal huntingtin, we tested RE1/NRSE occupancy in cells and mice depleted of endogenous huntingtin (Zuccato et al., unpublished manuscript). The finding of increased REST/NRSF occupancy in the absence of huntingtin provided further evidence that wild-type huntingtin controls the activity of the RE1/NRSE silencer at genomic level, and thus affects the transcription of many RE1/NRSE-controlled neuronal genes. We also concluded that RE1/NRSE is a target of wild-type huntingtin activity and that a loss of function of wild-type huntingtin underlies the REST/NRSF–RE1/NRSE dysfunction observed in HD.

4. BDNF is reduced in HD models

A large number of studies have found reduced BDNF levels in HD, which implies that this may put the target striatal neurons and possibly the corticostriatal circuitry at risk (Table 1). This hypothesis is supported by the fact that the neuronal degeneration in human HD mainly affects the striatum and cerebral cortex (Reiner et al., 1988; Rosas et al., 2003), and cortical atrophy may be present even before the onset of symptoms (Rosas et al., 2005). Reduced BDNF levels have also been observed in HD neural cell models, as well as genetic and pathogenetic mouse models of HD.

These observations led us to hypothesise a non-cell autonomous mechanism of neurodegeneration that makes striatal neurons vulnerable because of the reduced delivery of BDNF from cortical afferents. This mechanism may operate with other non-cell autonomous mechanisms, such as abnormalities in corticostriatal neurotransmission (Zeron et al., 2002; Cepeda et al., 2003; Li et al., 2004), and with cell-autonomous processes such as the formation of aggregates (Gu et al., 2005).

4.1. Reduced BDNF in HD cell and animal models

A first indication of a specific molecular defect in BDNF protein and mRNA levels in HD came from experiments on striatum-derived ST14A cells overexpressing full-length wild-type or mutant huntingtin which showed that, although cells overexpressing wild-type huntingtin produce more BDNF protein (see Section 3.2), the production of both BDNF mRNA and protein in mutant huntingtin cells was less than that in control cells (Zuccato et al., 2001). These initial data clearly showed that this molecular phenotype was not due to a generalised sick cell state but related to huntingtin function. A similar decrease was also reported in mutant huntingtin knock-in cells obtained from heterozygous and homozygous huntingtin knock-in mice in which a 109 CAG triplet repeat was inserted into the endogenous mouse huntingtin gene (Trettel et al., 2000; Zuccato et al., 2001). It was also found that the level of BDNF protein is decreased after the transient expression of huntingtin exon 1 bearing different CAG repeat lengths in an immortalised striatum-derived cell line (Canals et al., 2004). The same study found that cells transfected with exon 1 of mutant huntingtin containing 47 or 72 CAG repeats had a similarly reduced BDNF content, which suggested that the CAG size in this range does not exacerbate the BDNF phenotype. However, 103 CAG repeats significantly affected BDNF protein content only when large amounts of the protein were expressed, thus suggesting that, in this system, the decrease in BDNF depends not only on the number of CAG repeats but also on the level of transgene expression (Canals et al., 2004). However, mutant huntingtin knock-in cells stably expressing physiological mutant huntingtin levels showed a decrease in BDNF content, possibly indicating that the stable expression (but not overexpression) of mutant huntingtin is sufficient to affect BDNF protein levels (Zuccato et al., 2001, 2003).

Consistent with the *in vitro* data, reduced BDNF protein and mRNA levels have also been observed *in vivo* in the brain (specifically the cortex) of a large number of mouse models of HD. In particular, decreased BDNF protein levels have been found in the cortex, striatum and hippocampus of YAC mice bearing full-length huntingtin with 72 glutamines (YAC72 mice; Zuccato et al., 2001), whereas, as discussed in Section 3.2, there is more BDNF in mice overexpressing the normal protein. In the same YAC72 mice, human mutant huntingtin is expressed under its own promoter, which leads to a developmental and tissue-specific expression pattern that is similar to that seen in the case of endogenous huntingtin

Table 1
BDNF levels in HD cells, animal models and human brain tissue

2000	BDNF is reduced in striatum but not in a small number of specimen from human HD cortex (Ferrer et al., 2000)
2001	BDNF mRNA and protein are increased in ST14A cells stably overexpressing full-length wild-type huntingtin (Zuccato et al., 2001)
	BDNF mRNA and protein are decreased in ST14A cells overexpressing mutant huntingtin (Zuccato et al., 2001)
	BDNF protein is progressively reduced in $Hdh^{7/7}$,$Hdh^{109/7}$ and $Hdh^{109/109}$ knock in cells (Zuccato et al., 2001)
	Reduced BDNF mRNA and protein in the cortex, striatum and hippocampus of asymptomatic YAC mice aged 9 months (Zuccato et al., 2001)
	Early induction in cortical BDNF mRNA after striatal lesion by quinolinate, kainate and 3-nitropropionic acid (Canals et al., 2001)
	BDNF mRNA and protein are reduced in a small number of specimen from human HD cortex (Zuccato et al., 2001)
2002	Reduced BDNF mRNA levels in the cerebellum of R6/2 mice (Luthi-Carter et al., 2002)
2003	BDNF mRNA is progressively reduced in $Hdh^{7/7}$, $Hdh^{109/7}$ and $Hdh^{109/109}$ knock in cells (Zuccato et al., 2003)
	Cortical and striatal BDNF protein levels are reduced in transgenic HD mice (N-171 82Q) (Duan et al., 2003)
	Reduced BDNF protein levels in total brain of R6/2 mice (Zhang et al., 2003)
	BDNF protein is decreased in the cortex and striatum of $Hdh^{109/109}$ mice aged 5 months (Gines et al., 2003)
	BDNF mRNA is reduced in heterozygous Hdh^{ex5} knock-out mice (Zuccato et al., 2003)
	Cortical BDNF protein is decreased after 6 weeks of striatal quinolinate injections (Fusco et al., 2003)
2004	BDNF vesicle transport is progressively reduced in $Hdh^{7/7}$, $Hdh^{109/7}$ and $Hdh^{109/109}$ knock in cells (Gauthier et al., 2004)
	BDNF vesicle transport is enhanced in NG108-15 neuroblastoma cell transiently overexpressing full-length wild-type huntingtin, but not full-length mutant huntingtin (Gauthier et al., 2004)
	Reduced BDNF protein levels in M213 neural stem cells expressing mutant huntingtin exon 1 with different CAG lengths (Canals et al., 2004)
	Reduced cortical BDNF mRNAs in asymptomatic YAC mice aged 3 months (Hermel et al., 2004)
	BDNF protein is reduced in the striatum but not in the cortex of R6/1 mice (Spires et al., 2004)
	BDNF level is not altered in the cortex and striatum of R6/1 mice (Canals et al., 2004)
	R6/1/BDNF+/− double mutants mice show worsening of the HD phenotype (Canals et al., 2004)
	BDNF protein is reduced in striatum but not in human HD cortex (Gauthier et al., 2004)
	Emx-$BDNF^{KO}$ mice display a behavioural and neuropathological phenotype similar to that seen in mouse models of HD (Baquet et al., 2004)
2005	Cortical BDNF mRNA levels are reduced in R6/2 mice during disease progression (Zuccato et al., 2005a,b)
	Cortical BDNF mRNA is induced early after striatal lesions by quinolinate, kainite and 3-nitropropionic acid (Rite et al., 2005)
2006	Reduced BDNF secretion in $Hdh^{109/109}$ knock-in cells (Borrell-Pages et al., 2006)
	Reduced BDNF gene transcription in the cortex, striatum and hippocampus of R6/1 mice; increased BDNF protein in the cortex (Pang et al., 2006)
	Unaltered BDNF mRNA and protein levels in the cortex and striatum of conditional exon 1 HD94 mice (Gines et al., 2006)
	BDNF protein is not significantly modified in the brain of $Hdh^{109/109}$ mice aged 3 months; BDNF protein levels are reduced in blood from the same mice (Borrell-Page et al., 2006)
	BDNF protein level is reduced in blood samples from 3-nitropropionic acid-treated monkeys (Borrell-Pages et al., 2006)
	Transgenic overexpression of BDNF in the cerebral cortex delays disease phenotypes in R6/1 mice (Xu et al., 2006)
	BDNF mRNA is reduced in the cortex of BAC mice expressing full length human mutant huntingtin (Gray et al., 2006)
	Loss of BDNF mRNA expression in layer V cortical neurons in R6/2 mice (Wang et al., 2006)
	BDNF protein was reduced by about 40–45% in the hippocampus, cortex and striatum of $Hdh^{109/109}$ mice aged 2 months (Simmons et al., 2006)

Evidence from the literature.

(Hodgson et al., 1999). Nine-month-old YAC72 transgenic mice with no disease symptoms had 30% less cortical BDNF protein than their littermates, which indicates that BDNF deficit may occur before the onset of symptoms (Zuccato et al., 2001). Another study found reduced BDNF mRNA levels in YAC72 mice from the age of 3 months, thus further confirming that BDNF gene transcription can be affected before the onset of disease symptoms in mice (Hermel et al., 2004). As a consequence, reduced BDNF protein levels were found in the striatum of the same mice (Zuccato et al., 2001). A 40% reduction in BDNF content has also been detected in the hippocampus, a finding that is consistent with observations of impaired spatial memory in HD mice, as well as reports of hippocampal cell proliferation and neurogenesis deficits (Lazic et al., 2004; Gil et al., 2005; Grote et al., 2005). Although preliminary, these data may have a clinical correlation insofar as HD patients show cognitive abnormalities (Schmidtke et al., 2002).

Bacterial artificial chromosome (BAC)-mediated transgenesis has recently been used to develop mouse models expressing full-length mutant huntingtin with 103 glutamine repeats (BACHD) under endogenous huntingtin regulation. Interestingly, BACHD brains show a significant deficit in BDNF transcription at 6 months, the same age as the onset of rotarod deficits (Gray et al., 2006).

Other studies have shown reduced BDNF mRNA and protein levels in other HD mice transgenic for the N-terminal portion of the mutant protein. Zhang et al. (2003) have reported a 50% reduction in BDNF protein in total brain from 12-week-old symptomatic mice expressing a 63 amino acid N-terminal fragment of mutant huntingtin with 150 polyglutamines (R6/2 line) (Mangiarini et al., 1996), and Wang et al. (2006) have more recently found that, at the same age, the corticostriatal neurons located in layer V (which have projections to the striatum) show a 20% decrease in perikarial BDNF mRNA. We have made time-course analyses of BDNF mRNA levels in the same mice and, in line with the rapid disease progression in this frequently studied model (subtle motor and learning deficits appear after approximately 4–5 weeks and the animals usually die after 13–14 weeks), found reduced BDNF mRNA levels in the cerebral cortex from early pre-symptomatic stages (Zuccato et al., 2005a). Luthi-Carter et al. (2002) have reported that the same mice also show reduced BDNF gene transcription in the cerebellum from 8 weeks of age,

possibly leading to cerebellar dysfunction and altered motor coordination.

