Increased Cortical Expression of Two Synaptogenic Thrombospondins in Human Brain Evolution

Thrombospondins are extracellular-matrix glycoproteins implicated in the control of synaptogenesis and neurite growth. Previous microarray studies suggested that one gene of this family, thrombospondin 4 (THBS4), was upregulated during human brain evolution. Using independent techniques to examine thrombospondin expression patterns in adult brain samples, we report ~6-fold and ~2-fold greater expression of THBS4 and THBS2 messenger RNA (mRNA), respectively, in human cerebral cortex compared with chimpanzees and macaques, with corresponding differences in protein levels. In humans and chimpanzees, thrombospondin expression differences were observed in the forebrain (cortex and caudate), whereas the cerebellum and most nonbrain tissues exhibited similar levels of the 2 mRNAs. Histological examination revealed THBS4 mRNA and protein expression in numerous pyramidal and glial cells in the 3 species but humans also exhibited very prominent immunostaining of the synapse-rich cortical neuropil. In humans, additionally, THBS4 antibodies labeled β-amyloid containing plaques in Alzheimer’s cases and some control cases. This is the first detailed characterization of gene-expression changes in human evolution that involve specific brain regions, including portions of cerebral cortex. Increased expression of thrombospondins in human brain evolution could result in changes in synaptic organization and plasticity, and contribute to the distinctive cognitive abilities of humans, as well as to our unique vulnerability to neurodegenerative disease.

Keywords: gene expression, human evolution, neuroanatomy, plasticity, primates, synaptogenesis

Introduction

The advent of genome sequencing and related technologies has made it possible to characterize the genetic changes that occurred in human evolution in unprecedented and remarkable detail. Using these techniques to compare humans with chimpanzees and other mammals, several changes in the DNA sequences of genes have been identified that could affect brain structure and function in humans (e.g., Gilbert et al. 2005; Rockman et al. 2005; Varki and Altheide 2005; Pollard et al. 2006). In addition, a number of gene expression studies in brain and other tissues have identified differences in expression levels between humans and nonhuman primates, indicating that on the order of 100–300 genes underwent expression changes in cerebral cortex in the human lineage following the divergence of humans and chimpanzees (reviewed by Preuss et al. 2004). These gene-expression differences could be involved in human phenotypic specializations, such as our unique cognitive and behavioral abilities or our increased susceptibility to certain neurodegenerative diseases like Alzheimer’s disease (AD), which appears to be specific to humans (Walker and Cork 1999; Olson and Varki 2003). Furthermore, comparisons of messenger RNA (mRNA) levels in homologous brain regions of humans and other closely related primate species can also provide important clues for identifying previously unknown phenotypic differences in the cellular and histological organization of the human brain (Preuss et al. 2004).

We took as the starting point for this investigation the gene for thrombospondin 4 (THBS4), which showed several times higher expression in the adult cortex of humans compared with chimpanzees and macaques in preliminary microarray data (Cáceres et al. 2003). In vertebrates, the thrombospondins (abbreviated THBSs or TSPs) are a family of 5 genes known as THBS1–4, COMP (cartilage oligomeric matrix protein). These genes code for large extracellular-matrix glycoproteins (named also as THBS1–4 and COMP, but without italics to distinguish proteins from genes) that mediate cell adhesion, proliferation, motility, and cytoskeletal organization, through interactions with different matrix constituents, growth factors, and integrins and other cell-surface receptors (for recent reviews, see Adams 2004; Adams and Lawler 2004; Elzie and Murphy-Ullrich 2004). The nervous-system functions of these proteins have not been extensively investigated, although THBS4 and other thrombospondins have been reported to influence neurite growth (Osterhout et al. 1992; Chamak et al. 1994; Arber and Caroni 1995), and in rodent brains THBS4 has been localized to synapse-rich territories (Arber and Caroni 1995). Recent studies using cell-culture and knockout mouse models, however, show that THBS1 and THBS2 are astrocyte-secreted factors that induce synapse formation (Christopherson et al. 2005), and subsequent studies indicate that all thrombospondins, including THBS4, can elicit synapse formation (Eroglu et al. 2005).

