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Changes in alternative brain-derived neurotrophic factor transcript expression in the developing human prefrontal cortex

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Abstract

In this study, we determined when and through which promoter brain-derived neurotrophic factor (BDNF) transcription is regulated during the protracted period of human frontal cortex development. Using quantitative real-time polymerase chain reaction, we examined the expression of the four most abundant alternative 5′ exons of the BDNF gene (exons I, II, IV, and VI) in RNA extracted from the prefrontal cortex. We found that expression of transcripts I–IX and VI–IX was highest during infancy, whereas that of transcript II–IX was lowest just after birth, slowly increasing to reach a peak in toddlers. Transcript IV–IX was significantly upregulated within the first year of life, and was maintained at this level until school age. Quantification of BDNF protein revealed that levels followed a similar developmental pattern as transcript IV–IX. *In situ* hybridization of mRNA in cortical sections showed the highest expression in layers V and VI for all four BDNF transcripts, whereas moderate expression was observed in layers II and III. Interestingly, although low expression of BDNF was observed in cortical layer IV, this BDNF mRNA low-zone decreased in prominence with age and showed an increase in neuronal mRNA localization. In summary, our findings show that dynamic regulation of BDNF expression occurs through differential use of alternative promoters during the development of the human prefrontal cortex, particularly in the younger age groups, when the prefrontal cortex is more plastic.

Introduction

Brain-derived neurotrophic factor (BDNF) belongs to the nerve growth factor family of neurotrophins, which also includes nerve growth factor, neurotrophin-3, and neurotrophin-4. It was first isolated from porcine brain (Barde *et al.*, 1982), and has since been shown to support the survival, growth and differentiation of new neurons during early neural development, as well as maintaining neuronal populations and connections later in life in many different species. BDNF has been detected in the adult mammalian hippocampus, cortex, cerebellum, and basal forebrain, areas that are vital for learning, memory, and higher cognitive function (Timmusk *et al.*, 1993; Lauterborn *et al.*, 1996; Weickert *et al.*, 2003; Sathanoori *et al.*, 2004; Pruunsild *et al.*, 2007). Studies in the developing rat and mouse forebrain showed that BDNF mRNA is most highly expressed in the hippocampus, is robustly expressed in the cortex, but is expressed at very low levels or not at all in the striatum (Hofer *et al.*, 1990; Timmusk *et al.*, 1993, 1994a; Dennis & Levitt, 2005).

During development, BDNF expression is more abundant in the brain than in other tissues, and it shows a steady increase during postnatal life (Ernfors *et al.*, 1990; Hofer *et al.*, 1990; Wetmore *et al.*, 1990; Friedman *et al.*, 1991; Timmusk *et al.*, 1994a). Animal studies have demonstrated that BDNF plays a particularly important role postnatally during critical periods of cortical development, when it is required for the formation of ocular dominance columns in the visual cortex (Cabelli *et al.*, 1997; Mandolesi *et al.*, 2005). Although less is known about the role of BDNF during human cortical development, we have previously reported differential postnatal patterns of BDNF mRNA expression in distinct functional regions of the human brain (Webster *et al.*, 2002, 2006). In the dorsolateral prefrontal cortex (DLPFC), expression levels of BDNF mRNA and its high-affinity receptor, trkB-TK+, are significantly increased in the teenager/young adult period, but both can show a significant decline in the elderly (Romanczyk *et al.*, 2002; Webster *et al.*, 2002, 2006; Weickert *et al.*, 2003). Whereas BDNF mRNA expression did not change significantly

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during development in the hippocampus and primary visual cortex, BDNF and trkB-TK+ mRNA levels were highest during the neonatal period in the temporal cortex (Webster *et al.*, 2002, 2006). However, the molecular control of differential temporal and regional expression of BDNF in the human cortex is not well understood. In this study, we determined whether distinct DNA elements could be implicated in the temporal control of BDNF gene expression though differential promoter usage.

The structure of the BDNF gene is complex. The rodent BDNF gene was initially described as consisting of four transcription start sites that produced alternative 5′ noncoding exons spliced onto one common 3′ exon encoding the prepro-BDNF protein, as well as containing two separate polyadenylation sites in the 3′ end of the mRNA (Timmusk *et al.*, 1993). Through this alternative splicing and use of the two alternative polyadenylation sites, eight unique BDNF transcripts can be generated (Timmusk *et al.*, 1993). A recent analysis of the rodent BDNF gene structure by Aid *et al.* (2007) reported the presence of at least eight 5′ noncoding exons (four of which are novel) and one common 3′ coding exon. The structure of the human BDNF gene is continually being elucidated. The most recent description of the human BDNF gene structure added five additional 5′ exons/transcription start sites (Pruunsild *et al.*, 2007) to the eight already described by Liu *et al.* (2005), and reported the existence of endogenous antisense BDNF transcripts (Pruunsild *et al.*, 2007). The generation of a number of alternative 5′ transcripts enables upregulation of BDNF in response to distinct environmental demands, and may allow for differential subcellular targeting as well as region-specific expression in the brain (Pattabiraman *et al.*, 2005; Malkovska *et al.*, 2006; Pruunsild *et al.*, 2007; Chiaruttini *et al.*, 2008). For example, in rodents, BDNF transcript III–V (human: IV–IX) expression has been reported to vary diurnally (Berchtold *et al.*, 1999); moreover, administration of certain epileptic-inducing and antidepressant drugs to rodents can enhance global or specific expression of BDNF transcripts in the hippocampus (Timmusk *et al.*, 1993, 1994b; Dwivedi *et al.*, 2006; Chiaruttini *et al.*, 2008). Previous studies found anatomically restricted expression of different BDNF alternative transcripts in the brain (Timmusk *et al.*, 1993; Liu *et al.*, 2006; Aid *et al.*, 2007), and recently, different transcripts have been shown to be targeted to particular subcellular regions of neurons in the rodent and mouse hippocampus (An *et al.*, 2008; Chiaruttini *et al.*, 2008).

Although previous studies have reported developmental changes in human BDNF expression, changes in specific BDNF transcripts have yet to be described. The human DLPFC is important for abstract thinking, working memory, and planning ability, and has a protracted period of development and maturation, lasting up to two decades of life in humans (Giedd *et al.*, 1999; Sowell *et al.*, 1999). Thus, by establishing when specific BDNF promoters are used during the development of the frontal cortex, we can begin to elucidate the differential mechanisms involved in BDNF expression at different phases of human life. In the current study, we examined the change in gene expression of four conserved BDNF alternative transcripts across postnatal developmental stages in the human DLPFC.

Materials and methods

Cohorts

Sixty cases from normal individuals ranging from 71 days to 49 years in age were obtained from the University of Maryland Brain and Tissue Bank for Developmental Disorders (UMBB; NICHHD contract no. NO1-HD8-3283; 38 males and 22 females, 30 African Americans, 29 Caucasians and one Hispanic). The demographic data for each subject are listed in Table 1. Cohorts were matched for postmortem interval (PMI), brain pH and, as much as possible, for gender. Moreover, the cases within each age group did not differ according to PMI, brain pH, or RNA integrity (RIN) (see below).

RNA extraction and cDNA synthesis

Total RNA was extracted from ∼300 mg of frozen tissue, using a modified version of the TRIZOL Reagent method (Life Technologies Inc., Grand Island, NY, USA), as previously described (Kozlovsky *et al.*, 2004). RIN was assessed with high-resolution capillary electrophoresis (Agilent Technologies, Palo Alto, CA, USA). Four micrograms of total RNA was then used in a 50 μL reverse transcriptase reaction to synthesize cDNA using the SuperScript First-Strand Synthesis System for real-time polymerase chain reaction (RT-PCR) (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol.

