Neuroscience 144 (2007) 1109–1119

POSTNATAL ALTERATIONS IN DOPAMINERGIC MARKERS IN THE HUMAN PREFRONTAL CORTEX

C. S. WEICKERT,[a] M. J. WEBSTER,[b] P. GONDIPALLI,[a] D. ROTHMOND,[a] R. J. FATULA,[a] M. M. HERMAN,[a] J. E. KLEINMAN[a] AND M. AKIL[a]*

[a]*Clinical Brain Disorders Branch, Intramural Research Program, National Institute of Mental Health, National Institutes of Health, 9000 Rockville Boulevard, Building 10, CRC6-5340, Bethesda, MD 20892, USA*

[b]*Stanley Foundation Laboratory of Brain Research, Department of Psychiatry, Uniform Services University of Health Sciences, Bethesda, MD 20892, USA*

Abstract—Dopamine in the prefrontal cortex plays a critical role in normal cognition throughout the lifespan and has been implicated in the pathophysiology of neuropsychiatric disorders such as schizophrenia and attention deficit disorder. Little is known, however, about the postnatal development of the dopaminergic system in the human prefrontal cortex. In this study, we examined pre- and post-synaptic markers of the dopaminergic system in postmortem tissue specimens from 37 individuals ranging in age from 2 months to 86 years. We measured the levels of tyrosine hydroxylase, the rate limiting enzyme in dopamine biosynthesis, using Western immunoblotting. We also examined the gene expression of the three most abundant dopamine receptors (DARs) in the human prefrontal cortex: DAR1, DAR2 and DAR4, by *in situ* hybridization. We found that tyrosine hydroxylase concentrations and DAR2 mRNA levels were highest in the cortex of neonates. In contrast, the gene expression of DAR1 was highest in adolescents and young adults. No significant changes across age groups were detected in mRNA levels of DAR4. Both DAR1 and DAR2 mRNA were significantly lower in the aged cortex. Taken together, our data suggest dynamic changes in markers of the dopamine system in the human frontal cortex during postnatal development at both pre-and post-synaptic sites. The peak in DAR1 mRNA levels around adolescence/early adulthood may be of particular relevance to neuropsychiatric disorders such as schizophrenia in which symptoms manifest during the same developmental period.

Key words: development, dopamine receptors, tyrosine hydroxylase, cerebral cortex, adolescence, aging.

During postnatal development, sweeping changes in neurotransmitter systems including glutamate, GABA, serotonin, dopamine and acetylcholine occur in the cerebral cortex of primates (Brooksbank et al., 1981, 1982; Goldman-Rakic and Brown, 1982; Rakic et al., 1986; Lidow et al., 1991b; Lidow and Rakic, 1992; Perry et al., 1993; Tarazi et al., 1999). Of these neurotransmitter systems, dopamine (DA) is of particular interest in relation to the development of cognitive abilities subserved by the prefrontal cortex. The primate prefrontal cortex (PFC) receives a dense DA innervation (Levitt et al., 1984; Gaspar et al., 1989; Berger et al., 1991) and DA has been shown to be necessary for normal performance on working memory tasks in both human and non-human primates (Williams and Goldman-Rakic, 1995; Dreher et al., 2002; Mattay et al., 2002; Gao and Goldman-Rakic, 2003). Alterations in DA markers in the PFC have been characterized during postnatal development in non-human primates. For example, refinements in the DA innervation of prefrontal pyramidal neurons, lamina-specific alterations in density of DA afferents as well as a dramatic increase in cortical DA content have been reported to occur during postnatal development in monkeys (Goldman-Rakic and Brown, 1982; Lewis and Harris, 1991; Rosenberg and Lewis, 1994). However, we know very little about changes in DA markers in the *human* PFC during postnatal development. In this study, we sought to address this question through the examination of presynaptic and postsynaptic markers of the DA system in the postmortem human PFC from infancy to old age.