Brain BDNF levels have also been tested with conflicting results in another transgenic mouse line, R6/1, created at the same time as R6/2. R6/1 mice show slower disease progression because of the smaller amount of expressed mutant huntingtin (Mangiarini et al., 1996). Spires et al. (2004) reported that BDNF protein levels were reduced in R6/1 striatum but not in the cerebral cortex at the age of 5 months, whereas Canals et al. (2004) found no deficiency in striatal BDNF protein levels at the age of 6 months. The latter authors suggested that the unchanged BDNF levels in R6/1 mice may be due to the low transgene level, and it is true that cells expressing low levels of an exogenous mutant huntingtin tract do not show a reduction in BDNF protein content (Canals et al., 2004). However, we have found that mutant huntingtin knock-in cells expressing pathological huntingtin at physiological (low) levels show a significant reduction in BDNF content (Zuccato et al., 2001, 2003). More recently, Pang et al. (2006) have reported similar BDNF protein levels in the striatum and hippocampus of 5-month-old controls and R6/1 mice, but increased levels were found in the frontal cortex, and in the same study, they found reduced BDNF mRNA levels in the striatum, anterior cortex and hippocampus. As suggested by the authors themselves, these conflicting findings may be explained by the different methods used for BDNF protein quantification. Spires et al. (2004) used Western blotting, which differentiates mature BDNF (which decreased) from the immature form (which remained unmodified), whereas Canals and Pang used ELISA, which is more quantitative but does not distinguish mature and immature BDNF. It is possible that the striatal level of mature BDNF protein is significantly decreased but those of the much more abundant immature BDNF remain largely unchanged (Pang et al., 2006). Moreover, the reduced levels of BDNF mRNA in striatal neurons (which transcribe little or no BDNF) probably also affect the still uncertain BDNF levels in R6/1 mice, and so further investigations are necessary.

An additional study by Duan et al. (2003) found that BDNF protein levels were significantly decreased by 70–80% in the striatum and cortex of 3-month-old HD mice expressing a 517 amino acid N-terminal portion of huntingtin with 82 polyglutamine repeats driven by a mouse prion protein promoter (Schilling et al., 1999; Duan et al., 2003). BDNF levels have also been analysed in knock-in mice expressing physiological levels of one or two doses of mutant huntingtin bearing 111 CAG repeats: immunoblots showed a less intense BDNF band in striatal and cortical extracts from homozygous mutant huntingtin knock-in mice aged 5 months (Gines et al., 2003). Data from Borrell-Pages et al. (2006) indicating a small although non significant reduction in BDNF protein levels in total brain samples taken from 3-month-old homozygous knock-in mice further support BDNF deficits in this model of HD. Support for an early BDNF reduction in the brain of mutant huntingtin knock-in mice comes from a study by Simmons et al. (2006), who found that BDNF protein was reduced by 40–45% in the hippocampus, cortex and striatum of 2-month-old homozygous knock-in mice.

With a few exceptions that require further investigation, this evidence together clearly speaks in favour of reduced BDNF level in HD cells and animal models, and opens up the possibility that a similar dysfunction may be present in the human disease.

4.2. Less BDNF exon II promoter activity in HD

As described in Section 3, wild-type (but not mutant) huntingtin stimulates BDNF gene transcription by acting at the level of BDNF promoter II, whereas the presence of a pathological CAG expansion in huntingtin abolishes the ability to sustain BDNF gene transcription in HD. This is shown by the fact that reduced BDNF mRNA II levels are found in ST14A cells overexpressing full-length mutant huntingtin (Zuccato et al., 2001), as well as in heterozygous and homozygous mutant huntingtin knock-in cells (Zuccato et al., 2003). Furthermore, reporter gene assays confirm that BDNF exon II promoter is 60% less active in cells overexpressing mutant huntingtin than in parental cells (Zuccato et al., 2001).

Early *in vivo* data support these observations and indicate that BDNF mRNA II levels are much reduced in the cerebral cortex and hippocampus of pre-symptomatic YAC mice expressing human full-length mutant huntingtin (Zuccato et al., 2001), and similar findings were reported in an independent study of the same YAC mice at 3 months of age (Hermel et al., 2004). Finally, cortical BDNF mRNA II levels are 25% less in 8-week-old R6/2 mice than in controls and 60% less in 12-week-old symptomatic R6/2 mice (Zuccato et al., 2005a). Importantly, similar analyses by other authors have shown a significant depletion of wild-type huntingtin in 7-week-old R6/2 mice that parallels the timing of the reduced BDNF mRNA II level, thus suggesting that the decreased transcription from BDNF exon II promoter in this model may be due to the reduced level of endogenous huntingtin (Zhang et al., 2003).

The mechanisms by which BDNF exon II promoter activity is reduced in HD have been described in Section 3.3. As previously indicated, the RE1/NRSE silencer is the target of wild-type huntingtin on BDNF promoter II, and the wild-type protein inhibits its silencing activity by recruiting the REST/NRSF transcription factor (which binds and activates the silencer) in the cell cytoplasm (Zuccato et al., 2003). We have reported that increased REST/NRSF occupancy occurs at the RE1/NRSE within BDNF exon II in HD cells and animal models, as well as in the cerebral cortex of HD subjects, and this leads to increased activity of the silencer and reduced BDNF mRNA II levels (Zuccato et al., 2003; Zuccato et al., unpublished manuscript).

As said in Section 3.2, reduced levels of wild-type huntingtin decrease transcription from BDNF exon II promoter, thus indicating that a loss of function of wild-type huntingtin is responsible for the reduced activity of BDNF promoter II in HD. Unlike those of promoter II, the activities of promoters linked to exon III and IV are not influenced by wild-type huntingtin levels. However, the transcriptional activities of BDNF mRNA III and IV are affected in HD cells and mice,

possibly because of an increase in the toxic activity of the mutant protein on BDNF gene transcription (Zuccato et al., 2001, 2005a).

4.3. Less BDNF exon III and IV promoter activity in HD

In addition to BDNF exon II, which has been extensively described above, short regions flanking promoters I, III and IV have been thoroughly characterised in terms of their regulatory elements of gene transcription. Information about these promoter exons is given below, followed by a summary of experiments indicating the deleterious effect of mutant huntingtin on their transcriptional activity and speculations concerning the underlying mechanisms.

Early studies indicated that BDNF promoter I is physiologically activated at low levels and stimulated by the administration of KA, which evokes calcium signals through different subtypes of glutamate receptors (Zafra et al., 1990; Metsis et al., 1993). For this reason, BDNF exon I was originally defined as the inducible brain-specific promoter (Timmusk et al., 1993; Metsis et al., 1993). Recent studies by Liu et al. (2006) and Aid et al. (2007) have shown that BDNF promoter I is subject to physiological activation, as the mRNA transcribed from it can be detected in the cerebral cortex, cerebellum, hippocampus, thalamus and brain stem (Liu et al., 2006; Aid et al., 2007), but little is known about the mechanisms regulating the transcriptional activation of BDNF promoter I. It is known that it has distal CRE-like sequences, and a proximal calcium responsive element (CaRE) that is overlapped by an upstream stimulatory factor (USF) binding element (Tabuchi et al., 2002). The proximal element is bound by cAMP/Ca^{2+} responsive element binding protein (CREB) and upstream stimulatory factor 1 and 2 (USF1/USF2), and responds to Ca^{2+} signals evoked via L-type voltage-dependent Ca^{2+} channel (L-VDCC) and NMDA (Tabuchi et al., 2000, 2002).

More robust attempts have been made to elucidate the structure and activity of BDNF exon III promoter, which is characterised by the three Ca^{2+} responsive elements CaRE1, CaRE2 and CaRE3/CRE. These regulatory elements are stimulated by Ca^{2+} signals evoked by *N*-methyl-D-aspartate glutamate receptor (NMDA-R) and involve cyclic adenosine 3′,5′ monophosphate (cAMP) responsive element (CRE) binding protein (CREB), together with CaM kinase IV (Shieh et al., 1998; Tao et al., 1998). Moreover, CaRE1 and CaRE3/cAMP responsive element (CRE) are bound by the newly identified neuronal calcium responsive transcription factor (CaRF), whereas CaRE2 activity depends on the binding of transcription factor USF1/USF2 (Tabuchi et al., 2002; Chen et al., 2003b). Two interesting recent studies by Chen et al. (2003a) and Martinowich et al. (2003) have shown that methyl-CpG binding protein 2 (MeCP2), which binds methylated CpGs island on DNA and is involved in the long-term silencing of gene transcription, can selectively bind BDNF promoter III and repress BDNF gene transcription. Membrane depolarisation triggers the calcium-dependent phosphorylation and release of MeCP2 from BDNF promoter III, thus facilitating transcription. Mutations in the MeCP2 gene are the cause of Rett's syndrome (RTT), an X-linked progressive disorder characterised by arrested neurological development and subsequent cognitive decline. As MeCP2 plays a key role in the control of neuronal activity-dependent gene regulation, deregulation of this process when MeCP2 is mutated may contribute to the pathology of RTT (Chen et al., 2001).

Unlike the other BDNF promoters analysed above, BDNF exon IV promoter contains glucocorticoid-responsive elements (Schaaf et al., 2000, for a review), and its activity is influenced by thyroid hormone (Koibuchi et al., 1999) and corticosterone (Hansson et al., 2006). Additional findings indicate that CaM kinase II mediates the activation of BDNF promoter IV by Ca^{2+} influx. Transient transfection and overexpression experiments have shown that two nuclear isoforms of CaM kinase II (delta 3 and alpha B) specifically activate only promoter IV (Takeuchi et al., 2000). A more recent study by Takeuchi et al. (2002) has shown that MAPK kinase kinase (MEKK) and protein kinase A (PKA) can also upregulate the activity of BDNF promoter IV; in particular, CaM Kinase II and MEKK, respectively, activate the promoter linked to BDNF exon IV via c/EBP/beta and Sp1 transcription factors.

We started looking at the effects of mutant huntingtin on transcription from BDNF promoter I, III and IV in ST14A neural cells overexpressing the mutant protein. As expected on the basis of published data, BDNF mRNA I was not detected (Metsis et al., 1993), but we did find that transcription from BDNF promoter III and IV, which are physiologically subject to activation in the CNS, was significantly reduced in the presence of the mutant protein. Consequently, BDNF mRNA III and IV were also reduced in ST14A cells expressing mutant huntingtin, and their levels were also lower in heterozygous and homozygous mutant huntingtin knock-in cells (Zuccato et al., 2001).