The increased expression of THBS4 could, therefore, be an indication of important evolutionary changes in the synaptic organization and dynamics of the human brain. There are other possibilities, however. For example, the original microarray results could be false positives, or increases in mRNA might not be accompanied by increased protein levels (Preuss et al. 2004). In addition, THBS4 upregulation might affect not only the brain but many other tissues, and the additional protein synthesized in human cortex might not be localized in a manner consistent with a synaptogenic role, reflecting evolutionary forces acting on other functions. To address these alternatives, and in

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particular to determine which tissues and cell types were involved in thrombospondin expression changes in human evolution, we carried out a comprehensive analysis of thrombospondin gene-expression patterns in humans, chimpanzees, and macaque monkeys using multiple mRNA-based and protein-based techniques. Our results indicate that the mRNA and proteins of 2 thrombospondins, THBS4 and THBS2, were up-regulated in human cortical evolution. Moreover, comparative in situ hybridization and immunohistochemistry of THBS4, the thrombospondin most strongly upregulated in humans, show that it is expressed by numerous neurons and glia in all species examined, but that humans accumulate more THBS4 protein in the synapse-rich neuropil than do chimpanzees or macaques.

Materials and Methods

**Tissue Samples**

Tissue samples were obtained postmortem from a total of 58 adults of 4 different primate species: 26 humans (*Homo sapiens*), 13 common chimpanzees (*Pan troglodytes*), 13 rhesus macaques (*Macaca mulatta*), and 6 pigtail macaques (*Macaca nemestrina*). Detailed information about species, sex, age, cause of death, and origin of each sample is listed in Supplementary Table 1. Chimpanzee and macaque samples were dissected from individuals that died of natural causes or were euthanized for humane reasons and all procedures were carried out in accordance with Institutional Animal Care and Use Committees guidelines. Cortical tissue samples were taken primarily from the frontal pole (FP), anterior inferior temporal cortex (aIT), or temporal pole (TP) of each species, which correspond respectively to areas 10, anterior 21, and 38 in humans and macaques (Brodman 1909; Walker 1940), and to areas FE, the anterior part of TE, and TG in chimpanzees, which are likely homologous to areas 10, 21, and 38, respectively (Bailey et al. 1950).

**Combined Analysis of Microarray Data Sets**

Published human and chimpanzee gene-expression data from Affymetrix (Santa Clara, CA) arrays HG-U95Av1 and HG-U95Av2 (Enard et al. 2002; Cáceres et al. 2003; Khaitovich et al. 2004) or HG-U133Plus2 (Khaitovich et al. 2005) were analyzed in GCOS 1.3 (Affymetrix), after eliminating all the oligonucleotide probes with sequence differences to the human or chimpanzee genomes as previously described (Marquès-Bonet et al. 2004). Arrays employing the same platform (HG-U95A or HG-U133) were then normalized together by quantile normalization using the Bioconductor software package (Gentleman et al. 2004). To accurately quantify gene-expression levels, we included in the analysis only those probe sets that had 6 or more probes and that were detected (P or M detection call) in at least one of the arrays of a given tissue. For genes represented by multiple probe sets, the expression estimates for the different probe sets were averaged. In addition, the expression values of replicate arrays from the same individual were also averaged. Statistical comparisons were carried out using StatView software (SAS Institute, Cary, NC) and all probability values stated correspond to two-tailed tests.

**Real-Time Reverse Transcription PCR Quantification**

Total RNA was isolated from ~100 mg of frozen tissue by homogenization with TRIzol (Invitrogen, Carlsbad, CA) and was purified with the RNaseasy kit (Qiagen, Hilden, Germany). Real-time reverse transcription PCR (RT-PCR) was performed with ~400 ng of DNase I-treated RNA from each sample by using the SuperScript First Strand Synthesis kit (Invitrogen) and the iTaq SYBR Green Supermix with Rox (BioRad, Hercules, CA). To ensure that sequence differences between species did not affect the amplification, PCR primers were designed in regions of the 3' end of the gene mRNAs that were identical in the available human, chimpanzee, and rhesus macaque genome sequences (Supplementary Table 2). Gene-expression quantification was carried out in frontal and temporal cortex samples of 11 humans (Hs1, Hs2, Hs6–14), 5 chimpanzees (Pt4–8), 5 rhesus macaques (Mm1–3, Mm10, Mm11), and 5 pigtail macaques (Mm1–5), and heart samples of 5 humans (Hs15–19), 4 chimpanzees (Pt7–10), and 4 rhesus macaques (Mm12–15). For each sample, PCR amplification of the different thrombospondin genes was done in triplicate and 6 amplifications of the housekeeping gene β-actin were done to control for differences in initial complementary DNA (cDNA) concentration. Real-time RT-PCR results were analyzed using the Sequence Detector and Dissociation Curve programs (Applied Biosystems, Foster City, CA), and the number of cDNA molecules was calculated by comparison with a standard curve of known amounts of the corresponding PCR products, which were quantified with PicoGreen (Invitrogen).