Quantitative RT-PCR (qPCR)

Transcript levels in brain were measured by qPCR using an ABI Prism 7900 sequence detection system with a 384-well format. Control probes or housekeeping genes used to calculate the geometric mean were chosen from Applied Biosystems (Assays-on-Demand; Applied Biosystems, Foster City, CA, USA), and included cyclophillin A (cat. no. hs99999904-m1), glucuronidase beta (cat. no. hs99999908-m1), and succinate dehydrogenase complex, subunit A flavoprotein (cat. no. hs00188166-m1). The transcripts arising from the four best-characterized BDNF exons (Fig. 1) were chosen, and ABI probes were: BDNF I–IX (cat. no. hs00538277-m1), BDNF II–IX (cat. no. hs00538278-m1), BDNF IV–IX (cat. no. hs00380947-m1), and BDNF VI–IX (cat. no. hs00156058-m1). Each 10-μL PCR reaction contained 3 μL of cDNA, 0.5 μL of 20× primer/probe mixture, 5 μL of RT-PCR Mastermix Plus (Eurogentec, Seraing, Belgium) containing Hot Goldstar DNA Polymerase, dNTPs with dUTP, uracil-*N*-glycosylase, passive reference, and optimized buffer components, and 1.5 μL of diethyl pyrocarbonate deionized water. For transcripts I–IX, II–IX, and IV–IX, 3 ng/μL cDNA was used for the amplification. For transcript VI–IX, 30 ng/μL was used in the amplification, as transcript VI–IX is less abundant. Samples were run with an eight-point standard curve, using serial dilutions of pooled cDNA derived from RNA obtained from brain tissue (pooled from all cases). Several no-template controls, which produced no signal, were also included. PCR cycling conditions were: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 59°C or 60°C for 1 min. PCR data were obtained with sequence detector software (SDS version 2.0; Applied Biosystems). SDS software plotted real-time fluorescence intensity. The threshold was set within the linear phase of the amplicon profiles. Measurements for all samples were performed in triplicate. The geometric mean of the three housekeeping genes used was calculated as described in Vandesompele *et al.* (2002). None of the housekeeping genes varied across development (ANOVA, $P > 0.05$ for all housekeeping genes and geometric mean). The 2-ΔΔCT method was used for the analysis of gene expression (Livak & Schmittgen, 2001), where the adult group was set as the control group to which other developmental groups were normalized.

Western blotting

To measure BDNF protein expression, 40 mg of frozen tissue from the DLPFC was homogenized in 400 μL of homogenization buffer

Table 1. Brain cohort demographics

Group	Age	Gender	Race	pH	PMI	RIN
Neonate	71 days	F	H	6.36	21	5.2
Neonate	76 days	M	AA	6.60	28	9.1
Neonate	56 days	M	C	6.86	11	9.1
Neonate	54 days	M	AA	6.63	17	9.5
Neonate	73 days	M	C	6.12	24	5.5
Neonate	66 days	F	C	6.48	19	7.8
Infant	92 days	F	AA	6.54	14	9.1
Infant	188 days	F	AA	6.82	22	9.0
Infant	175 days	F	AA	6.47	18	7.3
Infant	139 days	M	AA	6.54	9	7.5
Infant	332 days	M	AA	6.87	18	8.6
Infant	198 days	M	AA	6.71	24	9.0
Infant	141 days	M	AA	6.81	5	9.4
Infant	196 days	M	AA	6.17	10	5.6
Infant	245 days	F	AA	6.58	21	7.5
*Infant	332 days	F	AA	6.38	10	
*Infant	118 days	M	C	6.36	19	
*Infant	301 days	M	AA	6.65	18	
Toddler	1 year 211 days	F	C	6.90	24	8.2
Toddler	4 years 232 days	M	C	6.92	18	7.9
Toddler	4 years 313 days	M	AA	6.74	19	9.1
Toddler	2 years 75 days	F	AA	6.89	11	8.3
Toddler	2 years 163 days	F	AA	6.74	22	8.3
Toddler	2 years 171 days	F	C	6.45	20	7.8
Toddler	2 years	M	AA	6.89	13	8.7
*Toddler	2 years 71 days	M	AA	6.64	27	
*Toddler	2 years 273 days	M	AA	6.16	14	
School age	5 years 144 days	M	C	6.74	17	8.6
School age	12 years 154 days	M	C	6.82	16	8.2
School age	12 years 353 days	F	C	6.85	18	8.9
School age	8 years 336 days	F	C	6.41	12	7.2
School age	7 years 306 days	M	AA	6.78	18	6.2
School age	11 years 198 days	F	C	6.44	12	8.6
*School age	6 years 320 days	M	C	6.05	18	
*School age	8 years 2 days	M	C	6.76	5	
*School age	8 years 50 days	F	AA	6.78	20	
Teenage	15 years	M	AA	6.76	13	6.2
Teenage	17 years 180 days	M	C	6.67	16	7.5
Teenage	17 years 300 days	M	C	6.80	12	9.2
Teenage	17 years 251 days	M	AA	6.83	16	8.5
Teenage	17 years 17 days	M	C	6.69	25	8.6
Teenage	17 years 138 days	M	C	6.84	19	7.5
*Teenage	16 years 250 days	F	C	6.81	16	
*Teenage	16 years 125 days	F	C	6.60	20	
Young adult	25 years 37 days	F	C	6.54	32	7.0
Young adult	22 years 185 days	M	C	6.75	12	8.4
Young adult	25 years 137 days	F	C	6.73	16	9.2
Young adult	22 years 334 days	M	AA	6.84	4	9.4
Young adult	21 years 341 days	M	C	6.96	13	8.6
Young adult	20 years 50 days	M	AA	6.50	18	8.6
Young adult	21 years 355 days	M	AA	6.25	7	7.5
Young adult	24 years 338 days	M	C	6.92	7	9.3
Adult	46 years 65 days	M	AA	6.75	18	8.1
Adult	42 years 342 days	M	C	6.49	18	8.2
Adult	35 years 363 days	M	C	6.73	13	8.7
Adult	38 years 231 days	M	AA	6.37	8	8.2
Adult	47 years 162 days	M	C	6.56	12	8.4
Adult	48 years 251 days	F	C	6.12	12	6.9
Adult	38 years 154 days	F	AA	6.98	19	8.2
Adult	49 years 79 days	F	AA	6.78	7	8.9

A, African American; C, Caucasian; H, Hispanic; M, male; F, female; PMI, postmortem interval; RIN, RNA integrity. *RIN values not applicable as samples were used only for protein.

(0.05 M Tris, pH 7.5, 50% glycerol, 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 0.015 mM aprotinin, 0.038 mM leupeptin, 0.030 mM pepstatin A, 0.028 mM E-64, 0.08 mM bestatin). Protein concentrations were determined using the Bradford protein assay (Sigma, Sydney, Australia). Equal amounts of protein (10 μg) were diluted in 2× Laemli buffer, boiled at 95°C for 5 min, and analysed by sodium dodecylsulfate polyacrylamide gel electrophoresis on 12% Bis/Tris gels (BioRad, Sydney, Australia). Proteins were transferred onto nitrocellulose membranes, and incubated with blocking solution [5% (w/v) non-fat milk, 0.1% (v/v) Tween-20 in phosphate-buffered