BDNF mRNA III and IV are reduced in animal models of HD. YAC mice expressing full-length mutant huntingtin show a reduction in BDNF mRNA III and IV levels starting at pre-symptomatic stages (Zuccato et al., 2001; Hermel et al., 2004). A similar pattern has been found in R6/2 mice, which express mutant huntingtin exon 1. In particular, BDNF exon IV mRNA level was the first to be affected (at 6 weeks of age), while defects in transcription from promoter III occurred only at very late stages (12 weeks of age) (Zuccato et al., 2005a).

The mechanism leading to the reduced expression of BDNF mRNA III and IV in HD is still unknown. However, an impaired CRE pathway has been observed (Sugars and Rubinsztein, 2003 for a review; Sugars et al., 2004) and, as BDNF promoter III has a CRE element, it is possible that a dysfunction in CRE activity may account for its reduced transcriptional activity. Various evidence indicates that crucial proteins in this event are CREB (which directly binds to the CRE element after phosphorylation by PKA at Ser133) and the co-activator binding protein CBP, which acts as a bridge between CREB and the transcriptional machinery. An important finding by Steffan et al. (2001) indicates that mutant huntingtin can interact with both the glutamine-rich activation domain and the acetyl transferase domain of CBP. They also found that a reduction in

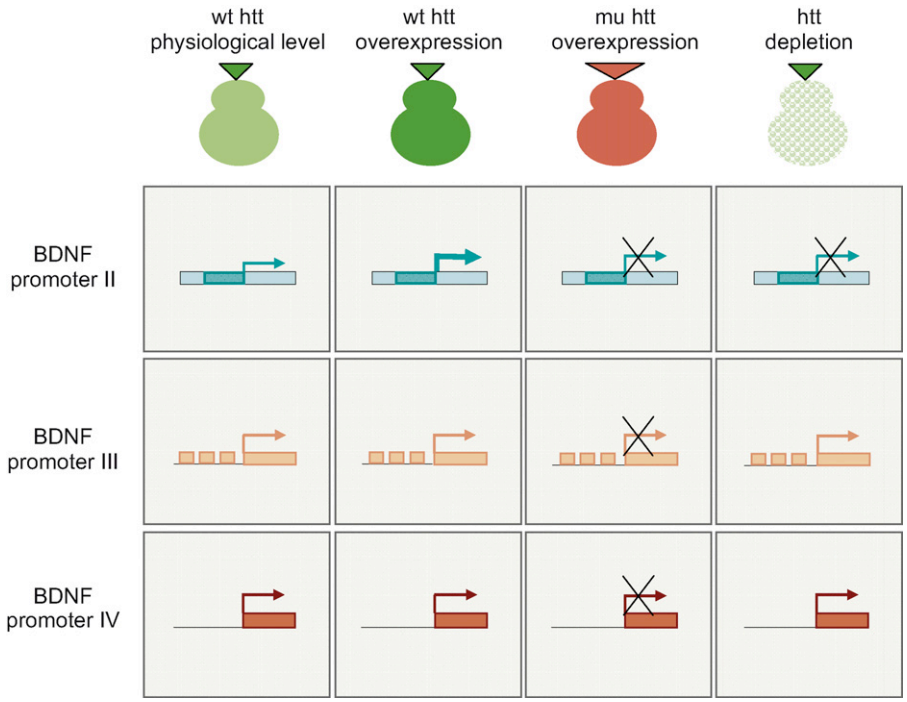

Fig. 3. The BDNF promoters, a read-out for wild-type and mutant huntingtin (wthtt and muhtt, respectively) activity. Transcription from BDNF exon II promoter is enhanced by wild-type huntingtin overexpression, whereas BDNF promoters III and IV transcriptional activity is unaffected. Mutant huntingtin overexpression reduces BDNF gene transcription from promoters II, III and IV. Reduced wild-type huntingtin levels causes reduction of BDNF gene transcription from promoter II, while transcriptional activity of promoters III and IV is unaffected. BDNF exon nomenclature is according to Timmusk et al., 1993.

the acetyltransferase activity of CBP causes a reduction in histone acetylation (Steffan et al., 2001), thus leading to a more compact chromatin structure that is less accessible to transcription factors, and potentially explaining the decrease in CRE-dependent transcription and reduction in BDNF mRNA III levels. Although early findings suggested that CBP can be sequestered into mutant huntingtin aggregates (Nucifora et al., 2001; McCampbell et al., 2000), a study by Yu et al. (2002) showed that altered CRE-dependent gene expression may be due to the interactions of soluble mutant huntingtin with nuclear CBP, rather than the depletion of this transcription factor by nuclear inclusions. CBP is therefore subtracted from the transcriptional machinery regulating the CRE element in BDNF promoter III. Reduced CREB phosphorylation (Gines et al., 2003; Giampa et al., 2006) and reduced cAMP levels (Gines et al., 2003) may also contribute to reduced transcription from BDNF exon III promoter in an HD background. Moreover, CRE-mediated transcription is also activated by co-factor TAFII-130, and evidence from Dunah et al. (2002) indicates that TAFII-130 interacts with mutant huntingtin, thus further impairing the transcriptional machinery at the CRE loci.

The reduced transcription from BDNF promoter linked to exon IV in HD (Zuccato et al., 2001, 2005a) may be explained on the basis of evidence showing that Sp1 participates in its activation (Takeuchi et al., 2002), whereas mutant huntingtin sequesters Sp1, thus blocking its physiological interaction with TAFII-130 and causing reduced transcriptional activity (Dunah et al., 2002; Li et al., 2002).

In conclusion, we suggest that reduced normal huntingtin activity is responsible for decreased transcription from promoter II, whereas reduced transcriptional activity at promoters III and IV reflects mutant huntingtin-induced toxicity (Fig. 3).

The above has potential therapeutic implications insofar as it suggests the usefulness of restoring BDNF levels in HD. The BDNF promoters can be used as reporter assays of huntingtin activity in order to identify the contribution of the activity of the mutant protein versus the loss of normal huntingtin function during HD progression. In particular, they can be used to develop reporter assays for the isolation of molecules that mimic wild-type huntingtin on BDNF exon II promoter. Such an assay would have the advantage of reflecting the activity of a much larger number of promoters located in neuronal genes and containing the RE1/NRSE element, thus anticipating the possibility that active compounds would restore transcription from a large number of RE1/NRSE controlled neuronal genes. In parallel, BDNF exon III and IV promoters can be used in reporter assays to identify drugs capable of reducing or blocking the ability of mutant huntingtin to inactivate BDNF gene transcription from the same promoters. These aspects will be discussed in Section 11.

5. BDNF vesicle transport is enhanced by wild-type huntingtin

Huntingtin is predominantly found in the cytoplasm of neurons and is enriched in compartments containing vesicle-associated proteins (DiFiglia et al., 1995). Moreover, wild-type huntingtin is antero- and retrogradely transported in rat sciatic nerve axons, which is consistent with a vesicle association

(Block-Galarza et al., 1997). Importantly, huntingtin interacts with the scaffolding proteins involved in axonal transport, such as huntingtin-associated protein 1 (HAP1), and with the dynactin protein of the motor complex responsible for the microtubule-mediated transport of vesicles (Block-Galarza et al., 1997; Gunawardena and Goldstein, 2004 for a review). Experimental reduction of wild-type huntingtin in *Drosophila melanogaster* causes defects in fast axonal trafficking (Gunawardena et al., 2003). A more recent study of embryonic striatal neurons taken from mice expressing only one copy of the wild-type allele or <50% of normal huntingtin levels further indicates that mitochondria became progressively immobilised (Trushina et al., 2004), thus confirming that normal huntingtin is involved in the transport of cell components. Studies by Gauthier et al. (2004) indicate that wild-type huntingtin is capable of sustaining BDNF vesicle transport in neurons.

5.1. Brief overview of BDNF transport and secretion in neurons

The molecular sorting, trafficking and release of BDNF vesicles have been at the centre of many studies, which have shown that it is a complex process involving different structural proteins and enzymatic activities.

BDNF is detected in large, dense core vesicles and brain synaptosomes (Fawcett et al., 1997; Michael et al., 1997), and transfection experiments using BDNF–green fluorescent protein (GFP) fusion constructs have indicated that it is packaged into secretory vesicles and transported to somato-dendritic compartments and axons (Haubensak et al., 1998; Moller et al., 1998; Kojima et al., 2001).

Like the other neurotrophins, BDNF is synthesised from a large precursor protein that is proteolytically cleaved intracellularly or extracellularly to yield the mature protein. Like most secreted proteins, BDNF is initially synthesised from its mRNA as a 32 kDa precursor protein (pro-BDNF) in the endoplasmic reticulum, and subsequently translocated to a series of intracellular organelles (including the Golgi complex, and immature and mature secretory vesicles), and finally secreted to the extracellular space in its 14 kDa mature form after proteolytic cleavage (Halban and Irminger, 1994).

The neurotrophins are usually secreted by a constitutive pathway, and then transported to the plasma membrane and continuously released into the extracellular space without any triggers. However, BDNF is secreted by passing preferentially into a regulated pathway following a variety of depolarisation stimuli (i.e. the secretion of BDNF is activity dependent) (see Lu, 2003, for a review). Recent findings suggest that a large amount of BDNF can also be secreted as pro-BDNF and then undergo extracellular cleavage by metalloproteinases and plasmin (Lee et al., 2001). A significant proportion of the processing of pro-BDNF to mature BDNF may therefore occur extracellularly, and so proteases on the cell surface or in extracellular space play a critical role in determining the proportion of the BDNF forms and thus the biological activity of the BDNF released after depolarisation.

Although only mature BDNF has been considered the biologically active form that elicits TrkB-dependent signalling (Huang and Reichardt, 2001), a number of recent studies have shown that, by binding the $p75^{NTR}$ receptor, pro-BDNF activates different intracellular pathways from those activated by mature BDNF, and initiates cell processes that may lead to the negative regulation of synaptic plasticity (Lee et al., 2001; Woo et al., 2005). Moreover, the interaction of pro-BDNF with $p75^{NTR}$ and the co-receptor sortilin leads to apoptotic cell death (Teng et al., 2005). The level of secreted pro-BDNF therefore may have profound implications for synaptic function and neuron survival.

New evidence has shown that motifs in the BDNF structure are crucial for BDNF sorting. The interaction between a sorting signal in the tertiary structure of mature BDNF and receptor carboxypeptidase E is required for BDNF activity-dependent release through the regulated secretory pathway (Lou et al., 2005), and disruption of this motif abolishes the activity-dependent release of endogenous BDNF by cortical neurons, thus affecting its intracellular distribution and trafficking (Lou et al., 2005).