**Western Blot Analysis**

Protein extraction was performed in 1% sodium dodecyl sulfate hypotonic buffer with protease inhibitors from ~100 mg of frontal-pole tissue from 3 humans (Hs2, Hs6, Hs17), 3 chimpanzees (Pt4, Pt6, Pt7), and 3 rhesus macaques (Mm2, Mm8, Mm9), and 60–120 μg of protein were electrophoresed through NuPAGE Novex 3–8% Tris-acetate gels and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen). Blots were blocked in 10% horse serum (for goat antibodies) or 5% nonfat powdered milk (for mouse and rabbit antibodies) and incubated with the primary antibody overnight at 4 °C. We used 2 different antibodies against THBS2, one monoclonal (Cat. no. 611150, BD Transduction Laboratories, San Jose, CA, 1:200 dilution) and one polyclonal (Cat. no. AF1635, R&D Systems, Minneapolis, MN, 1:200 dilution), and 3 polyclonal antibodies against THBS4 (Cat. no. 12D6, 15C11, and 4H9) (Enard et al. 2002; Khaitovich et al. 2005). Arrays employing the same platform (HG-U95A or HG-U133) were then normalized together by quantile normalization using the ImageQuant program (Molecular Dynamics, Buckinghamshire, UK). To obtain more accurate measurements, 3 different Western blots were analyzed, and thrombospondin protein levels were normalized by the β-tubulin average of each sample to control for loading differences.

**In Situ Hybridization**

In situ hybridization of THBS4 mRNA was carried out in both fixed and unfixed 16-μm-thick sections from the frontal polar cortex of 7 humans (Hs1, Hs6, Hs20–24), 6 chimpanzees (Pt11–16), 3 rhesus macaques (Mm16–18), and one pigtail macaque (Mm6) using methods detailed by O’Neil et al. (2004). Hybridization was performed overnight at 58 °C under high stringency conditions with 60% formamide and 2.5 ng/μl of digoxigenin-labeled riboprobes. Probes were generated by in vitro transcription from a 777-bp fragment of the 3’ coding region of the *THBS4* mRNA, which was amplified by RT-PCR using primers including a T7 and SP6 promoter (Supplementary Table 2). To control for nucleotide differences between the target sequences of each species, probe template was obtained from equimolar amounts of the PCR-amplified products of a human, a chimpanzee, and a rhesus macaque pooled together. After hybridization, sections were subjected to high stringency washes and to RNase digestion to eliminate any unbound probe. The bound probe was detected by an enhanced colorimetric reaction using an alkaline phosphatase-conjugated anti-digoxigenin antibody (De Block and Debrouwer 1993). Sections were then counterstained with Neutral Fast Red (Vector Laboratories, Burlingame, CA) or immunostained as described below with glial fibrillary acidic protein (GFAP) antibody (Cat. no. Z0334, Dako, Carpinteria, CA, 1:1000 dilution). Signal specificity was assessed by comparing sections hybridized with antisense and sense probe, the latter serving as a negative control.

**Immunohistochemistry**

Immunostaining for THBS4 protein was carried out on 50-μm-thick fixed sections and 5-μm-thick paraffin-embedded sections from FP of 11 humans (Hs1, Hs6, Hs21–29), 4 chimpanzees (Pt13–16), 4 rhesus macaques (Mm16–19), and one pigtail macaque (Mm6). After inactivation of endogenous peroxidase and blocking, sections were incubated in primary antibody for 1 h and stained with diaminobenzidine (DAB) solution using the LSAB+ kit (Dako). Selected sections were counterstained
for Nissl substance for analysis of the laminar localization of immunolabeling. Based on trial studies, we used the following dilutions of the same polyclonal antibodies against THBS4 used for Western blotting: R&D Systems antibody, 1:20 and 1:25; Santa Cruz Biotechnology antibody, 1:40; and Lawler 1259 antibody, 1:500. All 3 antibodies gave qualitatively similar patterns of staining, although results are illustrated with sections labeled with the R&D Systems antibody, which appeared to have the greatest specificity for THBS4 in the Western blots (Supplementary Fig. 1). No specific labeling was observed in the 3 types of negative controls that were carried out: omission of the primary antibody, substitution of nonimmune IgG at concentrations matched to the primary antibody, and matched serum controls. For double immunohistochemistry of THBS4 and β-amyloid (Aβ), sections were first incubated with the anti-THBS4 antibody and reacted with DAB. Then, sections were incubated with a mouse monoclonal antibody specific for amino acids 17-24 of the Aβ peptide (clone 4G8, Signet Laboratories, 1:2500 dilution) and reacted with nickel-enhanced DAB (Vector Laboratories).

