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THE TIMING OF NEURONAL LOSS ACROSS ADOLESCENCE IN THE MEDIAL PREFRONTAL CORTEX OF MALE AND FEMALE RATS

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Abstract—Adolescence is a critical period of brain maturation characterized by the reorganization of interacting neural networks. In particular the prefrontal cortex (PFC), a region involved in executive function, undergoes synaptic and neuronal pruning during this time in both humans and rats. Our laboratory has previously shown that rats lose neurons in the medial prefrontal cortex (mPFC) and there is an increase in white matter under the frontal cortex between adolescence and adulthood. Female rats lose more neurons during this period, and ovarian hormones may play a role as ovariectomy before adolescence prevents neuronal loss. However, little is known regarding the timing of neuroanatomical changes that occur between early adolescence and adulthood. In the present study, we quantified the number of neurons and glia in the male and female mPFC at multiple time points from preadolescence through adulthood (postnatal days 25, 35, 45, 60 and 90). Females, but not males, lost a significant number of neurons in the mPFC between days 35 and 45, coinciding with the onset of puberty. Counts of GABA immunoreactive cell bodies indicated that the neurons lost were not primarily GABAergic. These results suggest that in females, pubertal hormones may exert temporally specific changes in PFC anatomy. As expected, both males and females gained white matter under the PFC throughout adolescence, though these gains in females were diminished after day 35, but not in males. The differences in cell loss in males and females may lead to differential vulnerability to external influences and dysfunctions of the PFC that manifest in adolescence.

Key words: prefrontal cortex, adolescence, pruning, stereology.

INTRODUCTION

Adolescence, often defined as the period between puberty and adulthood, is a time characterized by neuroanatomical changes that coincide with an increased vulnerability to a variety of clinical disorders including depression, anxiety and schizophrenia (Spear, 2000; Steinberg, 2005; Paus et al., 2008). The prefrontal cortex (PFC), a region primarily involved in executive function and emotional regulation, continues to develop across much of the lifespan with adolescence as a critical period of development. In the human PFC, there is significant synaptic pruning during the adolescent period. Between early adolescence and adulthood, there is a decrease in dendritic spine density on PFC pyramidal neurons (Petanjek et al., 2011) and a decrease in synaptic density (Huttenlocher and Dabholkar, 1997). This period also coincides with an overall decrease in PFC volume (Gogtay et al., 2004; Lenroot and Giedd, 2006). Importantly, there is evidence for differences in the timing and trajectory of PFC development in males and females (Lenroot and Giedd, 2006).

Because of the difficulty in quantification and parcellation of the human PFC and given the homology between the rat and primate frontal cortex (Uylings et al., 2003), rat models for PFC development have been reliably used. Similar to primates, the rodent PFC continues to develop into adulthood, as both its volume (Van Eden and Uylings, 1985a) and laminar specificity (Van Eden and Uylings, 1985b) peak during the periadolescent period and then decrease until at least 90 days of age. Synaptic pruning and dendritic organization have been documented in the adolescent rodent medial prefrontal cortex (mPFC) as well. Specifically, between adolescence and adulthood, both male and female rats lose dendritic spines while only female rats lose a significant number of dendrites (Koss et al., 2014). In addition, while the density of PFC neurons projecting to the amygdala decreases between adolescence and adulthood (Cressman et al., 2010), there is an increase in the density of fibers innervating the mPFC from the amygdala (Cunningham et al., 2002).

Evidence from our laboratory indicates that changes in synaptic density and volume could be related to a loss of total neurons in the region. In the rat mPFC, the number of neurons decreases between adolescence and adulthood with neuronal loss being considerably greater in females (Markham et al., 2007). There is further evidence that in females, the actions of hormones secreted after puberty lead to this decrease in mPFC neuron number, as ovariectomy before puberty prevents this loss when neuron number is assessed in adulthood (Koss et al., 2015). This is similar to findings in the primary

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Abbreviations: ANOVA, analysis of variance; IL, infralimbic; mPFC, medial prefrontal cortex; NGS, normal goat serum; PBS, phosphate-buffered saline; PFC, prefrontal cortex; PL, prelimbic; TBS, tris-buffered saline.