In addition to the sorting motif in mature BDNF, the pro-BDNF sequence also has an impact on sorting, as a valine-to-methionine substitution in the prodomain of BDNF has been found. Amino acid substitution can influence various aspects of the biology of a given molecule and, in the case of BDNF, the valine-to-methionine substitution specifically impairs BDNF sorting from the Golgi complex by reducing the activity-dependent secretion of BDNF from neurons (Egan et al., 2003). This substitution is a recognised polymorphism of the human BDNF gene associated with memory impairment and increased susceptibility to neuropsychiatric disorders (Egan et al., 2003).

5.2. Wild-type huntingtin enhances BDNF vesicle transport: in vitro evidence

Data from Gauthier et al. (2004) indicate that wild-type huntingtin enhances the vesicle transport of BDNF along microtubules in mammalian cells. BDNF vesicle transport is increased by wild-type huntingtin overexpression and attenuated by reduced levels. The underlying mechanism involves HAP1, a huntingtin interactor that binds to proteins involved in vesicle transport, and the $p150^{Glued}$ subunit of dynactin.

A first indication of the possible role of huntingtin in BDNF vesicle transport came from the identification that endogenous wild-type huntingtin and BDNF co-localise in neuronal cells (Fusco et al., 2003; Gauthier et al., 2004). Next, the relationship between huntingtin and BDNF vesicle transport was approached by a series of *in vitro* experiments that included cells overexpressing wild-type huntingtin and cells in which endogenous huntingtin was reduced by means of siRNA techniques. The distribution and dynamics of BDNF vesicles were evaluated in real time by means of ultra-fast 3D videomicroscopy after the transfection of recombinant BDNF-eGFP, followed by deconvolution microscopy and the measurement of parameters such as the percentage of static vesicles, mean velocity and the pausing time of vesicles

(Gauthier et al., 2004). It was found that BDNF vesicles move faster in the presence of exogenous wild-type huntingtin and that their speed is reduced when the level of huntingtin is reduced. This finding led to the suggestion that the intracellular transport of BDNF-containing vesicles represents a novel function of wild-type huntingtin (Gauthier et al., 2004).

Further investigations by Gauthier et al. indicated that BDNF vesicle transport is mediated by microtubules, and that wild-type huntingtin enhances its efficiency. Molecular motors, such as kinesin and dynein, are proteins that move vital cargoes (vesicles, cytoskeleton, RNA, signalling proteins, and neuroprotective and repair molecules) on microtubule tracks. Within axons, vesicles from the cell body are transported anterogradely by kinesin motors to nerve terminals and synapses, whereas dynein and some kinesin motors intervene to transport and organelles in the retrograde direction. It was found that wild-type huntingtin enhances BDNF transport to both the tips of the neurite and the cell body, suggesting a possible role for huntingtin in both the anterograde and retrograde transport of BDNF (Gauthier et al., 2004).

Wild-type huntingtin binds huntingtin-associated protein 1 (HAP1) which in turn recruits the $p150^{Glued}$ subunit of dynactin (Block-Galarza et al., 1997; Engelender et al., 1997; Li et al., 1995, 1998; Schroer, 1996) and kinesin light chain (McGuire et al., 2005), which drive respectively retrograde and anterograde transport in neurons. Searching for the mechanism underlying the ability of wild-type huntingtin to sustain BDNF vesicle transport along microtubules, it was found that proteins of this complex, particularly huntingtin interactor HAP1, are essential: BDNF vesicle velocity decreased when HAP1 protein levels were reduced by siRNA, whereas its overexpression caused the formation of BDNF vesicle clusters in which wild-type huntingtin and the $p150^{Glued}$ subunit of dynactin are recruited to activate BDNF vesicle transport. Interestingly, truncated versions of wild-type huntingtin that do not interact with HAP1 did not increase BDNF vesicle transport (Gauthier et al., 2004).

6. Evidence of reduced BDNF transport in HD

To establish whether the presence of a polyglutamine expansion in huntingtin affects BDNF transport in HD, BDNF vesicle velocity was measured in heterozygous and homozygous mutant huntingtin knock-in cells in which 109 CAG repeats are inserted in the endogenous huntingtin locus, thus creating a situation similar to that found in HD patients. Further biochemical studies of mutant huntingtin knock-in cells, mice and HD postmortem tissues indicated that the complex driving BDNF vesicles is altered in HD. In particular, BDNF vesicle velocity is reduced in heterozygous and homozygous mutant huntingtin knock-in cells, which indicates that BDNF transport is altered when huntingtin contains an elongated polyglutamine expansion (Gauthier et al., 2004). The transport of mitochondria was unaffected in the same homozygous mutant huntingtin knock-in cells, thus indicating that wild-type huntingtin specifically enhances BDNF vesicle transport in this cell system. However, this is in conflict with the results of Trushina et al. (2004), who found that mitochondria transport was specifically affected in cells depleted of endogenous huntingtin and in cells bearing mutant huntingtin. These opposing results may be explained by the different cell systems used and raise the question as to whether defects in BDNF vesicle transport represent a selective phenotype in HD. To reinforce the selectivity of huntingtin involvement in the transport of BDNF vesicles, Gauthier et al. (2004) found that the proteins involved in other neurodegenerative diseases do not affect BDNF transport.

As previously mentioned, huntingtin is involved in the motor complex that includes HAP1 and the $p150^{Glued}$ subunit of dynactin (Block-Galarza et al., 1997; Engelender et al., 1997; Li et al., 1995, 1998; Schroer, 1996; Gauthier et al., 2004). The results of experiments using HD cells and mice, as well as human postmortem brain tissues, suggest that this motor complex is altered in HD. In particular, increased binding of mutant huntingtin to HAP1 reduced the association between HAP1/$p150^{Glued}$ dynactin and microtubules in heterozygous mutant huntingtin knock-in mice (Gauthier et al., 2004). This suggests that the mechanism controlling retrograde transport is altered in the presence of the polyglutamine expansion in huntingtin.

In addition, as most striatal BDNF comes from anterograde (and not retrograde) transport from the cerebral cortex, Gauthier et al. investigated whether the association between kinesin and microtubules is also reduced, and found this to be the case in *in vitro* experiments using homozygous mutant huntingtin knock-in cells. On the basis of the consideration that, in yeast two hybrid experiments, HAP1 may be pulled down with a human kinesin-like protein, it was suggested that the complex consisting of huntingtin/HAP1 and kinesin may be affected by the polyglutamine expansion, leading to impaired anterograde transport (Gauthier et al., 2004). As mentioned above, McGuire et al. (2005) showed that HAP1 interacts with kinesin light chain, a subunit of the kinesis complex that drives anterograde transport along microtubules. The disruption of HAP1/huntingtin interaction in the presence of the polyglutamine expansion can possibly alter the huntingtin/HAP1/kinesin light chain complex, further supporting the possibility of an impaired anterograde transport of BDNF vesicles in neurons. Although interesting, this study does not provide any direct evidence of defects in *in vivo* anterograde BDNF transport along the corticostriatal afferents, but there is *in vivo* evidence of a defect in retrograde transport in heterozygous knock-in mice. Interestingly, the mutant huntingtin knock-in mice and cells used to study the molecular mechanisms underlying the defect in BDNF vesicle transport show a significant reduction in BDNF protein and mRNA levels (Gines et al., 2003; Zuccato et al., unpublished manuscript), thus indicating that BDNF transport in the presence of the mutation may reflect the reduced availability of BDNF mRNA and protein in the cells and, in particular, in the cerebral cortex of the mice (see Section 4.1). However, the presence of reduced BDNF vesicle transport from the cortex to striatal targets in HD is supported by Western blot analyses showing no difference in the BDNF protein content of postmortem cortex from HD subjects and controls, whereas a smaller amount of BDNF has

been detected in autoptic striatum from other HD patients (Gauthier et al., 2004).

Subsequent findings have confirmed that BDNF vesicle transport is impaired in *in vitro* systems (Borrell-Pages et al., 2006) and provided an additional mechanism of BDNF sorting that includes mutant huntingtin and the heat DNAJ-containing protein 1b (HSJ1B). HSJB is an inhibitor of heat-shock protein cognate 70 kDa (hsc70), which removes clathrin from clathrin-coated vesicles (Cheetham et al., 1996). Clathrin is the main component of the protein coats decorating the cytoplasmic face of vesicles budding from the plasma membrane, the *trans*-Golgi network and endosomes (Gleeson et al., 2004), and is important for regulating vesicle secretion and endocytosis. BDNF, HSJ1B and clathrin co-localise at the *cis*-Golgi. The overexpression of HSJ1B positively regulates the sorting of BDNF-containing vesicles from the Golgi/*trans*-Golgi network, thus increasing BDNF release. Increasing levels of HSJ1B enhance the co-localisation of BDNF and clathrin, whereas reducing HSJ1B by siRNA dramatically decreases it (Borrell-Pages et al., 2006). Reduced HSJ1B levels have been found in HD patients, thus suggesting alterations in the formation of the clathrin coats on BDNF vesicles and impaired processing of BDNF at the Golgi, which may account for the reduced BDNF secretion (Borrell-Pages et al., 2006).

Although an *in vivo* defect in the transport of BDNF vesicles from cortex to striatum remains an interesting possibility for explaining the reduced supply of BDNF to striatal neurons and their selective vulnerability, further evidence is needed and being sought by a number of different groups.

7. BDNF levels in HD patients

As described above, analyses of BDNF levels in the striatum of HD mice have generated two different nonmutually exclusive data sets, indicating the possibility that reduced BDNF in striatum is the result of decreased BDNF production in the cortex and decreased transport of this neurotrophin from the cortex to striatum. The first envisions wild-type huntingtin participating in controlling the transcription of the BDNF gene in cerebral cortex by acting at the level of promoter II, thus allowing the correct production of BDNF protein and its anterograde transport to striatal targets via the corticostriatal afferents. Many HD mice analysed have shown a consistent decrease in BDNF mRNA and protein levels in the cerebral cortex (and lower BDNF protein levels in the striatum), due to reduced BDNF promoter II activity simultaneously to reduced transcription from BDNF promoters III and IV (Fig. 4). The second data set indicates a defect in BDNF vesicle transport as one of the primary events responsible for the depletion of BDNF in the striatum. According to this proposition, wild-type huntingtin controls the transport of BDNF from the cortex to striatum, and this transport is affected in HD (Fig. 4).