Results

Brain Expression of Thrombospondin Genes in Primates

To investigate thrombospondin expression patterns in human and chimpanzee brains, we first analyzed a combined set of the available microarray data from 6 different cortical and subcortical brain regions, after excluding all the oligonucleotide probes with sequence differences between the 2 species. Of the 5 thrombospondin genes, only THBS2, THBS4, and COMP met the criteria for minimum detectable expression in at least one of these regions. In the array data, COMP had equivalent expression levels in the different brain regions of both species. Consistent with previous results (Cáceres et al. 2005), THBS4 showed significantly higher levels in humans compared with chimpanzees in most cortical areas and the caudate nucleus (mean relative difference = 5.7) but not in the cerebellum (Fig. 1). THBS2 expression levels were also higher in humans than in chimpanzees in all brain regions examined except the cerebellum (Fig. 1), although the difference between species was smaller (mean relative difference = 2.3). When the variation in THBS4 and THBS2 expression levels across brain regions was analyzed, chimpanzees showed similar transcript levels of both genes throughout the brain (Fig. 1). In humans, however, THBS2 expression in every cortex and caudate region was significantly higher than in the cerebellum (pairwise Mann–Whitney tests: \( P < 0.05 \)), with a 3.7-fold difference on average. Likewise, there were significant differences in THBS2 expression between all forebrain regions and the cerebellum (pairwise Mann–Whitney tests: \( P < 0.05 \)), except for the anterior cingulate cortex, with a 2.0-fold difference on average. Therefore, although the numbers of individuals and brain regions available for analysis are limited, these results suggest an evolutionary upregulation of THBS4 and THBS2 in forebrain regions in adult humans.

To quantify gene-expression differences more accurately, transcript levels of thrombospondins in frontal and temporal cortex were measured by real-time RT-PCR using RNA samples from multiple adult humans, chimpanzees, and macaques. To normalize for differences in RNA content between samples, we used as a control a housekeeping gene, β-actin (ACTB), and quantified expression levels of thrombospondin relative to ACTB expression. In human cortex, there were on average 3.3–4.4 copies of THBS2 and THBS4 and 1.3–2.3 copies of THBS1 and THBS3, respectively, per \( 10^5 \) ACTB copies (Fig. 2). In contrast to the microarray probe data, however, COMP showed much lower expression levels in all species (0–0.5 COMP/\( 10^5 \) ACTB copies) and was virtually absent in humans (Fig. 2). Thrombospondin expression in the different cortical areas and different primate species was compared using repeated-measures 2-factor analyses of variance (ANOVAs) (Table 1). For all genes, there was a good correlation of transcript levels in frontal and temporal cortex across individuals of the 4 species, with no significant effect of cortical region. However, for THBS2, THBS3, THBS4, and COMP, significant differences in gene-expression levels between species were found. THBS4 and THBS2 displayed similar expression patterns in the RT-PCR as in the microarrays, with significantly more mRNA molecules present in humans than in chimpanzees and macaques in both frontal and temporal cortex (Bonferroni post hoc tests: \( P < 0.0001 \)), and no significant differences between the nonhuman primates (Fig. 2 and Supplementary Fig. 2). As in the microarray results, THBS4 showed the largest species differences with RT-PCR, humans exhibiting 6.7- and 17.9-fold higher levels than chimpanzees and macaques, respectively, whereas THBS2 expression was 2.0- to 5.6-fold higher in humans than in the other species (Fig. 2). To confirm that these differences were due to increased expression in humans, we reconstructed ancestral gene-expression levels (Supplementary Fig. 3). For both genes, the expression values estimated for the human-chimpanzee ancestor in frontal and temporal cortex fell outside the 95% confidence intervals of the human data, indicating that expression upregulation occurred in the human lineage following its separation from the chimpanzee lineage. Conversely, for
neither THBS4 nor THBS2 was there evidence of significant expression change in the lineage leading from the human-chimpanzee ancestor to modern chimpanzees.

Table 1
Repeated-measures 2-factor ANOVA of thrombospondin mRNA expression levels in frontal (FCx) and temporal (TCx) cortex of several primate species

