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Molecular evidence that cortical synaptic growth predominates during the first decade of life in humans

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ABSTRACT

Theories concerning the pathology of human neurodevelopmental disorders that emerge in adolescence, such as schizophrenia, often hypothesize that there may be a failure of normal cortical synaptic loss or pruning. However, direct evidence that synaptic regression is a major developmental event in the adolescent human cortex is limited. Furthermore, developmental work in rodents suggested that synaptic regression in adolescence is not a major feature of cortical development. Thus, we set out to determine when and to what extent molecular markers of synaptic terminals [synaptophysin (SYP), SNAP-25, syntaxin1A (STX1A), and vesicle-associated membrane protein 1 (VAMP1)] are reduced during postnatal human life spanning from 1 month to 45 years ($n = 69$) using several different quantitative methods, microarray, qPCR and immunoblotting. We found little evidence for a consistent decrease in synaptic-related molecular markers at any time point, but instead found clear patterns of gradual increases in expression of some presynaptic markers with postnatal age (including SNAP-25, VAMP1 and complexin 1 (CPLX1) mRNAs and 6/6 presynaptic proteins evaluated). A measure of synaptic plasticity [growth-associated protein of 43 kDa (GAP-43)] was elevated in neonates, and continued robust expression throughout life. Since CPLX1 protein is enriched in inhibitory terminals we also tested if the protein product of complexin 2 (CPLX2), which is enriched in excitatory neurons, is more specifically reduced in development. In contrast to CPLX1, which showed a steady increase in both mRNA and protein levels during postnatal development (both $r > 0.58$, $p < 0.001$), CPLX2 mRNA decreased from infants to toddlers ($r = -0.56$, $p < 0.001$), while CPLX2 protein showed a steady increase until young adulthood ($r = 0.55$, $p < 0.001$). Furthermore, we found that indices of the dendrites [microtubule associated protein 2 (MAP2)] and spines (spinophilin and postsynaptic density protein of 95 kDa (PSD95)] showed some evidence of reduction over time at the mRNA level but the opposite pattern, of a developmental increase, was found for PSD95 and spinophilin protein levels. Taken together, the postnatal changes in molecular components of synapses supports the notion that growth and strengthening of synaptic elements is a major developmental event occurring in the frontal cortex throughout childhood and that maintenance of steady state levels of synapse-associated molecules may predominate during human adolescence.

1. Introduction

The prefrontal cortex, which is markedly expanded and differentiated in the human brain, is essential for working memory, response inhibition, attention allocation and complex executive functions that enable us to inhibit inappropriate behaviors and thoughts, monitor our actions and plan and organize our future (Fuster, 1987; Arnsten and Li, 2005). Consequently the prefrontal cortex has been implicated in the pathophysiology of many uniquely human neuropsychiatric disorders, such as attention deficit hyperactivity disorder (ADHD), autism, schizophrenia, depression and bipolar disorder. Moreover, it is believed that these disorders may result from gene/environment interactions that disrupt the normal development of the brain. In particular, much attention has been given to the notion that disorders such as schizophrenia may result from the failure of normal synaptic regression that putatively occurs during adolescence (Bennett, 2008, 2009; Feinberg, 1982; Keshavan et al., 1994; McGlashan and Hoffman, 2000; Mirnics et al., 2001). This recurs as a theory for the developmental etiology of schizophrenia despite the fact that the primary literature demonstrating synaptic regression in the prefrontal cortex during human adolescence is limited (Huttenlocher, 1979, 1990) and despite inconsistent evidence for fewer synapses in the prefrontal cortex of subjects with schizophrenia (Eastwood

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Table 1
Demographics for the developmental cohort.