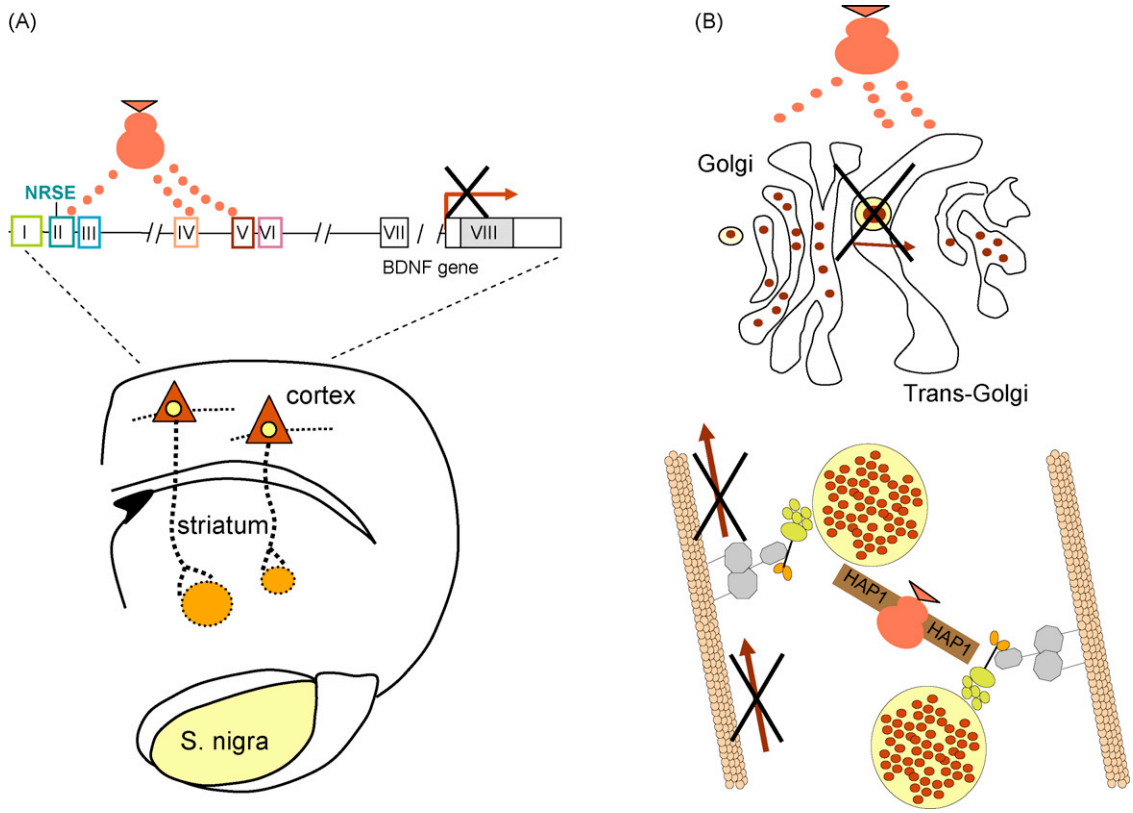

Fig. 4. BDNF in HD. Cortical BDNF trophic support is reduced in HD, contributing to striatal neurons vulnerability. BDNF dysfunction in HD is due to (A) reduction in cortical BDNF gene transcription, and (B) reduction in BDNF secretion: (top) decreased BDNF vesicles assembly and transport from Golgi to *trans*-Golgi network and (bottom) reduced BDNF vesicle movement along microtubules. In (A) BDNF gene structure is as in Liu et al., 2006 with exon IV and V corresponding to exon III and IV in Timmusk et al., 1993.

Despite the known difficulties in processing human postmortem material and the limited number of HD samples analysed so far, three studies have attempted to test BDNF levels in the human brain.

In the first, which was conducted in 2000, Ferrer et al. evaluated a small selection of samples and found decreased BDNF levels in striatum but not in the cerebral cortex, thus validating the second hypothesis. In particular, the parietal cortex, temporal cortex, hippocampus, caudate and putamen of four grade III HD subjects were analysed and compared with samples from six age-matched controls. Western blots indicated that the levels of mature BDNF protein (14 kDa) were between 53 and 82% less in the caudate and putamen of the HD subjects, but preserved in the cerebral (parietal and temporal) cortex and the hippocampus; immunohistochemical studies of the same tissue samples confirmed the reduced BDNF immunoreactivity in HD striatum (Ferrer et al., 2000). Although the BDNF signal was decreased in striatal neurons, BDNF labelling was maintained in scattered fibres. The authors suggested that the reduced BDNF protein levels in HD striatum could be due to selective defects in striatal neurons rather than reduced BDNF input from the cerebral cortex (Ferrer et al., 2000).

The findings of a second study by Gauthier et al. (2004) were in line with these data, showing that BDNF protein levels evaluated by Western blot in the cerebral cortex of 10 HD patients and 7 controls were reduced to about 50% in striatum, but not in the cerebral cortex; the negligible patient-to-patient variations indicated the highly homogenous nature of the patient cohort.

On the contrary, the third study (published by our group in 2001) found that the levels of BDNF protein (assessed by ELISA) and BDNF mRNA in cortex were consistent with those observed in the various transgenic mouse models of HD: there was a ~50% decrease in BDNF levels in the frontoparietal cortex of two HD subjects (grade II) in comparison with two age-matched controls (Zuccato et al., 2001).

It is highly likely that the differences in the results of these three studies were due to their different methods and the diversity of the analysed samples, and would be eliminated by analysing a larger number of samples. To this end, we are extending the study to a larger cohort of HD and control subjects, and our preliminary results indicate a statistically significant reduction in cerebral cortex BDNF mRNA and protein levels in HD, thus supporting the notion of impaired BDNF production in human HD cortex as a consequence of an expanded CAG tract inside the HD gene (Zuccato et al., unpublished manuscript).

8. BDNF depletion enhances neuropathology and disease onset in HD mice

Previous data indicate that the deficit in striatal BDNF in HD may be due to reduced BDNF gene transcription in the cerebral cortex or reduced BDNF vesicle transport (or both). As described in Section 2.3, the downregulation of endogenous cortical BDNF highlights a link between cortical BDNF levels and specific aspects of HD attributable to decreased BDNF levels (Baquet et al., 2004). To investigate whether the downregulation of endogenous BDNF can influence disease onset and/or progression, R6/1 mice were crossed with heterozygous BDNF knock-out mice to generate double mutants expressing mutant huntingtin exon 1 and only one allele of BDNF (Canals et al., 2004). These R6/1 bdnf$^{+/-}$ mice showed an earlier onset of motor dysfunction with significant motor alterations 6–8 weeks before R6/1 mice, and the progression of the movement deficits was more rapid and severe. It was also found that, in comparison with R6/1 mice, the double mutant mice had more tremors at rest and sudden movements that resembled chorea (Canals et al., 2004).

At neuropathological level, reducing BDNF levels in an HD background causes a reduction in striatal volume. This has been attributed to the loss of striatal neurons, which was observed in the R6/1 bdnf $^{+/-}$ mice at 30 weeks of age. As no striatal cell loss was reported in the R6/1 line (Mangiarini et al., 1996), it was suggested that, in combination with the presence of overexpressed mutant huntingtin, the lack of one BDNF allele contributes to selective striatal degeneration. In particular, immunohistochemical findings indicate that the deficit in endogenous BDNF in double-mutant mice reduces the number of DARPP-32-positive cells, particularly the enkephalin-positive neurons (the most affected population in HD); furthermore, the administration of BDNF to the same double-mutant mice increases the number of enkephalin-positive neurons (Canals et al., 2004).

Another study of R6/1 mice by Pineda et al. (2005) examined the effect of decreased BDNF levels at the level of the nigrostriatal system, which accounts for dopaminergic input to the striatum. These afferents also provide BDNF to striatal targets, accounting for about 5–10% of striatal BDNF (as mentioned above, most comes from the cortical afferents). In comparison with R6/1 mice, R6/1 bdnf $^{+/-}$ mice have more aggregates in the substantia nigra pars compacta, and significantly less striatal dopamine and 3,4-dihydrophenylacetic acid (DOPAC) levels. In association with the reduced dopamine receptor expression in R6/1 bdnf $^{+/-}$, this finding suggests that the decreased BDNF levels in HD may cause dysfunctions in the nigrostriatal dopaminergic system. Changes in locomotor activity after stimulating the dopaminergic system with amphetamine (a drug that induces dopamine release) indicate that double-mutant mice are less active that R6/1, which suggests that reduced BDNF levels in the presence of mutant huntingtin may cause dopaminergic dysfunction, and thus possibly contribute to the motor alterations associated with human HD (Pineda et al., 2005). BDNF supplements may help restore dopaminergic activity, which could be beneficial in reducing such motor symptoms (Pineda et al., 2005).

More recently, R6/1 mice have been crossed with mice overexpressing BDNF. BDNF overexpression in the cerebral cortex improved their behavioural performance, thus slowing the progression of the disease (Xu et al., 2006).

9. TrkB levels in HD

In order to exert its biological activity, BDNF binds to TrkB receptors, and a number of studies have indicated that TrkB is

modulated after neuronal damage: for example, TrkB mRNA is increased in cortical and striatal neurons after excitotoxic lesions (Canals et al., 1999; Checa et al., 2001), and reduced TrkB levels have been associated with neurodegeneration in AD (Connor et al., 1996, 1997; Allen et al., 1999; Ferrer et al., 1999; Soontornniyomkij et al., 1999; Savaskan et al., 2000). A recent report indicates that mutant huntingtin affects TrkB levels in HD (Gines et al., 2006) by showing that TrkB protein levels are reduced in mutant huntingtin knock-in cells and HD mouse models. TrKB protein levels were lower in the striatum but not the cerebral cortex of (i) 5-month-old R6/1 mice expressing mutant huntingtin exon 1, (ii) 9-month-old homozygous mutant huntingtin knock-in mice, and (iii) 17-month-old HD94 mice, a conditional mouse model of HD expressing tetracycline-regulated exon 1 mutant huntingtin with 94 glutamines. Decreased striatal TrkB protein levels were associated with reduced mRNA expression in the HD94 mice (Gines et al., 2006), but the inactivation of mutant huntingtin rescued TrkB expression to control levels, thus indicating that the continuous expression of mutant huntingtin may be required to reduce TrkB expression, and that this reduction is reversible if mutant huntingtin is inactivated (Gines et al., 2006). In addition, TrkB expression seemed to depend on the length of the CAG tract in huntingtin exon 1, further confirming that a polyQ-dependent mechanism may underlie the decrease in TrkB expression (Gines et al., 2006).

A dramatic reduction in TrkB receptors has also been found in striatum from three HD patients (including one with juvenile-onset HD), and reduced TrkB levels were detected also in cortical samples from four HD subjects, which contrasts with results obtained in HD mouse models showing unaffected cortical TrkB levels (Gines et al., 2006). Further investigations are required to understand the extent and consistency of the TrkB downregulation, and its level in the early stages of the disease.