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species effect</th>
<th>Cortical region effect</th>
<th>Interaction</th>
<th>FCx versus TCx</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>df</td>
<td>P</td>
<td>F</td>
</tr>
<tr>
<td>THBS1</td>
<td>0.46</td>
<td>3,22</td>
<td>0.7152</td>
<td>2.00</td>
</tr>
<tr>
<td>THBS2</td>
<td>25.90</td>
<td>3,22</td>
<td>&lt;0.0001</td>
<td>1.22</td>
</tr>
<tr>
<td>THBS3</td>
<td>28.33</td>
<td>3,22</td>
<td>&lt;0.0001</td>
<td>3.72</td>
</tr>
<tr>
<td>THBS4</td>
<td>32.29</td>
<td>3,22</td>
<td>&lt;0.0001</td>
<td>2.17</td>
</tr>
<tr>
<td>COMP</td>
<td>5.07</td>
<td>3,22</td>
<td>0.0081</td>
<td>2.67</td>
</tr>
<tr>
<td>All combined</td>
<td>13.22</td>
<td>3,22</td>
<td>&lt;0.0001</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Figure 2. Quantification of mRNA expression levels of the 5 thrombospondin genes in frontal and temporal cortex of different primate species by real-time RT-PCR. Graphs represent the average number of copies of each thrombospondin (THBS) mRNA for 10^3 B-actin (ACTB) mRNA copies in the y axis, with the standard error indicated by error bars. Species included were humans (Hs), chimpanzees (Pt), rhesus macaques (Mn), and pigtail macaques (Mn). Expression levels in each individual sample are provided in Supplementary Figure 2.

Additional gene-expression differences between species were found for THBS3 and COMP (Table 1). THBS3 mRNA was significantly elevated in pigtail macaques compared with rhesus macaques, humans, or chimpanzees (Bonferroni post hoc tests: P < 0.0001), with levels in chimpanzees significantly lower than in the other 2 species (Bonferroni post hoc tests: P < 0.05). COMP transcript was not detected in human cortex and was expressed at low levels in nonhuman primates (Fig. 2), although no significant differences between species were found in the post hoc comparisons. Consistent with this, phylogenetic analysis of gene-expression changes (Supplementary Fig. 3) suggests that THBS3 was downregulated in the lineage leading to chimpanzees and upregulated in the lineage leading to pigtail macaques, and that COMP was downregulated in the human lineage (although the difference was statistically significant only for frontal cortex). Despite these other changes, when the expression levels of the 5 genes were combined, humans showed significantly more total thrombospondin mRNA in the cortex than the other primates (Table 1; Bonferroni post hoc tests: P < 0.01).

Expression levels of each gene were generally very consistent between individuals of the same species (Supplementary Fig. 2). Nevertheless, to make sure that differences in thrombospondin expression levels between species were not affected by unequal sex or age sample composition, we examined the effect of these factors in humans, the species for which we had the largest sample. No significant differences in transcript levels between males (n = 6) and females (n = 5) were found for any of the thrombospondins genes. Also, there were no significant differences in THBS2 and THBS4 expression levels between the young (30–31 years, n = 3), middle-aged (40–48 years, n = 5), or old (74–87 years, n = 3) individuals, although both THBS1 and THBS3 did show slightly higher levels in young versus middle-aged or old humans.

THBS4 and THBS2 Expression in Nonbrain Tissues
To compare expression levels of THBS4 and THBS2 between humans and chimpanzees in tissues other than brain, we analyzed the available microarray data from heart, kidney, liver, and testis (Khaitovich et al. 2005). The mRNAs of both genes were detected in most of these tissues, with the exception of kidney and liver, where THBS4 was not expressed. In contrast to forebrain regions, nonbrain tissues had comparable expression levels of THBS4 and THBS2 in humans and chimpanzees and there were no significant differences between species (Fig. 3A). The only exception was testis, which showed significantly higher levels of THBS4 (1.7-fold) and THBS2 (1.4-fold) in humans than in chimpanzees (Fig. 3A). We performed additional real-time RTPCRs of THBS4 and THBS2 in heart samples from humans, chimpanzees, and rhesus macaques (Fig. 3B). Consistent with the microarray results, humans and chimpanzees had similar
numbers of mRNA molecules of the 2 genes in heart, and considerably more than in cortex (20–60 \( THBS/10^3\) \( ACTB\) copies). In macaques, however, \( THBS4\) and \( THBS2\) expression levels in heart were lower than in humans and chimpanzees (Mann–Whitney tests: \( P < 0.05\)).

**Differences in \( THBS4\) and \( THBS2\) Protein Levels in the Brain**

To determine whether the observed upregulation of \( THBS4\) and \( THBS2\) in cerebral cortex results in increased protein levels, we carried out Western blot analysis with samples from the FP of humans, chimpanzees, and macaques (Fig. 4). Comparable results were obtained with 3 different antibodies for \( THBS4\). Well-defined bands at the expected molecular weight (103.5 kDa) appeared in the human samples in all cases, whereas only very faint bands corresponding to \( THBS4\) protein were detected in the nonhuman primates (Fig. 4A). Average \( THBS4\) protein levels in humans were significantly higher than in nonhuman primates (Fig. 4B), with approximately an 8.0-fold and 30.8-fold increase compared with chimpanzees and macaques, respectively (Mann–Whitney tests: \( P < 0.05\)). Similarly, in Western blots for \( THBS2\), we observed the expected band of 129.0 kDa in the 3 species (Fig. 4B), and \( THBS2\) protein levels in humans were 1.4- to 1.5-fold higher than those of chimpanzees and macaques (Mann–Whitney tests: \( P < 0.05\)) (Fig. 4B).