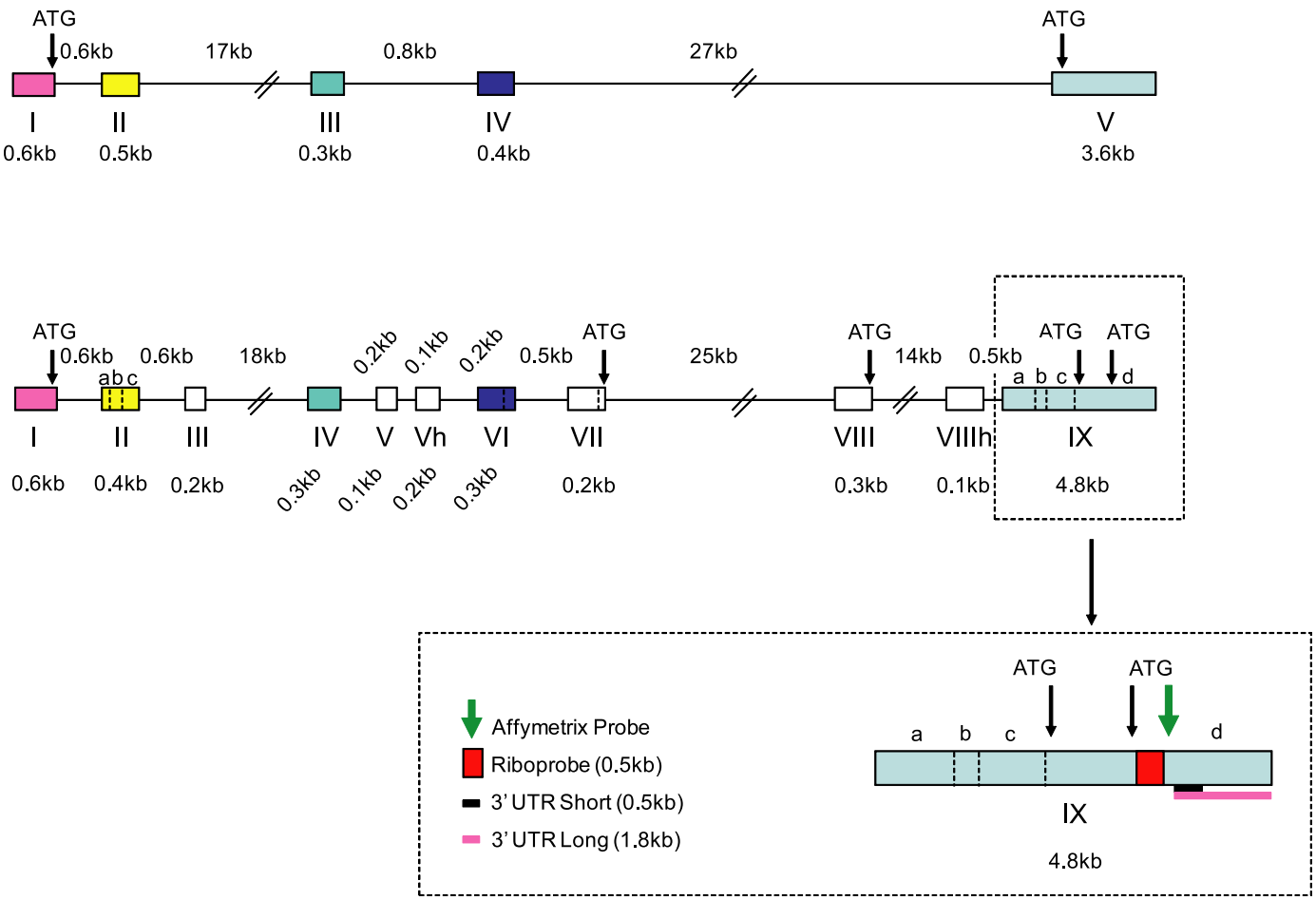

FIG. 1. Brain-derived neurotrophic factor (BDNF) gene structure. Schematic of the BDNF gene as originally described in rodents by Timmusk *et al.* (1993), consisting of four noncoding exons in the 5′-UTR and one 3′ coding exon. The most recent description of the genomic organization and nomenclature of the human BDNF gene (Pruunsild *et al.*, 2007) contains 10 noncoding exons in the 5′-UTR and one 3′ coding exon. The human gene contains multiple translational (ATG) start sites. Exons with the letter h are specific to the human BDNF gene and are not conserved across species. The insert box details the primers/probes used in our current study and previous studies (Webster *et al.*, 2002), as well as the two polyadenylation sites giving rise to short and long BDNF transcripts.

saline (PBST); room temperature; 1 h]. Membranes were incubated with a primary antibody for human BDNF [diluted 1 : 200 in 5% bovine serum albumin (BSA)–PBST; 4°C; overnight] (Santa Cruz, Heidelberg, Germany, cat. no. sc-546) or *β*-actin (diluted 1 : 10 000 in blocking solution; 4°C; overnight) (Chemicon International, Sydney, Australia; cat. no. MAB1501). Membranes were washed three times for 10 min each with PBST, and incubated with peroxidase-conjugated affinity-purified secondary antibodies [anti-rabbit (diluted 1 : 1000 in 5% BSA–PBST for BDNF) or anti-mouse (diluted 1 : 5000 in blocking solution for *β*-actin); room temperature; 1 h] (Chemicon International). After further washing, bound antibodies were incubated with enhanced chemiluminescence reagent (Millipore, Sydney, Australia) and visualized by chemiluminescence on a Versadoc Imaging System (BioRad). Bands were quantitated by densitometry using QUANTITY ONE 1-D analysis software v4.6.5 (BioRad).

Plasmid constructs

For construction of plasmids for riboprobe synthesis, DNA fragments specific for the alternative BDNF transcripts I–IX, II–IX, IV–IX and VI–IX were amplified from cDNA, using the following primer sets:

BDNF I–IX Forward, 5′-AACTTCTCACATGATGACTTC-3′;
BDNF II–IX Forward, 5′-TATCTCCAGGATCTAGCCACC-3′;
BDNF IV–IX-IX Forward, 5′-AGCAGCTGCCTTGATGGTTAC-3′;
BDNF VI-IX Forward, 5′-ATCGGAACCACGATGTGACTCC-3′; and
BDNF Common Reverse, 5′-ATCCAACAGCTCTTCTATCACG-3′.

Plasmids were constructed by annealing PCR products into PCRII using the PCRII TA cloning kit (Invitrogen), according to the manufacturer's instructions. Cloned inserts were verified by sequencing.

Riboprobe synthesis and in situ *hybridization*

To qualitatively determine the distribution of the alternative BDNF transcripts, frozen blocks of the DLPFC were sectioned in the coronal plane at 14 *μ*m thickness, mounted on gelatin-subbed slides, and stored (−80°C), and *in situ* hybridization was performed as previously described but with modifications (Weickert *et al.*, 2003). Briefly, riboprobes were synthesized from linearized plasmids using the SP6/T7 Riboprobe Combination System (Promega, Sydney, Australia), substituting rUTP for [^{35}S]rUTP (Perkin Elmer, Melbourne, Australia). Riboprobes were then purified by ammonium acetate/ethanol precipitation. Thawed sections were fixed briefly in 4% formaldehyde/phosphate-buffered saline, acetylated with acetic anhydride, delipidated in chloroform, and dehydrated in a series of ethanol solutions. *In situ* hybridization was performed overnight in humidified chambers at 55°C with the ^{35}S-labeled

riboprobes ($2.4–4 \times 10^6$ c.p.m. in 200 μL per section or 5 ng/mL) added to the hybridization buffer [50% formamide, 10 mM Tris–HCl (pH 7.5), 600 mM NaCl, 1 mM EDTA, 0.02% Ficoll, 0.02% BSA, 0.02% polyvinylpyrrolidone, 0.1% salmon sperm, 0.05% total yeast RNA, 0.005% yeast tRNA, 0.1% sodium thiosulfate, 25% dextran sulfate, 0.1% sodium dodecylsulfate, 100 mM dithiothreitol]. Coverslips were removed in 2× SSC, and the sections were then incubated with RNaseA solution for 30 min. Sections were washed twice in 2× SSC and incubated for 1 h in 2× SSC at 50°C. They were then rinsed twice with 0.2× SSC and incubated in 0.2× SSC for 1 h each at 55 and 60°C. Sections were then dehydrated in a series of ethanol solutions (containing 300 mM ammonium acetate) and air-dried. The sections were exposed to autoradiographic film (BioMax film; Kodak, Rochester, NY, USA) for 60 days. Images were scanned at 1200 d.p.i. using a CanoScan N676U. For silver grain detection of the four alternative BDNF transcripts, slides were dipped in emulsion (Kodak) following autoradiographic film exposure. Slides were then dried and left in the dark for approximately 4 months. Slides were developed in a D-19 developer (Kodak), and then Nissl-counterstained for nucleic acid.