Various lines of evidence indicate that BDNF and TrkB protein are subject to autocrine/paracrine regulatory signals on which their expression and distribution depend: e.g. exposure to high BDNF doses leads to the downregulation of TrkB receptors (Frank et al., 1996; Knusel et al., 1997; Sommerfeld et al., 2000). In order to verify whether reduced BDNF levels in HD might affect the expression of TrkB, the level of BDNF receptors was analysed in R6/1 and R6/1 bdnf$^{+/-}$ mice: no differences were detected in TrkB levels, thus suggesting that substantial deficits in BDNF do not exacerbate the reduction in TrkB induced by mutant huntingtin (Gines et al., 2006).

Taken together, the results of these studies suggest that corticostriatal disease may be influenced not only by reduced cortical BDNF production and transport but also by reduced TrkB levels, and this has to be borne in mind when designing therapeutic strategies based on BDNF administration. It was however surprising that, although TrkB levels were reduced in the R6/1 mice, the administration of exogenous BDNF improved their neuropathological phenotype, thus suggesting that the remaining TrkB molecules were still capable of efficiently transducing BDNF-dependent cell signalling (Canals et al., 2004).

10. A role for BDNF polymorphisms?

As stated in Section 5.1, a valine-to-methionine substitution at position 66 (Val66Met BDNF) is a known polymorphism of the human BDNF gene that is located in the 5′ pro-BDNF sequence encoding the precursor peptide (pro-BDNF), which is proteolytically cleaved to form the mature protein. This BDNF polymorphism does not affect mature BDNF protein function, but it has recently been shown that it dramatically alters the intracellular trafficking and packaging of pro-BDNF, and consequently the regulated secretion of the mature peptide (Egan et al., 2003; Chen et al., 2004).

The BDNF Val66Met polymorphism is highly conserved across species and relatively common in the human population with a prevalence for heterozygotes of 20–30%, and a prevalence for the homozygote of ∼4% (Neves-Pereira et al., 2002; Egan et al., 2003; Hariri et al., 2003; Sen et al., 2003). A number of genetic linkage and behavioural studies have shown that this polymorphism is associated with neuropsychiatric disorders, including AD (Ventriglia et al., 2002), PD (Momose et al., 2002), bipolar disorders (Neves-Pereira et al., 2002; Sklar et al., 2002), depression (Sen et al., 2003), obsessive compulsive disorder (Hall et al., 2003) and schizophrenia (Neves-Pereira et al., 2005), as well as with normal personality traits (Itoh et al., 2004).

While this review was submitted, an article from Del Toro et al. (2006) was published describing the effects of mutant huntingtin on the intracellular trafficking and release of the two forms of BDNF. It was found that mutant huntingtin does not impair the transport from the endoplasmic reticulum to the Golgi apparatus both of Val66Val BDNF and Val66Met BDNF. Instead, it specifically alters the post-Golgi trafficking of BDNF vesicles. In particular, the post-Golgi trafficking of Val66Val BDNF was significantly blocked in mutant huntingtin cells, whereas the transport of Val66Met BDNF was not affected. These data clearly indicate that the mutant protein affects solely the trafficking of Val66Val BDNF form, without causing a major retention of Val66Met BDNF in the Golgi apparatus (Del Toro et al., 2006).

Given this information and the extensive evidence relating BDNF to HD, the BDNF gene has been tested as a potential modifier of the age of HD onset. It is known that the length of the polyglutamine expansion inversely correlates with the age of onset in the case of a small and large number of CAG repeats, but its predictive value is weaker in the intermediate range (Wexler et al., 2004). It has consequently been postulated that other factors affect the course of the disease. Soon after the discovery of the HD gene, it was hypothesised that familial factors other than CAG repeats may account for a proportion of the variance in the age of onset (Andrew et al., 1993; Duyao et al., 1993; Snell et al., 1993), and the search for genetic modifiers of HD onset and progression represents a major part of current HD research efforts. It has in fact been demonstrated that a few modifier genes, such as the GluR6 kainate receptor (GRIK2) (Rubinsztein et al., 1997), NMDA receptor subunit 2B (GRIN2B) (Arning et al., 2005), and ubiquitin carboxy-terminal hydrolase L1 (UCHL1) (Metzger et al., 2006a) account for a proportion of the variance.

On the basis of these considerations and data showing that the age of onset is modulated by reduced BDNF levels in HD mice (Canals et al., 2004; Baquet et al., 2004), four studies have analysed the effect of the BDNF Val66Met polymorphism.

A first study by Alberch et al. (2005) involved 122 HD patients and 95 controls of Spanish ancestry, and indicated a later age at disease onset in patients with the Val66Met polymorphism, although this was restricted to the group of patients with between 42 and 49 CAG repeats. A more recent study by Di Maria et al. (2006) tested the effect of the Val66Met variant in a larger group of 255 Italian patients, and found no association between the Val66Met genotype and differences in age at disease onset. In this study, RE1/NRSE variants were not identified in the screening procedure use (Di Maria et al., 2006). An independent study of larger samples of HD patients from North America (228 with an extremely young and 329 with an extremely old onset of HD) also found no correlations with BDNF variants (Kishikawa et al., 2006), and similar results have been obtained by Metzger et al. (2006b) in 980 unrelated European HD patients.

The failure to revealing any significant effect of BDNF Val66Met on age at the time of onset of HD has generated discussion concerning the impact of the BDNF deficit on the disease. As the Val66Met polymorphism influences BDNF transport from the Golgi region to appropriate secretory granules, and not its transcription or biological activity, we believe that the lack of an association indicates that the defect in BDNF transport has no impact on age at disease onset, although it may still have an effect on disease progression. The possibility that BDNF transcriptional activity is present presymptomatically and may therefore affect age at onset and/or disease progression remains open.

11. BDNF: a therapeutic path for Huntington's disease?

A number of studies have shown that BDNF is a potent neurotrophic factor for GABA-ergic striatal neurons. When administered to cultured striatal neurons, it increases their cellular GABA content, the frequency of parvalbumin and calbindin neurons, and the levels of neuropeptide Y and somatostatin (Mizuno et al., 1994; Ventimiglia et al., 1995). In addition, a study by Nakao et al. (1995) showed that BDNF promoted the survival of DARPP-32 positive neurons and, as DARPP-32 is a specific marker of MSN maturation, it was suggested that BDNF plays a role in the maturation of striatal neurons. In particular, it was found that BDNF stimulated the morphological differentiation of DARPP-32-containing neurons by increasing the length of their neurites, the number of branching points on the neurites, and the soma area. Later studies showed that *in vitro* maturation of a large subset of medium-sized striatal spiny neurons requires BDNF, which regulates the expression of DARPP-32 and cyclic AMP-regulated phosphoprotein, 21 kDa (ARPP-21) (or calbindin), both of which are necessary for the differentiation of striatal neurons (Ivkovic et al., 1997; Ivkovic and Ehrlich, 1999).

Additional studies in which BDNF protein was administered *in vivo* by means of direct brain infusion have demonstrated that the neurotrophin increases the mRNA expression of striatal neuropeptides (Croll et al., 1994; Sauer et al., 1994; Arenas et al., 1996).

Various lines of evidence now indicate that BDNF deficit contributes significantly to the pathogenesis of HD, thus suggesting that it may make a valuable candidate co-therapeutic agent in HD. Promising suggestions of the potential benefits of BDNF came from *in vitro* studies by Saudou et al. (1998), who reported that cultured mammalian neurons bearing the mutant protein were prevented from dying when BDNF was administered, and, more recently, Zala et al. (2005) have confirmed these early data by demonstrating that the lentiviral delivery of BDNF to primary neurons expressing mutant huntingtin protects against the cell death induced by the mutant protein. Other data concerning the potential *in vivo* benefits of BDNF come from R6/1 mice treated intrathecally via a minipump (Canals et al., 2004), in which it was found that BDNF increased the expression of enkephalin as well as the number of enkephalin-expressing striatal cells, the most affected cells in HD (Canals et al., 2004).

Despite these promising results, BDNF supplementation raises a number of different problems. The major problem is how to assess the amount of BDNF that reaches the affected neurons, and this is compounded by the fact that BDNF is relatively unstable and only a small amount can cross the blood-brain barrier (Betz et al., 1989; Knusel et al., 1992; Pardridge, 2002). If the amount of BDNF is too small, it may not be sufficient to produce the required effects; if it is too large, it may be dangerous because, besides regulating survival, differentiation and maintenance of neuron-specific characteristics, BDNF is also important in modulating activity-dependent neuronal plasticity, which are essential for the functional and structural refinement of neuronal circuits, as well as learning and memory (see Binder and Scharfman, 2004, for a review). Uncontrolled BDNF administration may interfere with these mechanisms and even give rise to serious side effects such as epileptic activity (Binder et al., 2001). It has also been reported that high BDNF levels may downregulate the expression of TrkB receptors, thus reducing the signalling pathway activated by BDNF and therefore blocking any possible beneficial effect (Frank et al., 1996; Knusel et al., 1997; Sommerfeld et al., 2000). It is probably for these reasons that subcutaneous and intrathecal BDNF administrations have met with little clinical success (BDNF study group Phase III, 1999; Ochs et al., 2000).

In an attempt to overcome such problems, improved methods allowing a local and regulated supply of BDNF have been developed, including *in vivo* and *ex vivo* gene transfer, and other therapeutic approaches aimed at stimulating the synthesis of endogenous BDNF are under investigation. In this context, a number of drugs that enhance BDNF production in the brain are being studied, as the production of BDNF mimetics. Finally, interesting new perspectives have arisen from the observation that physical exercise and diet markedly increase endogenous BDNF levels in the hippocampus and cerebral cortex.

11.1. BDNF delivery via viral vectors and engineered cells

In a first attempt to improve neurotrophin delivery, BDNF and other trophic factors (ciliary neurotrophic factor (CNTF) and glial derived neurotrophic factor (GDNF) have been incorporated into viral vectors (including adenovirus and lentivirus) aimed at allowing the direct intracerebral infection of endogenous striatal neurons. These experiments have provided evidence that neurotrophins are significantly protective against excitotoxic lesions (Anderson et al., 1996; De Almeida et al., 2001; Mittoux et al., 2002; Zala et al., 2004) and one phase I clinical study involving a small number of HD patients administered CNTF-releasing microencapsulated cells no side effects were observed over a period of 2 years (Emerich, 1999; Bachoud-Levi et al., 2000; Bloch et al., 2004).