**Localization of \( THBS4\) mRNA and Protein in Frontal Cortex**

To further characterize the evolutionary changes in thrombospondin expression, we examined the distribution of gene products by in situ hybridization and immunohistochemistry in sections from frontal cortex. We focused on \( THBS4\), the thrombospondin showing the greatest expression differences between humans and nonhuman primates.

In situ hybridization of \( THBS4\) mRNA yielded qualitatively similar labeling patterns in the frontal cortex of humans, chimpanzees, and macaques (Fig. 5A–C). In all species, \( THBS4\) expression was predominantly neuronal, and numerous stained cell bodies with the distinctive morphology of pyramidal neurons were present (Fig. 5D–E). Astrocytes and oligodendrocytes were also labeled, however. Double labeling by in situ hybridization for \( THBS4\) transcript and immunocytochemistry for GFAP, a selective marker for astrocytes (Eng et al. 2000), revealed large \( THBS4\)-expressing astrocytes in layer 1 and deeper layers (Fig. 6A), as well as numerous GFAP-negative oligodendrocytes in the white matter (Fig. 6B). Finally, we observed labeled endothelial cells in blood vessel walls within the cortical gray matter (Fig. 6C). Therefore, \( THBS4\) mRNA was expressed by various cell types in the cortex of adult primates, and there were no obvious differences in the distribution of \( THBS4\)-expressing cells between species.

Consistent with the pattern of mRNA expression, immunohistochemistry with multiple \( THBS4\) antibodies in humans, chimpanzees, and macaques labeled numerous cortical pyramidal cells (Fig. 7), along with some glial cells, which were especially evident in layer 1 and in the white matter, as well as endothelial cells associated with blood vessels. Significantly, however, all the humans we examined consistently exhibited much denser labeling of the neuropil surrounding cell bodies in the gray matter than did chimpanzees or macaques (see especially Fig. 7A–C). This intense human neuropil staining was not observed with any of the different negative control procedures employed.

In addition, 2 humans (Hs21 and Hs25), aged 41 and 78 years, respectively, and classified as normal controls by the supplying institution, exhibited small, dark patches of \( THBS4\) immunoreactivity in the gray matter that resembled the amyloid-rich plaques that accumulate in human aging and especially in AD. Subsequent \( THBS4\) immunostaining of frontal and hippocampal cortex samples from 3 Alzheimer’s patients (Hs27–29) revealed even more numerous plaque-like patches than the normal cases (Fig. 8A). To confirm that \( THBS4\) immunoreactivity was associated with the \( \beta\)-amyloid (A\( \beta\)) plaques, we double labeled frontal and hippocampal tissue sections from Hs21 and the AD cases with \( THBS4\) antibodies and with antibody 4G8, a specific marker for A\( \beta\)-containing plaques (Wisniewski et al. 1989) (Fig. 8B). As shown in Figure 8C, patches that were strongly stained for \( THBS4\) were also immunoreactive for A\( \beta\).

**Discussion**

Our comparative studies of humans, chimpanzees, and macaques indicate that the expression of 2 thrombospondin
genes, \textit{THBS4} and \textit{THBS2}, and their corresponding proteins, was increased in the forebrain during human evolution. Additionally, we found that \textit{THBS4} is expressed by large numbers of neurons and glia in the frontal cortex of all these species but that humans exhibit unusually strong staining for \textit{THBS4} protein in the synapse-rich neuropil. This is the first study to relate changes in gene-expression levels in human evolution to changes in the localization of proteins within the brain, and illustrates how comparative genomics can guide the discovery of distinctive features of human brain organization.

\textit{Evolutionary Upregulation of THBS4 and THBS2 Expression in Adult Human Forebrain}

Gene-expression changes have long been thought to play an important role in determining the phenotypic differences between humans and chimpanzees (King and Wilson 1975). One possible evolutionary advantage of regulatory changes versus changes in gene-coding sequences is that the former could affect one or a few tissues, instead of all the tissues where the gene is expressed, with the less widespread effects resulting in fewer deleterious consequences for the organism (Carroll 2005). Data on thrombospondin gene-expression levels, although currently available for a relatively small set of brain regions and nonbrain tissues, suggest that the human upregulation of \textit{THBS4} and \textit{THBS2} involved primarily the brain, and particularly the forebrain.