Statistical analysis

To exclude measurement errors, we conducted outlier detection of the triplicates obtained from the qPCR raw data by determining the percentage variance between each of the replicates. If one replicate showed > 3% variance in cycle threshold (CT), the replicate was removed and duplicate measures were used. If they still showed > 3% variance, the entire sample was removed from the cohort (usually one or two cases and at most three cases, all from different age groups, were dropped). As a test for population outliers, calculated data points for each sample that was greater than two standard deviations from the mean for that age group were also excluded from both mRNA and protein analysis (one case was dropped from analysis of transcript II–IX and two cases from that of transcript VI–IX). Data are presented as mean + SEM. Statistical analyses were conducted using STATISTICA 7 (StatSoft Inc., 2000, STATISTICA FOR WINDOWS, Tulsa, OK, USA). One-way ANOVA was used to assess significance according to age group. BDNF mRNA and protein levels were also correlated with pH, PMI and RIN values to determine whether any relationships among BDNF and demographic variables or sample characteristics existed. ANOVAs were followed up with Fisher least significant difference *post hoc* analysis to assess the significance among the developmental groups. A P-value < 0.05 was considered to be statistically significant.

Results

Demographic variables and mRNA controls

The postmortem pH values ranged between 6.12 and 6.98, and did not vary across the age groups ($F_{6,43} = 1.36$, $P = 0.25$) (Table 1). PMI ranged between 4 and 32 h, and did not significantly differ between the age groups ($F_{6,43} = 1.12$, $P = 0.37$). None of the individually measured 'housekeeper' control mRNAs, including cyclophillin, glucuronidase beta and succinate dehydrogenase complex, differed according to age group (all $F < 0.81$, all $P > 0.05$). Cyclophillin mRNA levels and the geometric mean of the 'housekeeper' mRNAs used for normalization were, as expected, negatively correlated with pH and RIN, showing that the control mRNAs serve as appropriate normalization factors for any individual variation in RNA quality that is related to brain pH and RIN values (Lipska *et al.*, 2006). The geometric mean of the housekeeper mRNAs showed no significant change across development as analysed by one-way ANOVA ($F_{6,43} = 0.72$, $P = 0.64$) (Fig. 2). Gender had no impact on the expression of the housekeeping genes.

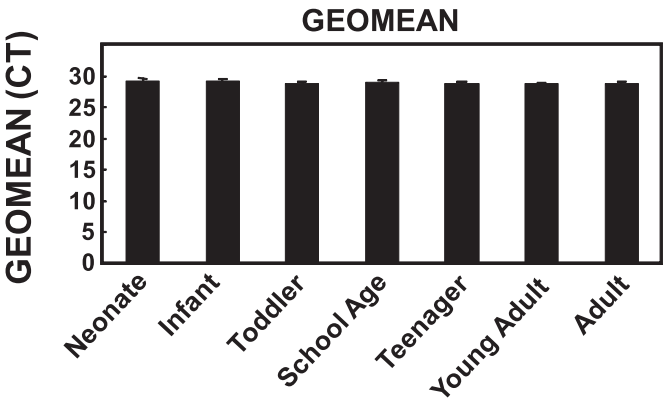

FIG. 2. Expression of the geometric mean (geomean) across development. The geometric mean was calculated from the CT values of the housekeeping genes encoding cyclophillin, glucuronidase beta, and succinate dehydrogenase complex, subunit A. Values are presented as mean + SEM.

Expression of BDNF transcript I–IX is highest during the first year of life

Expression of transcript I–IX changed significantly across development (ANOVA: $F_{6,43} = 6.09$, $P = 0.00011$), higher expression being observed at the earlier stages of life ($P < 0.05$) (Fig. 3A). Gene expression reached a maximum at infancy, and after this stage, expression levels of transcript I–IX subsequently declined ($P < 0.0005$). The level of I–IX mRNA found in the school age group was maintained well into adulthood ($P > 0.05$).

Expression of BDNF transcript II–IX is low at birth and increases during the first few years of life to peak in toddlers

Analysis of transcript II–IX expression across development revealed a unique expression pattern (ANOVA: $F_{6,41} = 2.81$, $P = 0.02$). Expression of transcript II–IX was lowest in the neonate group, but increased gradually during the first few years of life to reach a peak in the toddler group (infants > neonates, $P < 0.05$; toddlers > infants, $P = 0.0002$) (Fig. 3B). Transcript expression was subsequently reduced in the school age group to levels comparable to those of the infants ($P < 0.05$, comparing toddlers to school age), where it was maintained from school age to adulthood (all comparisons, $P > 0.05$).

Expression of BDNF transcript IV–IX is highest in infants and toddlers

Transcript IV–IX expression levels were modulated significantly across the developing DLPFC (ANOVA: $F_{6,43} = 2.52$, $P = 0.035$). Interestingly, changes in gene expression of transcript IV–IX revealed a developmental pattern that was similar in some respects to expression of both transcript I–IX and transcript II–IX, particularly during the earlier points in development. Like that of transcript II–IX, expression of transcript IV–IX was lowest in the neonate group (Fig. 3C). Expression levels then quickly increased within the next 6 months to reach a maximum at infancy ($P = 0.002$), like those of

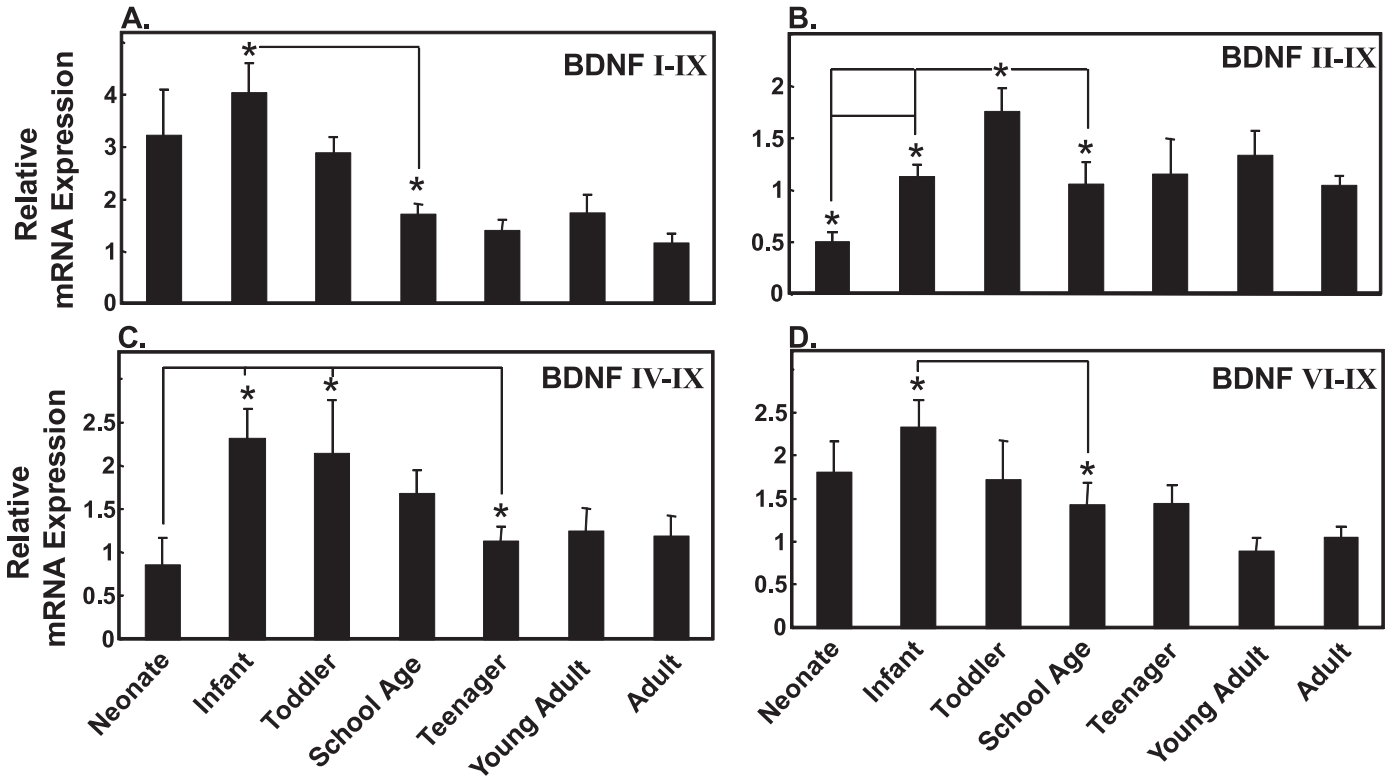

FIG. 3. mRNA expression of alternative brain-derived neurotrophic factor (BDNF) transcripts across development. Expression of (A) transcript I–IX, (B) transcript II–IX (C) transcript IV–IX and (D) transcript VI–IX was measured by quantitative real-time polymerase chain reaction for the seven developmental groups. Data are expressed relative to the adult age group as ΔΔCT, and presented as mean + SEM. Significance between groups was $*P < 0.05$ or lower.