PCR cohort	Neonate	Infant	Toddler	School age	Adolescent	Young adult	Adult
n	9	13	9	7	8	9	8
Age, mean years (range)	0.18 (0.11–0.24)	0.53 (0.25–0.91)	2.8 (1.6–4.8)	9.6 (5.4–12.9)	16.92 (15–17.8)	23.2 (20.1–25.4)	43.4 (36–49.2)
PMI, mean hours (SEM)	22 (1.88)	17.5 (1.76)	22 (3.21)	14.3 (1.91)	15.5 (1.86)	13.7 (2.75)	13.4 (1.6)
pH, mean (SEM)	6.56 (0.05)	6.61 (0.05)	6.7 (0.06)	6.69 (0.07)	6.75 (0.03)	6.67 (0.08)	6.6 (0.09)
RIN, mean (SEM)	8.4 (0.4)	8.3 (0.19)	8.2 (0.16)	8.4 (0.25)	7.8 (0.33)	8.6 (0.28)	8.2 (0.21)
Sex (M/F)	5/4	8/5	4/5	3/4	6/2	6/3	5/3
Race	6AA,2C,1H	10AA, 3C	5AA,4C	1AA, 6C	2AA, 6C	4AA, 5C	4AA, 4C

Protein cohort	Neonate	Infant	Toddler	School age	Adolescent	Young adult	Adult
n	11	14	10	9	8	9	8
Age, mean years (range)	0.18 (0.11–0.24)	0.53 (0.25–0.91)	2.8 (1.6–4.8)	9 (5.4–12.9)	16.92 (15–17.8)	23.2 (20.1–25.4)	43.4 (36–49.2)
PMI, mean hours (SEM)	22.5 (1.54)	17 (1.72)	21 (2.98)	15 (1.56)	15.5 (1.86)	13.7 (2.75)	13.4 (1.62)
pH, mean (SEM)	6.5 (0.06)	6.6 (0.05)	6.6 (0.08)	6.6 (0.09)	6.75 (0.03)	6.67 (0.08)	6.6 (0.09)
Sex (M/F)	6/5	9/5	5/5	5/4	6/2	6/3	5/3
Race	6AA,4C,1H	11AA, 3C	6AA,4C	3AA, 6C	2AA, 6C	4AA, 5C	4AA, 4C

Array cohort	Neonate	Infant	Toddler	School age	Adolescent	Young adult	Adult
n	7	8	6	6	6	6	6
Age, mean years (range)	0.17 (0.11–0.24)	0.46 (0.25–0.91)	3 (1.6–4.8)	9.7 (5.4–12.9)	17 (15–17.8)	23 (20.1–25.4)	42 (36–49.2)
PMI, mean hours (SEM)	22.7 (6.34)	17.6 (7.69)	25.7 (9.56)	14.7 (5.43)	16.8 (4.71)	12 (5.4)	13.8 (5.34)
pH, mean (SEM)	6.6 (0.05)	6.69 (0.05)	6.74 (0.07)	6.73 (0.06)	6.79 (0.02)	6.75 (0.08)	6.68 (0.09)
RIN, mean (SEM)	8.96 (0.12)	8.6 (0.23)	8.2 (0.23)	8.7 (0.16)	7.97 (0.43)	9 (0.14)	8.4 (0.14)
Sex (M/F)	5/2	6/2	3/3	3/3	5/1	4/2	4/2
Race	6AA,1C	6AA, 2C	3AA,3C	1AA, 5C	2AA, 4C	3AA, 3C	4AA, 2C

PMI, postmortem interval; pH, brain tissue pH levels; RIN, RNA integrity number; SEM, standard error: M, male; F, female; AA, African American; C, Caucasian; H, Hispanic.

et al., 2000; Glantz and Lewis, 1997; Glantz et al., 2000; Halim et al., 2003; Honer et al., 1999; Karson et al., 1999; Perrone-Bizzozero et al., 1996; Scarr et al., 2006; Weickert et al., 2004; Fung et al., in press-a).