Tyrosine hydroxylase (TH) is the rate limiting enzyme in DA biosynthesis and TH immunoreactivity has been used as a marker of the density of DA afferents to the cerebral cortex in both human and non-human primates (Lewis et al., 1988; Gaspar et al., 1989; Williams and Goldman-Rakic, 1993; Zecevic and Verney, 1995; Akil et al., 1999). Alterations in the density of TH-immunoreactive axons during the postnatal development of monkey PFC have been reported (Rosenberg and Lewis, 1994; Lewis et al., 1998). However, changes in TH levels in the *human* dorso-lateral prefrontal cortex (DLPFC) as a function of postnatal development have not been described.

The most obvious postsynaptic markers of the DA system are its receptors. There are five known G protein-coupled receptors (dopamine receptors (DAR) 1 through 5) expressed in the human PFC (for review see Missale et al., 1998). Although developmental changes in all DARs may occur, mRNA levels of three of these receptors DAR1, DAR2 and DAR4, have been shown to be the most abundant in the adult human PFC (Meador-Woodruff et al., 1996) and therefore lend themselves to quantitative comparisons across age groups. Moreover, these three receptors are of particular interest in psychiatric illness because of their known role in mediating executive function (Floresco et al., 2006) and their interaction with antipsychotic medications (Seeman, 1992; Hall et al., 1994; Beischlag et

*Corresponding author. Tel: +1-301-451-1450; fax: +1-301-402-2588.
E-mail address: makil@mail.nih.gov (M. Akil).

Abbreviations: DA, dopamine; DAR, dopamine receptor; DLPFC, dorso-lateral prefrontal cortex; PFC, prefrontal cortex; PMI, postmortem interval; TBS-T, Tris-buffered saline 1×, 0.05% Tween; TH, tyrosine hydroxylase.

0306-4522/07$30.00+0.00
doi:10.1016/j.neuroscience.2006.10.009

al., 1995; Meador-Woodruff, 1995). Although patterns of gene expression of DARs have been described in the monkey (Lidow et al., 1998) and in the aged human PFC (Meador-Woodruff et al., 1996), they have not been examined in the human PFC throughout the lifespan.

In this study, we used Western blotting and an antibody directed against TH and *in situ* hybridization with riboprobes specific for the DAR1, DAR2 and DAR4 transcripts in postmortem PFC specimens from six age groups: neonates, infants, adolescents, young adults, adults and aged. Characterizing changes in the levels of these pre- and post-synaptic markers of the DA system as a function of postnatal development in the human PFC is important for understanding the development of normal human cognition and how it might go awry in neuropsychiatric disorders with a neurodevelopmental component such as schizophrenia and attention deficit disorder.

EXPERIMENTAL PROCEDURES

Tissue collection and processing

Brain specimens were obtained through the Office of the Medical Examiners of the District of Columbia and were processed in the Section on Neuropathology of the Clinical Brain Disorders Branch as previously described (Kleinman et al., 1995). In each case, one cerebral hemisphere was cut coronally into 1–2 cm slabs and flash frozen in a mixture of dry ice and isopentane (1:1, V:V). The middle one-third (in the rostral–caudal dimension) of the middle frontal gyrus was selected from fresh frozen coronal blocks and sectioned on a cryostat at a thickness of 14 μm. Tissue sections were thaw-mounted onto gelatin-coated microscope slides and stored at −80 °C. For protein extractions, 1 to 2 g of tissue immediately adjacent to the middle frontal gyrus was obtained. A pie-shaped wedge was carefully dissected to minimize the inclusion of white matter and the frozen tissue was thoroughly mixed by pulverization. In order to facilitate cytoarchitectural identification of Brodmann's area 46 (BA 46 which we will also refer to as DLPFC), every 50th tissue section was stained for Nissl substance with Thionin. Tissue specimens in each set of experiments were selected according to tissue quality and availability. Consequently, not every case was included in every experiment. Brain pH was measured as previously described (Romanczyk et al., 2002).

Cohort description

Nine neonates (4 months and younger), five infants (5–12 months), eight adolescents (14–18 years), eight young adults (20–24 years), eight adults (34–43 years) and seven aged individuals (63–86 years) were included in this study (see Table 1). The cohorts for each experiment contained five cases or more per age group and did not show statistically significant group differences in either pH or postmortem interval (PMI) (ANOVA $P>0.05$). All available clinical information for each case was carefully and independently reviewed by two board-certified psychiatrists (M.A. and J.E.K.). Whenever possible, collateral information about the subjects was obtained from telephone interviews with surviving relatives of the deceased. Subjects with a history of neuropsychiatric disorders or substance abuse were excluded. Cases with significant neuropathological abnormalities or with neuropathology consistent with Alzheimer's disease were also excluded.