Later studies confirmed that the viral delivery of BDNF can be protective in animal models of HD. In an elegant study, Bemelmans et al. intrastriatally injected adenovirus encoding BDNF in rats that were lesioned 2 weeks later with quinolinic acid (QUIN), a toxin that induces striatal neuron death by means of an excitotoxic process. One month after the lesion, histological analyses revealed that striatal neurons were protected only in the rats treated with the BDNF adenovirus. The QUIN induced lesions were 55% smaller in the BDNF adenovirus-treated mice, and survival of striatal GABA-ergic neurons was increased, thus indicating that transferring the BDNF gene is of therapeutic value (Bemelmans et al., 1999). In a subsequent study, BDNF was delivered to the striatum in the QUIN rodent model of HD by means of an adeno-associated viral (AAV) vector: the rats received a unilateral intrastriatal injection of AAV-BDNF and were lesioned with QUIN 3 weeks later. A significant reduction in striatal neuron loss was observed 2 weeks after the lesion, thus indicating that BDNF may provide neuronal protection in this rodent model of HD (Kells et al., 2004).

An alternative approach adopted in parallel to the development of the lentiviral delivery of BDNF has been to engineer cells to express and continuously release the desired transgene at safe doses. In a first attempt, immortalised rat fibroblasts genetically engineered to secrete BDNF were implanted in the rat striatum 7 days before the striatal infusion of excitotoxic quantities of an NMDA-receptor agonist. Analysis of striatal damage 7 days after the lesion revealed that BDNF-secreting fibroblasts offered no protection (Frim et al., 1993). A later study showed that BDNF had only limited ability to protect the striatum from damage due to an excitotoxic lesion by transplanting putative neural stem cells that had been genetically modified to overexpress BDNF in the striatum of rats, which were injected with quinolinic acid in the same area 1 week later. One month after the lesion, striatal degeneration, lesion size and the loss of DARPP-32 positive neurons were only slightly improved by the BDNF-secreting cells (Martinez-Serrano and Bjorklund, 1996). Subsequent attempts have been more successful probably because lower and safer BDNF doses have been released (Perez-Navarro et al., 1999, 2000; Ryu et al., 2004), including a particularly interesting study by Ryu et al. (2004). The authors investigated the ability of transplanted BDNF-overexpressing bona fide neural stem cells taken from human fetal brain to protect animals after 3-nitropropionic acid (3NP) administration, which causes widespread neuropathological deficits similar to those seen in HD. The animals receiving the intrastriatal cell implantation 1 week before 3NP treatment showed significantly improved motor performance and less striatal neuron damage, whereas those transplanted 12 h after 3NP treatment did not show any improvement in motor performance or any protection of striatal neurons from the toxicity induced by 3NP (Ryu et al., 2004).

Although increasing BDNF expression by means of viral vectors and BDNF-engineered cells has led to encouraging results, there are still a number of problems to be overcome, as overexpression of the transgene can cause toxic effects and many of the vectors are toxic *per se*. Furthermore, and more importantly, the invasiveness of the approach makes it difficult to apply in patients. Consequently, current experiments are aimed at regulating transgene expression by using inducible promoters, or developing techniques that will increase endogenous BDNF levels.

11.2. BDNF mimetics

Despite the improvements that have been made to strategies for local neurotrophin production and delivery, many difficulties still remain. Unfortunately, neurotrophins have a number of suboptimal pharmacological properties, including poor stability with a serum half-life of minutes or less, poor oral bioavailability, and restricted CNS penetration (Poduslo and Curran, 1996; Saltzman et al., 1999; Pardridge, 2002). One potentially powerful approach to overcoming these limitations is to develop synthetic, small-molecule neurotrophin mimetics with favourable stability and tissue penetration profiles that act via targeted neurotrophic receptors. Researchers are therefore developing small-molecule BDNF analogues with improved pharmacokinetic properties and an ability to penetrate the blood brain barrier to an appreciable extent. The primary source for these studies is the three-dimensional structure of BDNF, which has been used as a template to design cyclic peptides that mimic BDNF making use of loops 1, 2 and 4, which are required for binding to TrkB receptors. Preliminary studies have demonstrated that such BDNF mimetics act as BDNF agonists that promote the survival of cultured sensory neurons, although their drug-like properties, particularly their proteoliyic stability, their ability to function via TrkB receptors, and their capacity for crossing the cell membrane, still require analysis (O'Leary and Hughes, 2003; Fletcher and Hughes, 2006). A considerable amount of work remains to be done, but this approach may represent a valuable step towards the development of BDNF mimetics for clinical use.

11.3. Drugs that increase BDNF levels and their effectiveness in HD

BDNF has received considerable attention for being modulated in the brain by antidepressant treatments (drugs,

electroconvulsive shocks, physical exercise), although there is still no direct evidence that endogenous BDNF plays a role in the mechanism of antidepressant drug action. The antidepressants known to affect BDNF levels are selective serotonin reuptake inhibitors (SSRIs) (Badawy and Morgan, 1991; Nibuya et al., 1996; Tardito et al., 2006) and lithium (Chuang, 2004). Furthermore, memantine and riluzole (a non-competitive inhibitor of ionotropic glutamate NMDA receptor) (Marvanova et al., 2001; Mizuta et al., 2001; Katoh-Semba et al., 2002), and cystamine and cysteamine, have recently been shown to increase BDNF levels and their effects on HD have been analysed.

11.3.1. SSRIs

SSRIs facilitate the signalling of serotonin by inhibiting its reuptake. However, although alterations in monoamine metabolism take place soon after drug administration, the clinical antidepressant effect develops slowly over several weeks of continuous treatment. This suggests that changes in monoamine metabolism alone cannot explain the entire effect, and indicates that antidepressants may act by evoking adaptive changes in intracellular signal transduction and synaptic connectivity (Nestler et al., 2002), which would make neurotrophins (particularly BDNF as a key regulator of neurite outgrowth and synaptic plasticity) potential mediators of the plastic changes induced by antidepressants. It is important to remember that HD patients are frequently exposed to antidepressant treatments, and specific studies have been carried out in an attempt to clarify the variability in their responses to such treatments.

Serotonin may have protective effects on striatal and cortical neurons by activating cyclic AMP and CREB signals, which also lead to BDNF expression; other target genes of cyclic AMP-CREB signalling that may play a role in the neuroprotective effect of SSRIs include Bcl-2 and NFKB (Freeland et al., 2001; Tardito et al., 2006). A first attempt to test the effect of SSRIs on mouse models of HD involved the administration of paroxetine 5 mg/(kg day) to HD-N171-82Q mice, which was found to delay the onset of behavioural symptoms and increase life span (Duan et al., 2004). Significant impairment of the behavioural phenotype was observed specifically at the level of motor function (Duan et al., 2004), and the weight loss previously reported in this model occurred significantly more slowly than in vehicle-treated HD mice. Histological analyses also revealed a decrease in brain atrophy. HD-N171-82Q are normally hyperglycemic but paroxetine treatment reduced blood glucose levels, thus providing evidence that, in addition to neurodegenerative processes, it improves glucose metabolism in HD. Paroxetine also increased survival even when administered after the onset of motor dysfunction (Duan et al., 2004), thus suggesting the possibility that HD patients may benefit from SSRIs after they become symptomatic.

These findings open the way to studies of the effects of paroxetine in human HD patients, but previous studies have found no clinical benefit with the use of other SSRIs. There is a single case report of fluoxetine exacerbating chorea (Chari et al., 2003) and, although another study found it a useful antidepressant, it failed to provide any substantial clinical benefit to non-depressed HD patients (Como et al., 1997).

11.3.2. Lithium

Lithium induces the expression of BDNF and the subsequent activation of TrkB in cortical neurons (Fukumoto et al., 2001). The mechanisms underlying this effect include a lithium-mediated reduction in excitotoxicity as a result of increased glutamate uptake, and the regulation of a number of signal transduction intermediates such as myo-inositol, protein kinase C, phosphotidylinositol-3 kinase (PI-3K)/protein kinase B (AKT), ras-mitogen-activated protein kinase (MAPK), glycogen synthase kinase (GSK)-3alpha and -3beta, and calcium. Early studies by Chuang et al. indicated that a subcutaneous lithium chloride (LiCl) injection for 16 days before QUIN infusion considerably reduces the size of QUIN-induced striatal lesions (Wei et al., 2001). It was later found that it can protect against polyglutamine toxicity in cell lines (Carmichael et al., 2002) by inhibiting GSK-3beta, which is involved in apoptotic cell death, and increasing beta-catenin whose overxpression protects cells from mutant huntingtin-induced toxicity (Carmichael et al., 2002).

One year later, on the basis of lithium's reported neuroprotective and anti-depressive properties, other studies determined whether chronic LiCl treatment affects the progression of the phenotype in R6/2 mice, but found that it had variable effects on motor behaviour and did not improve survival (Wood and Morton, 2003). More recently, a study by Senatorov et al. (2004) has suggested that lithium may be neuroprotective in the QUIN-injection model of HD because of its ability to inhibit apoptosis and induce neuronal and astroglial progenitor proliferation or migration from the subventricular zone (SVZ).

11.3.3. Memantine and riluzole

Memantine is a medium-affinity non-competitive *N*-methyl-D-aspartate receptor antagonist that has been clinically used as a neuroprotective agent to treat AD and PD. At clinically relevant doses, it markedly increases BDNF and TrkB mRNA levels in rat brain (Marvanova et al., 2001), and its effects on BDNF mRNA were reflected in changes in BDNF protein levels.

Like memantine, riluzole (a neuroprotective drug commonly used in amyotrophic lateral sclerosis (ALS)) acts by blocking glutamatergic neurotransmission in the CNS. Interestingly, it has also been found to upregulate the levels of a number of key neurotrophic factors, including BDNF and glial-derived neurotrophic factor (GDNF) (Mizuta et al., 2001; Katoh-Semba et al., 2002)

These data suggest that the anti-excitotoxic activity of memantine and riluzole is accompanied by an increase in the endogenous BDNF production in the brain, and so a 2-year, multicentre open-label study of 27 HD patients was carried out in order to investigate the effectiveness of memantine (up to 30 mg/day) in delaying disease progression. The results suggest that memantine treatment may be useful in doing so (Beister et al., 2004), and another open-label trial has found that riluzole causes transient motor improvement in human HD patients (Seppi et al., 2001; Huntington Study Group, 2003).