Several studies, including MRI (Reiss et al., 1996; Giedd et al., 1999; Sowell et al., 1999), fMRI (Adleman et al., 2002; Klingberg et al., 2002), electrophysiological (Hudspeth and Pribram, 1992), neuropsychological (Levin et al., 1991) and glucose metabolism (Chugani et al., 1987) studies indicate that the human prefrontal cortex may not be functionally mature until early adulthood. This delayed functional development of the frontal cortex may be due to a protracted development of neurons and glia, and to a delay in myelination (Yakovlev and Lecours, 1967; Giedd et al., 1999) or due to 'synaptic pruning'. Analysis of synaptic development in the frontal cortex indicates that there is an increase in the density of synapses in the first postnatal year that reach levels as much as 150% of the adult (Huttenlocher, 1979, 1990; Huttenlocher and Dabholkar, 1997). The density of synapses then appears to decline throughout childhood and adolescence to reach levels found in the mature brain by mid adolescence (Huttenlocher, 1994; Huttenlocher and Dabholkar, 1997). Using synaptophysin, considered a proxy for synaptic density (Walaas et al., 1988; Masliah et al., 1989, 1990, 1993; Eastwood et al., 1994), several studies examined mRNA levels in the human cortex (Webster and Weickert, 2004) and hippocampus (Eastwood et al., 2006) and found no evidence for a decrease in synapses during the adolescent period. While one study found that synaptophysin protein levels peak in the human cortex during childhood (but not in infancy as may have been predicted from the synaptic density studies) and then decrease in adolescence; they did not find a corresponding decrease in the postsynaptic protein, PSD95 (Glantz et al., 2007). Thus, the synaptic components involved, and the time course for synaptic growth and regression have not been clearly established in the postnatal human brain. Using real-time PCR (RT-PCR) and protein analysis we have focused the current study on genes associated specifically with synaptic number and synaptic vesicles. We have examined synaptophysin and members of the SNARE complex (SNAP-25, syntaxin, and synaptobrevin [vesicle-associated membrane protein: VAMP]) as indices of presynaptic function and complexin 1 and 2 as synaptic indices enriched in inhibitory and excitatory synapses respectively. We also examined growth-associated protein of 43 kDa (GAP-43) which is a synaptic plasticity-associated protein present in the presynaptic terminals but is not associated with the vesicles, and also several postsynaptic markers, including microtubule associated protein 2 (MAP2), PSD95 and spinophilin. In addition we have used microarray data, to survey the developmental expression profiles of all the genes associated with the synapse (as identified by GO categories) in the human prefrontal cortex.

2. Experimental procedures

2.1. Cases

A total of 69 cases ranging in age from 6 weeks to 49 years were obtained from the NICHHD Brain and Tissue Bank for Developmental Disorders (NICHHD Contract number NO1-HD8-3283). The cases included 42 males and 27 females and were grouped a prior as neonates (0.1–0.24 years), infants (0.25–0.9 years), toddlers (1–5 years), school age (5–12 years), teenagers (15–17 years), young adults (20–25 years) and adults (35–49 years, Table 1). The initial cohort of 45 cases used for the microarray project was expanded before the PCR and protein experiments were conducted. All subjects were free of neurological and psychiatric symptoms at the time of death.

2.2. RNA and protein extraction

Total RNA was extracted from the gray matter of the middle frontal gyrus (Brodman's area 46) using the standard Trizol extraction procedure, and was then purified through a Qiagen RNA miniKit column (Quiagen Inc, Valencia, CA, USA) according to the manufacturers protocol. RNA was processed through the Affymetrix preparation protocol (www.affymetrix.com), hybridized to HG-U133 version 2.0+ (GeneChips, Affymetrix, CA, USA). Protein was extracted from approximately 100 mg of pulverized tissue from an adjacent section of BA46, weighed, placed in sterilized glass homogenizer, thawed on wet ice and homogenized as previously described (Weickert et al., 2003). Crude protein was quantified using the Bradford method and stored at −80 °C.

2.3. Microarray analysis

Microarray experiments were conducted as described previously (Weickert et al., 2009) on a subset of 45 cases, with representatives from all age groups. Hybridized arrays were subjected to rigorous quality control including analysis of 5′ 3′ ratios (included range 0.40–0.79), percent present (included range 37–47%),