transcript I–IX, and then, as with transcript II–IX, high levels of expression were found in the toddler age group. Transcript IV–IX expression gradually declined, reaching significantly lower levels in the adolescent group than in infants and toddlers ($P < 0.05$), with intermediate expression being seen in the school age group. Adolescent expression levels of IV–IX mRNA were then maintained into adulthood.

Expression of BDNF transcript VI–IX peaks within the first year of life

The gene expression pattern of transcript VI–IX varied significantly across development (ANOVA: $F_{6,39} = 2.93$, $P = 0.018$). During the earlier stages of development, the gene expression profile of transcript VI–IX was very similar to that of transcript I–IX. Expression of transcript VI–IX was highest in infancy ($P < 0.05$), but showed a subsequent developmental decline in the school age group ($P < 0.05$). After the teenage years, transcript VI–IX expression was further attenuated into adulthood (Fig. 3D). In the young adult and adult groups, expression of transcript VI–IX was significantly lower than in the infant group ($P < 0.01$).

Mature BDNF protein levels peak during infant development

In our next approach, we examined the changes in BDNF protein expression across development. We found immunoreactive BDNF bands migrating at about 28 kDa and 14 kDa at all ages. Figure 4A shows the immunoreactive band in the 14 kDa range from a representative western blot, where samples were assayed in a blinded and randomized order. Infants showed the highest levels of BDNF protein expression, followed by toddlers. Internal controls of a 1 : 1 mixture of infant–adult DLPFC homogenates were run as a correction for gel-to-gel variation. β-Actin was used as a loading control, and did not vary across development (ANOVA: $F_{6,43} = 0.79$, $P = 0.59$) (Fig. 4D). Protein expression of the mature form of BDNF varied across development, reaching a peak in infancy (Fig. 4B) (ANOVA: $F_{6,43} = 5.15$, $P = 0.0005$). This trend is consistent with the developmental pattern observed for transcript IV–IX, although the 14 kDa BDNF protein levels were found to correlate with mRNA expression of each individual alternative transcripts ($P = 0.05$) as well. As with transcripts I–IX, II–IX, IV–IX, and VI–IX, an increase in BDNF protein expression was observed from neonates to infants ($P = 0.0001$). Interestingly, the increase in BDNF protein was sustained in the toddler group, even while the I–IX and VI–IX mRNAs were decreasing. This sustained BDNF protein level may be explained by the maintained transcript IV–IX expression or by the increase in transcript II–IX expression in toddlers as compared to infants. Thereafter, protein expression decreased, reaching a plateau in the school age group. Expression further decreased in the adult group, where BDNF protein levels were reduced to levels comparable to those in the neonate group (toddler > adult, $P = 0.002$); this may reflect the fact that all BDNF transcripts appear to be at or near their lowest level of expression in the adult group. Quantitation of the prepro-BDNF (28 kDa) protein (Fig. 4C) showed a developmental pattern similar to that of the mature BDNF protein (ANOVA: $F_{6,52} = 2.40$, $P = 0.04$), with significant correlation ($r = 0.30$, $P = 0.04$), although the magnitude of developmental change was more subtle for prepro-BDNF. The calculated ratio between the prepro-BDNF and mature BDNF proteins did not change significantly according to age group (ANOVA: $F_{6,52} = 0.82$, $P = 0.56$).

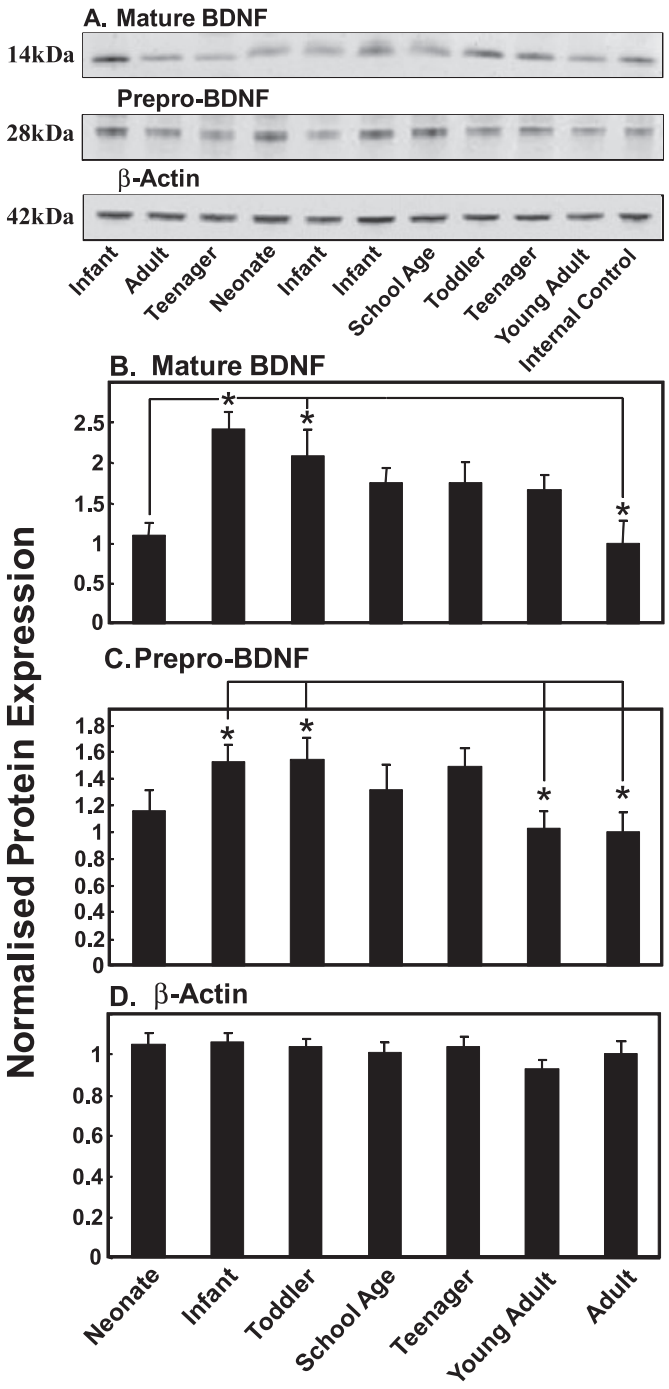

FIG. 4. Expression of brain-derived neurotrophic factor (BDNF) protein across development. (A) A representative western blot is presented, containing the seven developmental groups of interest immunoprobed for the 14 kDa mature BDNF protein and the 28 kDa prepro-BDNF protein. β-Actin was probed as a loading control, and an internal control was used for normalization between western blots. (B–D) The 14 kDa BDNF protein (B), the 28 kDa prepro-BDNF protein (C) and β-actin (D) were quantitated by densitometry. Data are expressed relative to the adult age group, and presented as mean + SEM. Significance between groups was $^*P < 0.05$ or lower.