11.3.4. Cystamine and cysteamine

It has been suggested that transglutaminases (TGases) play a critical role in the pathogenesis of CAG trinucleotide repeat disorders because they cross-link huntingtin and catalyse the formation of aggregates. As TGase activity is increased in HD brain, they represent an attractive target for possible therapeutic intervention in HD (Gentile and Cooper, 2004; Hoffner and Djian, 2005, reviews)

Early findings indicated that cystamine is a competitive inhibitor of TGase activity. It limits the aggregation of proteins with an expanded polyglutamine tract (Igarashi et al., 1998; De Cristofaro et al., 1999), and has also been shown to decrease apoptosis in cultured cells exposed to glutamate or an N-terminal fragment of mutant huntingtin with an associated decrease in TG activity (Ientile et al., 2003; Zainelli et al., 2005). In the light of these exciting *in vitro* results, a number of trials have been carried out in animal models of HD. Cystamine protects against 3NP striatal lesions in mice (Fox et al., 2003) and, more importantly, improved behaviour and survival in two independent therapeutic trials in R6/2 mice (Dedeoglu et al., 2002; Karpuj et al., 2002), limiting the decrease in brain weight, brain volume and neuronal atrophy (Dedeoglu et al., 2002). More recent findings by Van Raamsdonk et al. (2005) indicated that cystamine reduces striatal volume loss and neuronal atrophy in YAC128 mice, but does not reverse progressive motor dysfunction or the downregulation of striatal marker DARPP-32, whose expression is significantly reduced in this model. However, other recent evidence suggests that the improved survival and motor function in cystamine-treated R6/2 mice may not be solely due to TGase inhibition because R6/2 mice not expressing tissue transglutaminase also benefit from cystamine administration (Bailey and Johnson, 2005).

Other beneficial effects of cystamine include the inhibition of caspase-3 activity, increased cell levels of the anti-oxidant glutathione and cysteine (Lesort et al., 2003; Fox et al., 2003), and an increase in the expression of heat-shock proteins (Karpuj et al., 2002). Furthermore, recent findings have linked it and its reduced cysteamine form (a drug approved by the Food and Drug administration, FDA) to BDNF secretion (Borrell-Pages et al., 2006), thus opening up the possibility that the neuroprotection observed in treated animals may be due to a cystamine-mediated increase in BDNF secretion (Borrell-Pages et al., 2006). In their elegant study, Borrell-Pages et al. found that cystamine increases the levels of heat-shock protein DnaJ-containing protein 1b (HSJ1B), which are low in HD patients. HSJ1B inhibits the polyQ-induced death of striatal neurons and neuronal dysfunction, a neuroprotective effect that involves stimulation of the BDNF secretory pathway through the formation of clathrin-coated vesicles containing BDNF, and so it has been suggested that cystamine increases BDNF secretion from the Golgi, and that this effect is blocked by reducing HSJ1B levels or by overexpressing transglutaminase.

Borrell-Pages et al. further demonstrated that cystamine and cysteamine are both neuroprotective in HD mice insofar as they enhance BDNF levels in the brain of homozygous mutant huntingtin knock-in mice and R6/1 mice genetically modified to express only one functional BDNF allele (Borrell-Pages et al., 2006). The authors also showed that cystamine or cysteamine injections increase serum BDNF in mutant huntingtin knock-in mice with respect to control mice that, however, according to Radka et al., 1996 is barely detectable in mouse serum. Similarly, 3NP-treated macaques show an increase in serum BDNF levels after cysteamine treatment (Borrell-Pages et al., 2006). Finally, they found that the cysteamine-induced increase in brain and serum BDNF levels was still detectable 12 weeks after continuous treatment, which suggests that the efficacy of treatment with repeated cysteamine doses is unlikely to decrease over time. Tolerated cystamine doses have been evaluated in HD patients, thus strengthening the case in favour of using cystamine and cysteamine as a therapeutic approach to HD whose efficacy may involve increasing BDNF levels.

11.4. New perspectives for increasing endogenous BDNF

Other therapeutically interesting perspectives have emerged from the observation that physical exercise and diet cause a marked increase in BDNF levels in rat brain, particularly the hippocampus and cerebral cortex. Early studies showed that dietary restriction (DR) and physical exercise can have profound effects on brain functions and vulnerability to injury and disease (see Mattson et al., 2003, for a review; Spires et al., 2004).

11.4.1. Diet

DR promotes neuronal survival by enhancing resistance against cell stress (Yu and Mattson, 1999; Guo et al., 2000), reducing oxidative damage (Dubey et al., 1996), stimulating the production of new neurons (neurogenesis), and improving synaptic plasticity (see Mattson et al., 2003, for a review).

Data in mouse models of AD, PD and HD indicate that DR can protect neurons against neurodegeneration, suggesting that dietary changes may reduce disease severity (Mattson et al., 2003). When rats were kept on a periodic fasting/dietary restriction regimen for several months before the administration of 3NP acid, their motor function improved and more striatal neurons survived (Bruce-Keller et al., 1999).

Animal data suggest that BDNF signalling plays a role in the beneficial effects of DR, exercise and cognitive stimulation on brain aging. When rats or mice are kept on an intermittent fasting/dietary restriction regimen, BDNF levels increase in several brain regions, including the hippocampus, cerebral cortex and striatum (Lee et al., 2002; Duan et al., 2001b). BDNF seems to play a particularly important role in the excitoprotective effect of DR as the infusion of a BDNF blocking antibody into the lateral ventricle of rats and mice significantly attenuated the neuroprotective effect of DR in the kainate model of seizure-induced hippocampal damage (Duan et al., 2001a,b).

Recent findings indicate that HD mice with lower BDNF protein levels in the cerebral cortex and striatum, and the formation of aggregates and cell apoptosis in the brain, show delayed disease onset and increased survival when kept on a DR regimen (Duan et al., 2003). DR reduces brain atrophy and the

formation of huntingtin aggregates, and diminishes caspase activation, thus apparently blocking the toxic effects elicited by mutant huntingtin. As BDNF protein levels are increased in the cerebral cortex and striatum of HD mice on DR, they may contribute to the improvement. DR may therefore be considered a potential early strategy (before the development of symptoms) for counteracting HD phenotypes and restoring normal brain BDNF levels.

11.4.2. Environmental enrichment

It has been shown that environmental enrichment markedly delays the onset and progression of HD in transgenic mice. It involves providing the mice with environments containing regularly changed, complex and stimulating objects. The impact of such a strategy was reported for the first time in 2000, when it was shown that R6/1 mice exposed to environmental enrichment experienced a delayed disease onset and slower rate of disease progression, and had improved behavioural performances on motor tests (Van Dellen et al., 2000). Further studies have indicated that environmental enrichment also slows disease progression in the more severe R6/2 mouse model of HD (Hockly et al., 2002), a well as in N171-82Q transgenic HD mice (Schilling et al., 2004).

Environmental stimulation delays the onset of cognitive deficits (see Van Dellen et al., 2005, for a review), and its beneficial effects have also been demonstrated by studies of HD patients (Sullivan et al., 2001), in whom a more fertile environment leads to increased self-awareness and self-esteem, and improved physical, mental and social functioning, even in late-stage HD (Sullivan et al., 2001).

The mechanisms by which these beneficial effects are mediated are still unclear, but there are a number of plausible possibilities. Enrichment is associated with increased synaptic signalling and the stimulation of second messenger systems; it also has an effect on neuronal morphology, as it is associated with increased spine density. There is evidence that environmental enrichment or physical exercise upregulate the transcription of genes encoding neuronal proteins that are important for neuronal plasticity, learning and memory (Rampon and Tsien, 2000). In particular, environmental enrichment upregulates neurotrophins such as BDNF and NGF in the hippocampus and cortex (Falkenberg et al., 1992; Young et al., 1999; Pham et al., 1999a,b; Keyvani et al., 2004).

Recent studies of R6/1 transgenic mice have shown that environmental enrichment rescues striatal and hippocampal BDNF protein deficits (Spires et al., 2004). As the antero-medial cortical levels of BDNF protein were unaffected, it was hypothesised that the enrichment rescued the cortical-striatal transport of BDNF. An independent study using the same model found that voluntary physical exercise delayed the onset of HD and the decline in cognitive ability (Pang et al., 2006). These observations in R6/1 mice require further confirmation in other HD mouse models, but suggest that the beneficial effect of enrichment may be partially mediated by increased BDNF levels. Recent findings indicate that neurogenesis is impaired in R6/1 and R6/2 mice (Phillips et al., 2005; Lazic et al., 2006). The stimulatory role of enrichment and BDNF on neurogenesis suggests that this may be one avenue for the therapeutic effects of environmental stimulation.

11.4.3. Isolation of novel drugs acting on the RE1/NRSE

The mechanisms by means of which huntingtin controls BDNF gene transcription and transport have now been elucidated (see Sections 3.3.2 and 5.2), thus providing novel downstream targets that can be exploited by drug-screening strategies whose final goal is to isolate drugs capable of sustaining BDNF production and transport. In our opinion, a first possible target for the selective high-throughput screening of novel compounds able to increase BDNF production is the BDNF promoter. The transcriptional complex to be targeted for such a purpose is represented by RE1/NRSE, the silencer of gene expression within BDNF exon II promoter, and REST/NRSF, the transcription factor that activates its silencing activity (Zuccato et al., 2003). RE1/NRSE is located within the promoter region of a broader spectrum of neuronal genes, and modulating this target with the final aim of inhibiting its silencing activity could lead to the isolation of compounds that are not only capable of increasing BDNF levels, but would also have widespread pro-transcriptional activity on a number of neuronal genes (Patent: ‘‘Method for the identification of drugs useful in Huntington’s disease therapy’’, Italy, no. MI2002A000809 dep. 17-04-2002; Europe, no. 03712609.1; USA, no. 511 665, dep. 18-10-2004). An assay has recently been produced and validated in our laboratory, and pilot compound screening has identified structurally related analogues that are active at concentrations in the low nanomolar range (Rigamonti et al., unpublished manuscript).

12. Conclusion

The findings described in this paper indicate that BDNF is essential for maintaining the corticostriatal pathway, and the cortical BDNF is required for the survival and differentiation of striatal neurons under both physiological and pathological conditions. More importantly, they show that the normal huntingtin protein, whose mutation causes HD, is involved in the physiological control of BDNF synthesis and transport in the brain: it sustains cortical BDNF gene transcription and drives BDNF vesicle sorting in neuronal cells. Both processes are simultaneously disrupted in HD. The mechanisms underlying these different properties have been the subject of intensive investigation and, although more data are necessary, it is already clear that BDNF is one of the critical factors missing in HD. We have described several lines of evidence indicating that BDNF levels are reduced in the brains of HD patients who, in addition to being affected by the toxicity of mutant huntingtin, seem to be characterised by decreased normal huntingtin activity, which may reduce cortical BDNF gene transcription. Finally, we have shown that the RE1/NRSE BDNF locus (as well as many other RE1/NRSE loci in the genome) is the first identified target of wild-type huntingtin activity. It is therefore possible that increasing endogenous BDNF production or supplying exogenous BDNF may have therapeutic effects if applied in an appropriate spatio-temporal context.

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