Several mechanisms could account for the observed increase in \textit{THBS4} and \textit{THBS2} gene products. Sherwood et al. (2006) have found that frontal cortex has a 46% higher glia/neuron ratio in humans than in chimpanzees, and suggest that this difference could account for some of the gene-expression changes reported by comparative microarray studies. An increase in the proportion of cells expressing \textit{THBS4} and \textit{THBS2} is unlikely to account for much of the evolutionary changes in the expression of these genes, given the magnitude of the expression changes (at least 2-fold for \textit{THBS2} and 6-fold for \textit{THBS4}). Furthermore, inspection of sections labeled for \textit{THBS4} mRNA (Fig. 5) or protein (Fig. 7) does not indicate major differences in the numbers or types of cells expressing \textit{THBS4} in humans, chimpanzees, and macaques. This suggests that the larger amounts of \textit{THBS4} gene products detected in the forebrain of humans compared with nonhuman primates result primarily from the regulation of gene expression at the cellular level.

Higher levels of transcription could be achieved by gene duplication in the human lineage. Sequencing data, however, indicate that \textit{THBS4} and \textit{THBS2} are present only in single copies in the human genome, at 5q14 and 6q27, respectively (http://genome.ucsc.edu/). Therefore, we consider that the increase in \textit{THBS4} and \textit{THBS2} expression in humans likely involved regulatory changes in a cis-acting motif or a transcription factor, and that the regulatory change may have affected transcription of both genes. This is suggested by the similarity of \textit{THBS4} and \textit{THBS2} expression patterns across humans and chimpanzees in the brain (Fig. 1) and in nonbrain tissues (Fig. 3), and by the fact that the upregulation of both genes involves a restricted set of tissues (cortex, caudate, and testis, among the tissues we examined). Previous studies have already shown that regions of the adult mouse brain with a common origin during development, such as the forebrain, tend to share similar gene-expression patterns (Zapala et al. 2005), and that in humans and mice the tissue with gene-expression patterns most similar to the forebrain is the testis (Guo et al. 2005; Shyamsundar et al. 2005).

\textit{Cellular Localization and Synaptogenic Effects of Thrombospondins}

We have found that \textit{THBS4} is expressed by multiple cell types in the frontal cortex of humans and nonhuman primates. The neuronal cell body and neuropil localization of \textit{THBS4} is consistent with data from the developing and adult mouse brain (Arber and Caroni 1995). We also observed some \textit{THBS4}-expressing endothelial cells associated with cortical blood vessels, as reported by Stenina et al. (2003). Unlike previous studies, however, we noted a large number of \textit{THBS4}-expressing glia,

\textbf{Figure 4.} Quantification of \textit{THBS4} and \textit{THBS2} protein levels in primate frontal cortex by Western blot analysis. \textit{A} Western blot results for \textit{THBS4} (103.5 kDa), \textit{THBS2} (129.0 kDa), and \textit{β}-tubulin (\textit{TUBB}) (49.8 kDa) using FP samples of 3 humans (Hs), 3 chimpanzees (Pt), and 3 rhesus macaques (Mm). For each protein, one representative blot is shown on top and the average band intensity from the 3 different blots quantified is shown below. In each blot, band intensities were normalized to those of one human case (Hs2). \textit{B} Average \textit{THBS4} and \textit{THBS2} protein levels in the FP of humans, chimpanzees, and rhesus macaques. The y axis corresponds to the average band intensities of the 3 individuals of each species relative to the human value, after normalization by \textit{TUBB} levels to control for protein loading differences. Error bars represent standard errors. Asterisks indicate the results of the Mann-Whitney test for the comparison of thrombospondin levels between humans and nonhuman primates. * \textit{P} < 0.05.
including both astrocytes and oligodendrocytes. The major histological difference between humans, chimpanzees, and macaques was the higher density of THBS4 protein labeling in the neuropil of humans, consistent with a role for thrombospondins in synapse formation. The neuropil consists of an intricate web of fine neuronal presynaptic and postsynaptic processes ensheathed by fine astrocytic processes (Derouiche et al. 2002), although it is not possible to determine from our present material whether the fine neuropil processes labeled with THBS4 antibody are of glial or neuronal origin (or both).