Pan-BDNF expression by microarray

We found that BDNF mRNA corresponding to the common 3′ coding region was differentially expressed according to age group (ANOVA, $P = 0.0002$) in a subset of our samples ($n = 45$). The region targeted by the microarray (Affymetrix probe in Fig. 1) was adjacent to the region of the transcript that we assayed in our earlier studies (riboprobe in Fig. 1) (Webster *et al.*, 2002; Weickert *et al.*, 2003). The overall expression of the pan-BDNF probe detected by microarray mirrored that of several of the alternative BDNF transcripts (I–IX, IV–IX, and VI–IX) and also that of the BDNF protein, especially with regard to the peak in expression at infancy.

Distribution of alternative BDNF transcripts in the DLPFC

To qualitatively examine the expression of the alternative BDNF transcripts in an anatomical context, we investigated the laminar distribution of the individual transcripts by *in situ* hybridization (Fig. 5). Expression of all four BDNF transcripts showed similar laminar patterning in all age groups (Fig. 5A). From observation of the autoradiographic films, expression of all four alternative BDNF transcripts was highest in the deeper cortical layers V and VI, where expression is clearly defined at all age groups. Layer I showed no expression at any age group, whereas moderate expression was observed in layer II, and fairly robust expression was seen in deep layer III, especially in neonates (Fig. 5A, layers II and III). Expression of BDNF in the mid-cortical layer (layer IV) was low in the younger age groups (neonates). In the neonate group, the lightly BDNF-labeled mid-cortex appeared to cover a fairly large cortical width, and thus the 'stripe' with low BDNF label is obvious (Fig. 5Ai–iv, layer IV). At the cellular level, we saw no distinct accumulation of silver grains over neuronal nuclei at the youngest ages (neonates and infants) (Fig. 5Bi–iv). In the older age groups, there appeared to be a 'thinner' and less distinctive layer of low to moderate BDNF expression in the mid-cortex on the film images (Fig. 5Axiii–xvi). At the cellular level, we found an apparent increase in BDNF mRNA-expressing neurons, represented by accumulation of silver grains over some neuronal nuclei (Fig. 5Bv–viii), especially for transcripts II–IX and IV–IX. In contrast, in the deeper cortical layers (V and VI), we observed intense and concentrated silver grain accumulation for all four BDNF transcripts over neurons in the youngest age group (Fig. 5Ci–iv) and fewer silver grains over neurons in the older age group (Fig. 5Cv–viii). The silver grain distribution was also more diffuse in layer V and VI pyramidal neurons with advancing age, indicative of BDNF mRNA possibly being targeted to areas beyond the immediate soma.

Discussion

Differential BDNF transcript expression in development

We have found significant changes in the gene expression of four major BDNF alternative transcripts in the developing human DLPFC. Transcripts I–IX, IV–IX and VI–IX show similar developmental expression patterns, with the highest expression in infancy. A unique finding of this current study was the developmental pattern observed in transcript II–IX expression. The expression level of transcript II–IX was highest in the toddler age group, and was delayed by about 2–3 years as compared to transcripts I–IX, IV–IX, and VI–IX.

All four transcripts show a reduction in gene expression during the school age years, and some show an even greater attenuation after school age. Our findings are in general agreement with those of others, who have reported similar developmental trends in the cerebral and frontal cortex of rodents and primates, with increasing BDNF mRNA or protein expression during early postnatal life (Hayashi *et al.*, 1997; Katoh-Semba *et al.*, 1997, 1998). Moreover, our findings are also in agreement with the developmental trends described for the temporal

cortex of humans, where we observed that BDNF mRNA expression peaked early postnatally (Webster *et al.*, 2006).

Interestingly, our expression patterns differ from those described for the rodent visual cortex, where transcripts I–V (human: I–IX) and II–V (human: II–IX) were near the limits of detection during postnatal development, whereas transcripts III–V (human: IV–IX) and IV–V (human: VI–IX) were more abundant and continued to increase up to adulthood (Pattabiraman *et al.*, 2005). However, in our previous study of pan-BDNF mRNA expression in the human visual cortex (Webster *et al.*, 2002), we found no obvious change in BDNF mRNA across the lifespan. As well as species differences, there may be regional differences in the developmental expression of BDNF. The dynamic regulation of BDNF gene expression found in this study and in some areas in our previous studies suggest that brain region-specific regulatory factors may activate promoter-specific BDNF expression during distinct stages of life (Webster *et al.*, 2002, 2006).

However, the early peaks in BDNF expression that we found in this study are in contrast to our previous findings, where BDNF expression was lowest in infancy and higher in the young adult and adult groups (Webster *et al.*, 2002). There could be two reasons for this discrepancy: (i) the use of a different cohort; or (ii) the method by which BDNF mRNA expression was assayed. We do not believe that differential targeting of the BDNF transcripts in our earlier study as compared to our current study is a likely explanation. When we examined BDNF mRNA expression by microarray using a pan-BDNF probe targeting the common 3′ coding region of exon IX, we found similar trends in pan-BDNF as compared with three out of four alternative transcripts. Additionally, the change in pan-BDNF mRNA expression was reflected in BDNF protein expression. Thus, disparity between the current study and our previous study is probably due to cohort differences. In our previous study, we combined the neonate and infant groups, and this may have limited our ability to detect the increased expression of BDNF specifically at infancy, as neonates often show the lowest expression of BDNF (transcripts II–IX and IV–IX and mature protein), whereas infants often show the highest. Furthermore, the toddler and school age groups, where we saw dynamic change in BDNF expression in the present study, were not available in the earlier cohort.