Western blotting

Pulverized frozen PFC tissue (100–130 mg) was thawed and homogenized over ice using a handheld homogenizer filled with extraction buffer (AEBSF 0.024%, aprotinin 0.005%, leupeptin 0.001%, pepstatin A 0.001%, glycerol 50%, Tris 0.6%) at the ratio of 1 g tissue to 10 ml buffer. Protein concentration was determined in each sample with the Bradford method (Bradford, 1976). Homogenized samples were aliquoted and stored at −80 °C. Aliquots were defrosted on wet ice before use. In each case, equivalent amounts of protein (20 μg) were aliquoted from homogenates and prepared for blotting by adding 25% by volume Tris–glycine SDS sample buffer 4× (Invitrogen Corporation, Carlsbad, CA, USA) and ddH_2O to normalize the loading volume for experimental conditions. All samples were then loaded onto 10% Tris–glycine acrylamide gels and electrophoresed at 120 V with MES Running Buffer 1×. After transferring the protein from the gel to nitrocellulose membranes (80 V for 105 min), the membranes were placed on a rocking platform in blocking solution (Tris-buffered saline 1×, 0.05% Tween (TBS-T)), 6% normal goat serum (Vector Laboratories, Burlingame, CA, USA) and 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) for 40 min at room temperature. The membranes were then incubated with gentle agitation for 72 h in 4 °C refrigerator with a primary antibody directed against TH (Chemicon International, Temecula, CA, USA, MAB318) at 1:1000 concentration in TBS-T with 3% normal goat serum and 2% bovine albumin. Following primary incubation, the membranes were washed with TBS-T then placed in 1:10,000 goat anti-mouse IgG H&L secondary antibody (Chemicon International, AP124P) with 4% normal goat serum for two hours at room temperature. The membranes were again rinsed in TBS-T, developed with ECL-Plus (Amersham Biosciences, Piscataway, NJ, USA) according to manufacturer's instructions and exposed to Kodak Bio-Max MR film (Eastman Kodak, Rochester, NY, USA) for several exposures times (ranging from 10 min to 1 h). Blots were rinsed and re-exposed with a primary antibody directed against β-actin (1:3000, Chemicon International, MAB1501) which served as a control for protein loading. Reported data are the average of two separate Western blotting experiments with the same subjects included in each experiment.

In situ hybridization

Six slide-mounted tissue sections containing BA46 from each case were hybridized with ^{35}S-labeled riboprobes for receptors DAR1, DAR2 or DAR4 (two tissue sections per riboprobe per case). We used cDNA templates for 1) $DAR1_{395}$, a 395 base pair riboprobe corresponding to GenBank accession # BC074978.2 base pairs 489–883; 2) $DAR2_{360}$, a 360 base pair corresponding to #NM000795.2 bp 826–1185; 3) $DAR4_{353}$, a 353 base pair riboprobe corresponding to GenBank accession #L12397 bp 3545–3898. All templates were sub-cloned from plasmids kindly provided by Olivier Civelli (UC Irvine). Inserts were sequenced and found to be 99–100% homologous to the deposited sequences (BLAST search). *In situ* hybridization was performed as previously described (Meador-Woodruff et al., 1996).

To control for between-experiment variations, tissue sections from all subjects were always processed in the same experiment. Also, sense strand control riboprobes for all three probes were hybridized under the same experimental conditions. After the *in situ* hybridization procedure, tissue slides, along with ^{14}C standards (American Radiolabeled Chemicals, Inc., St Louis, MO, USA) were exposed to Kodak autoradiographic film (BioMax MR) for 1–4 days.

Image analysis

Autoradiographic films were scanned using a Hewlett Packard Scanjet Plus flatbed at 300 dpi resolution. Autoradiographic images were analyzed using NIH Image (Rasband, NIH, v1.61). Measures of optical density were conducted blind to diagnosis, within the boundaries of BA46, in a field that is cut perpendicular to the pial surface in order to minimize distortions in relative