Evolutionary increases in levels of THBS4 and THBS2 could have important consequences for human synaptic and neuritic organization. Experimental results in model systems (Arber and Caroni 1995; Christopherson et al. 2005; Eroglu et al. 2005) suggest that increased thrombospondin levels could enhance plastic changes in the adult human cortex by contributing to a higher density of synapses, a higher rate of synaptic turnover, increased rates of neurite growth, or some combination of these. There are currently no data bearing on synaptic densities or rates of synaptic turnover or neurite growth in the cortex of humans compared with chimpanzees or other nonhuman primates. However, compared with macaques and other monkeys, the pyramidal neurons of human cortex are reported to have more complexly branched dendrites and a higher density of dendritic spines (Duan et al. 2003; Elston et al. 2006), spines being the sites where excitatory axon terminals synapse upon pyramidal cells. There is also genomic evidence suggesting that additional genes that could play roles in synaptic turnover and neurite growth were upregulated in human brain evolution.

Figure 5. Histological localization of THBS4 mRNA in primate frontal cortex by in situ hybridization. (A–C) Low-magnification photomicrographs of the frontal polar cortex of a human (A), a chimpanzee (B), and a rhesus macaque (C), showing the hybridization of the THBS4 antisense probe in unfixed, snap-frozen sections. (D–F) High-magnification photomicrographs of fixed tissue sections showing numerous pyramidal cells labeled for THBS4 mRNA in cortical layer 3 of a human (D) and a chimpanzee (E). Scale bars: (A–C) 250 μm; (D–F) 50 μm.

Figure 6. THBS4 mRNA expression in various cell types of human frontal cortex. (A–B) Gray and white matter sections double labeled by in situ hybridization for THBS4 mRNA (blue staining) and by immunocytochemistry for the astrocyte-specific marker GFAP (brown staining). (A) In gray matter, arrowheads denote THBS4-expressing astrocytes in layer 1 and in deeper layers of the cortex. Not all GFAP-immunoreactive cells were clearly labeled with the THBS4 antisense probe, however. The cells in layers 2 and 3 exhibiting blue label only were probably neurons. (B) In the white matter (WM), a large population of small cells labeled strongly for THBS4 mRNA but did not stain for GFAP (arrowheads). Based on their number and size, these were probably oligodendrocytes. (C) Endothelial cells in the wall of a cerebral blood vessel labeled by in situ hybridization for THBS4 mRNA are indicated by arrowheads. The red coloration of the vessel is from blood. Scale bars: (A–B) 50 μm; (C) 10 μm.
(Preuss et al. 2004). Evolutionary changes in molecular systems leading to greater plasticity in adult cortex could have profound effects on human cognition, by, for example, enhancing the ability to reorganize cortical networks in response to novel external circumstances or neurological insults (Neville and Bavelier 2002; Pascual-Leone et al. 2005).

**Thrombospondins, Aβ, and AD**

Our results indicate that THBS4 protein accumulates in Aβ-rich plaques in the frontal and hippocampal cortex of individuals with AD and nondemented control cases. A previous study reported that thrombospondin localizes to senile plaques in individuals with AD (Bué et al. 1992). Only 2 thrombospondins, THBS1 and THBS2, were well known at that time, however, and it is unclear which thrombospondins were labeled by the antibodies used in that study. Although the localization of thrombospondins to amyloid plaques does not necessarily implicate them in the pathogenesis of AD, the possibility that increased thrombospondin expression contributes to human vulnerability to AD deserves serious consideration. Aged nonhuman primates can develop amyloid plaques (Gearing et al. 1994; Poduri et al. 1994; Elfenbein et al. 2006), and aged squirrel monkeys have Aβ levels comparable with humans with AD (Rosen et al. 2006), yet as far as we know, AD occurs only in the human species (Walker and Cork 1999; Olson and Varki 2003). It is thus possible that one or more molecules that were modified in human evolution enhance Aβ toxicity in humans, and THBS4 and THBS2 could be among those molecules. Interactions between thrombospondins and Aβ have not been studied in detail but thrombospondin is reported to bind to Aβ fibrils (Kowalska and Badellino 1994) and Aβ and thrombospondins share a number of cell-surface receptors (Bamberger et al. 2003). Furthermore, THBS4 is thought to have proinflammatory effects by binding to the Mac-1 receptor (Pluskota et al. 2005), which is expressed by macrophages and microglia (Rotshenker 2003). Neuroinflammation is considered by some to contribute to Aβ neurotoxicity in AD and other neurodegenerative diseases (Schwab et al. 2004; Mrak and Griffin 2005). Finally, it is noteworthy that the upregulation of THBS4 and THBS2 in human evolution occurred in the cortex but not the cerebellum, for although the cerebellum is commonly a site of Aβ deposition in AD, it rarely shows the neurofibrillary tangles characteristic of the cortex (Braak et al. 1989).
Notes

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Supplementary Data

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

References


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