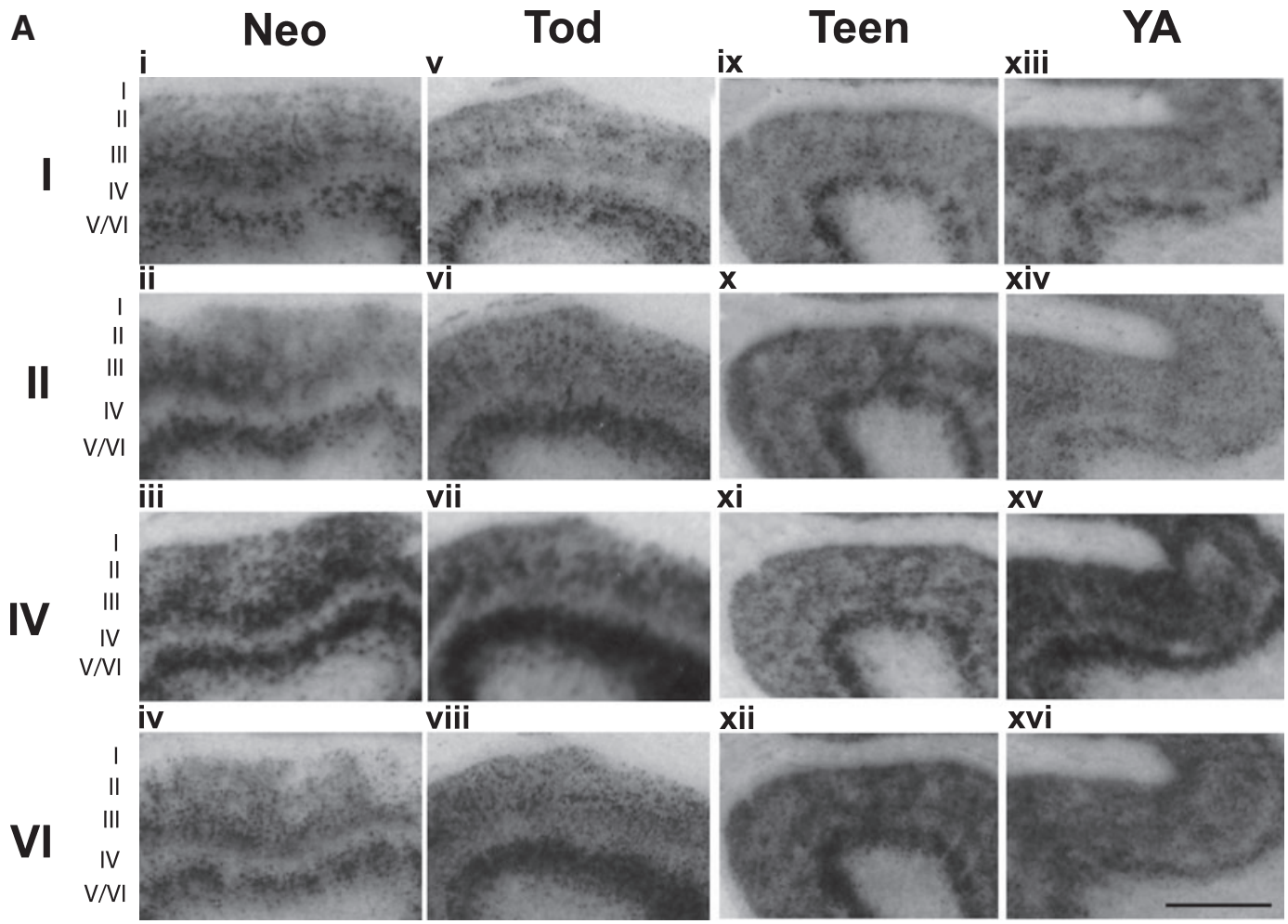

FIG. 5. Laminar distribution of alternative brain-derived neurotrophic factor (BDNF) transcripts in the dorsolateral prefrontal cortex (DLPFC). (A) Autoradiographic film images for specific BDNF transcripts after *in situ* hybridization are shown. Expression of transcripts I–IX, II–IX, IV–IX and VI–IX in the DLPFC of a neonate (Neo, i–iv), toddler (Tod, v–viii), teenager (Teen, ix–xii), and young adult (YA, xiii–xvi). BDNF mRNA hybridization to mid-cortical layers early in life is low, and expression is increased at older ages (note that this is most obvious in BDNF II–IX and IV–IX, in ii as compared to xiv, and in iii as compared to xv, respectively). Silver grain staining followed by Nissl counterstaining (small black dots over blue nuclei) of transcripts I–IX, II–IX, IV–IX and VI–IX in (B) layer IV and (C) layer VI of the DLPFC of the same neonate (Neo, i–iv) and young adult (YA, v–viii) from (A) are shown. Calibration bars: 3 mm (A); 75 μm (B); 75 μm (C); 20 μm (insets).

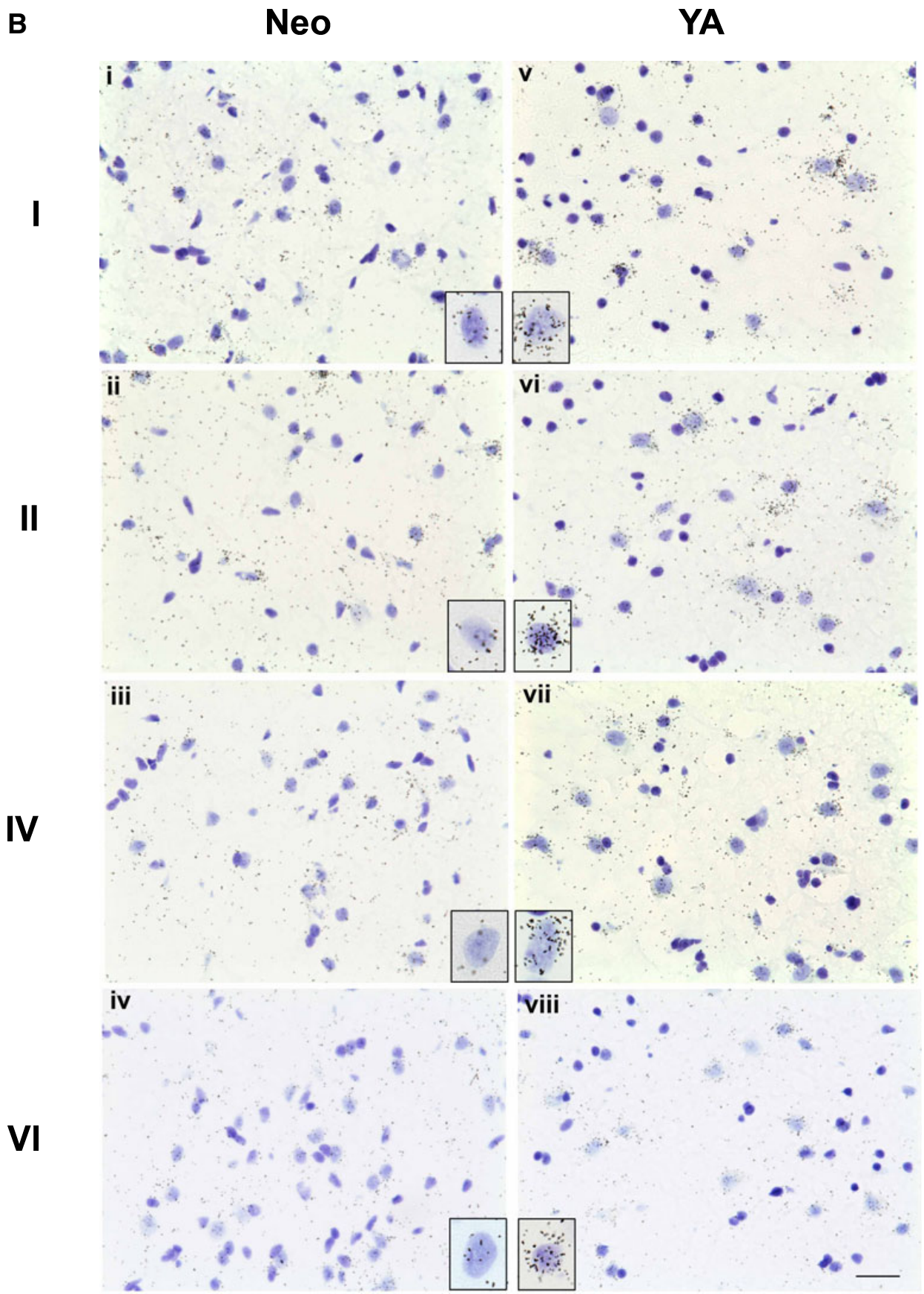

FIG. 5. Continued.

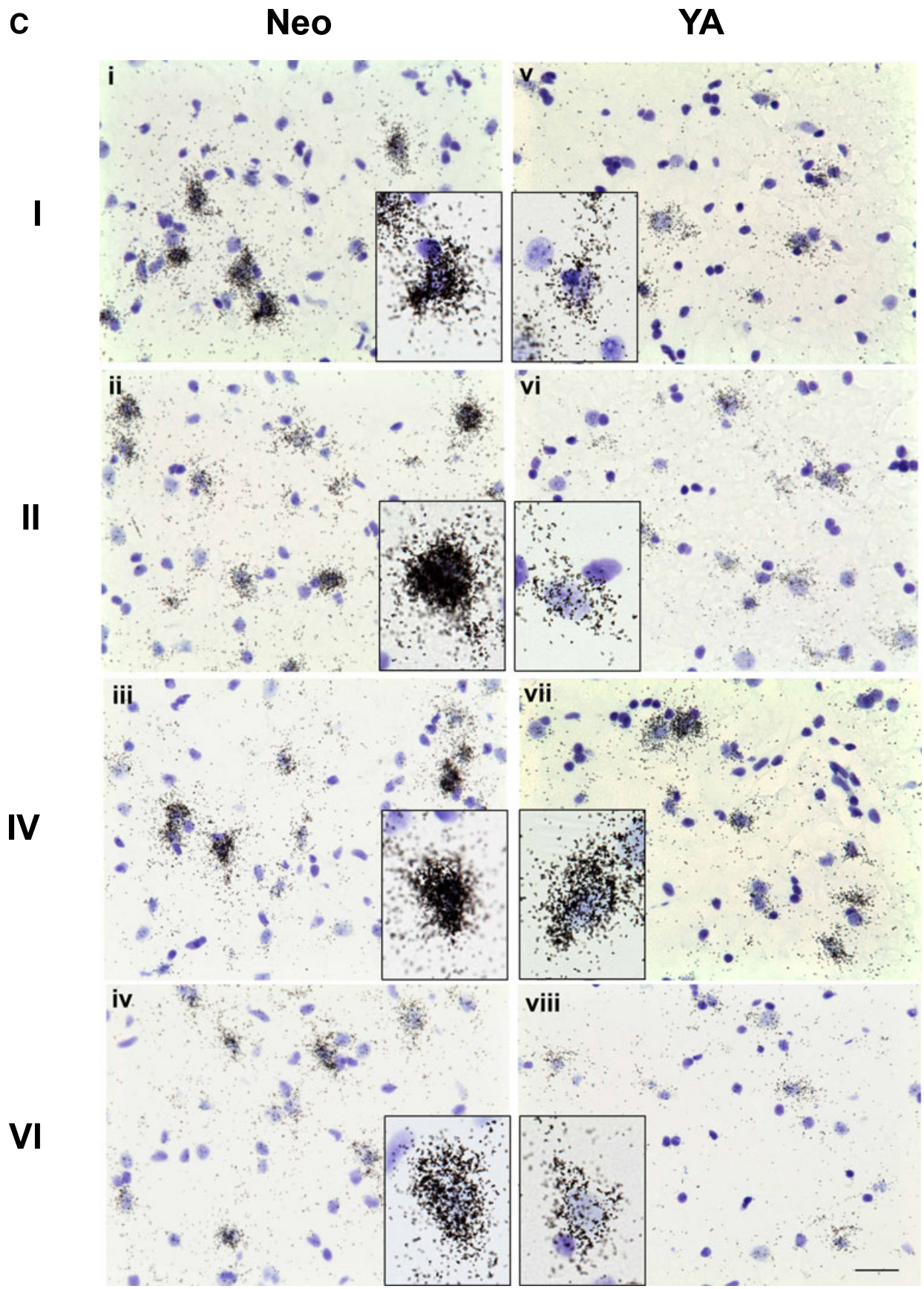

FIG. 5. Continued.

Targeting of BDNF transcript variants

The use of alternative BDNF transcripts enables targeted expression of BDNF in a developmental and anatomically specific manner, including biasing the subcellular localization of mRNA. In the rat visual cortex, both transcript III–V (human: IV–IX) and transcript IV–V (human: VI–IX) are expressed in the cell soma, and transcript IV–V can be expressed in the dendritic processes (Pattabiraman *et al.*, 2005). In the hippocampus, alternative BDNF transcripts with unique 5′ exons have been shown to localize to different cell layers in the dendrites following epileptogenic seizures (Tongiorgi *et al.*, 2004; Chiaruttini *et al.*, 2008). In a recent study, it was found that the 3′ region of mRNA can also influence subcellular targeting of the BDNF transcript; long 3′-UTR transcripts are targeted to the dendrite, where they help to maintain spines, and the short 3′-UTR transcripts are found in the cell body (An *et al.*, 2008). Interestingly, the authors showed that mouse exon II (human: exon II) containing mRNA was significantly enriched in the long form (An *et al.*, 2008). In our study, we observed decreases in the size of layer IV and increased BDNF signal in layer IV in the mature age group. As DLPFC layer IV is enriched in inhibitory neurons, which do not themselves express BDNF mRNA (Gorski *et al.*, 2003; Weickert *et al.*, 2003), we suggest that this may represent an increase in BDNF mRNA targeted to the apical dendrites of layer V pyramidal neurons, which extend into layer IV, as we find that the mRNA signal, while decreasing in the older age groups, also becomes less concentrated over the soma. Moreover, it is the BDNF transcript containing exon II and exon IV that appears to undergo the largest increase in expression in the mid-critical layers of the frontal cortex, the same transcript that is known to be preferentially targeted to dendrites (An *et al.*, 2008). The increased localization of BDNF mRNA to dendrites during maturation may act favorably to strengthen and support specific spines and synaptic function at targeted anatomical sties. However, we also detected an increase in BDNF mRNA-expressing neurons in the mid-cortical layers later in life, suggesting that the upregulation of BDNF in a subset of neurons in layer IV is developmentally delayed and contributes to the thinning of the BDNF-void zone during maturation. These neurons probably represent small excitatory pyramidal neurons, which are known to reside in layer IV (Levitt *et al.*, 1993).

BDNF and synaptogenesis

Our findings suggest that transcript-specific expression of BDNF may be acting as a regulator of neuronal morphology and synaptic plasticity during postnatal cortical development, when spine density formation and synaptic connectivity are changing. Previous studies have found a 1.5-fold increase in synaptic density in the frontal cortex during the first few years of human life, a time period that overlaps with the highest levels of all four BDNF transcripts and the highest level of BDNF protein. Synaptic density may decrease during adolescence and stabilize by young adulthood, when the cortex reaches structural maturity (Huttenlocher *et al.*, 1982; Huttenlocher, 1990; Bourgeois *et al.*, 1994; Huttenlocher & Dabholkar, 1997; Glantz *et al.*, 2007). Moreover, quantitation of synaptic density in layer III of the middle frontal gyrus almost mirrors the developmental pattern of BDNF mRNA expression, particularly of transcripts I–IX, IV–IX, and VI–IX.

BDNF is required during critical phases of cortical development. Previous studies have localized BDNF mRNA especially in the pyramidal cells of layer V/VI in human and primate cortex (Huntley *et al.*, 1992; Webster *et al.*, 2002; Weickert *et al.*, 2003). In our study, we found that the laminar distribution of four of the 5′ alternative BDNF transcripts mirrored that of the pan-BDNF probe, with the highest expression being seen in the deep cortical layers V and VI (Huntley *et al.*, 1992; Webster *et al.*, 2002). It should be noted that *in situ* hybridization was used in this study to determine the pattern of expression at various ages, and the findings are only qualitative so far. Previously, McAllister *et al.* (1995) showed that pyramidal neurons in cortical layer IV of the visual cortex respond to BDNF by increasing their dendritic arborization. Considering that BDNF mRNA and protein have been reported to be localized in the dendrites and soma (Tongiorgi *et al.*, 2004; An *et al.*, 2008; Chiaruttini *et al.*, 2008), our findings of increased BDNF being expressed robustly in layers V and VI may suggest that inputs from neighboring cortical neurons or cortical projection neurons that target layers V/VI are particularly BDNF responsive. Indeed, BDNF is required for the formation and maintenance of ocular dominance columns in the visual cortex (Cabelli *et al.*, 1997; Mandolesi *et al.*, 2005). Modulation of cortical BDNF levels inhibits the formation of ocular dominance patches in layer IV formed by thalamic input (Cabelli *et al.*, 1995, 1997). Thus, a change in the amount of BDNF synthesized impacts on remodeling of thalamic input, and, given that we found particularly low BDNF mRNA levels in cortical layer IV early in life, this suggests that dendrite-derived BDNF may be available in limiting amounts to thalamocortical inputs targeting layer IV of the DLPFC during infancy. Forebrain specific knock-outs of BDNF suggest that BDNF is critical for the maintenance but not the formation of cortical neuronal somal size and dendrite structure during postnatal development (Gorski *et al.*, 2003). Our data suggest that the first few years of life may represent critical phases of BDNF-regulated prefrontal cortical development in humans; however, more anatomically refined changes in the targeting of BDNF mRNA may also play a role in supporting specific synapses throughout life.

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Abbreviations

BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; CT, cycle threshold; DLPFC, dorsolateral prefrontal cortex; PBST, 0.1% (v/v) Tween-20 in phosphate-buffered saline; PMI, postmortem interval; qPCR, quantitative real-time polymerase chain reaction; RIN, RNA integrity; RT-PCR, real-time polymerase chain reaction.

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