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visual cortex where ovarian hormones after puberty were found to play a role in neuronal pruning (Nuñez et al., 2002). It is currently unclear whether these neuronal losses in the mPFC involve GABAergic interneurons or glutaminergic pyramidal cells, or a combination of both. However, one study showed that ovariectomized adult females have a greater density of parvalbumin-positive GABA neurons in the mPFC than that of intact females (Cholanian et al., 2014), suggesting the neuronal losses in intact females may detectably include GABA cells.

Counter to the pruning of many cellular components of the gray matter is the increase in white matter between the juvenile and adult periods. Myelination is known to continue well into adulthood in the corpus callosum of rats (Nuñez et al., 2000). Electron microscopic analysis of the posterior (splenium) corpus callosum shows that there is an increase in the number of axons that are myelinated between the juvenile period and adulthood, even while axons are pruned (Kim and Juraska, 1997). Furthermore the presence of ovarian hormones from puberty on leads to a lower number of myelinated axons in this region in early adulthood (Yates and Juraska, 2008). Like the posterior corpus callosum, the volume of the white matter under the PFC continues to increase between adolescence and adulthood in both male and female rats. However, a sex difference emerges at P90 with males having a larger white matter volume than females (Markham et al., 2007).

A critical question that remains unanswered is the trajectory of these neuroanatomical changes, specifically whether they occur gradually throughout the adolescent period or within a well-defined temporal window that may coincide with puberty. If puberty is a key component for these changes, there should be differences between males and females due to the difference in pubertal timing and the different hormones involved. Here we quantified neuron and glial cell number, along with the volume of the white matter under the PFC, in male and female rats. In addition to counts of all neurons in the mPFC, we stereologically counted the number of GABA immunoreactive interneurons. Most previous studies have compared two ages: juvenile/early adolescence and adult. Here we examine five ages from the juvenile period to puberty through adulthood in order to delineate when these anatomical changes occur.

EXPERIMENTAL PROCEDURES

Subjects

Subjects were the offspring of Long-Evans hooded rats obtained from Harlan Laboratories (Indianapolis, IN, USA) and bred in the vivarium in the Psychology Department at the University of Illinois. All animals were weaned on postnatal day (P) 24 and housed with same-sex littermates in pairs or triplets until sacrifice. Tissue from both male and female rats was collected at P25, P35, P45, P60 and P90 (n = 10–11 per group) for a total of 105 animals. P25 is during the preadolescent period, P35 approximates the period of pubertal onset in females, P45 approximates puberty onset in males, and P60 and 90 represent the transition from late adolescence to early adulthood. For each animal that was not sacrificed before pubertal onset, the day the animal reached puberty was recorded. For females, vaginal opening was used as a marker of puberty, since this coincides with surges of both luteinizing hormone and estrogen secretion (Castellano et al., 2011). For males, preputial separation was used for marking puberty, as this coincides with the major surge in androgens (Korenbrot et al., 1977). Each age group was comprised of animals from a minimum of five litters, and at each age, no more than two animals of the same sex came from the same litter. All animals were kept on a 12:12-h light–dark cycle with *ad libitum* access to food and water. All procedures were approved by the University of Illinois Institutional Care and Use Committee, and adhere to the National Institute of Health guidelines on the ethical use of animals.

Histology

Rats were given a lethal dose of sodium pentobarbital, and were perfused intracardially with 0.1 M phosphate-buffered saline (PBS) (pH 7.4) followed by 4% paraformaldehyde fixative in PBS. Brains were removed and post-fixed for an additional 24 h and were then cryoprotected in a PBS solution containing 30% sucrose for three days. Once a brain had sunk in sucrose, it was sliced coronally into 40-μm sections with a freezing microtome. Every fifth section containing PFC was temporarily placed into 0.1 M PBS and then mounted on gelatin-coated slides. Once dried, sections were stained with Methylene Blue/Azure II. Staining procedures were identical to those described in Markham et al. (2007).

Volume and cell number estimation

Parcellation of the mPFC and adjacent white matter was conducted as previously described by our laboratory (Markham et al., 2007) using the StereoInvestigator software program (MicroBrightField, Williston, VT, USA). Briefly, the ventral mPFC (prelimbic (PL) and infralimbic (IL) subregions) was identified based on the cytoarchitectonic criteria delineated in Van Eden and Uylings (1985a,b) (Fig. 1A). The boundary of the PL and the anterior cingulate was determined by the broadening of layer V cells and increase in density of layer III cells in the anterior cingulate region, along with a thin ‘‘empty band’’ that is visibly less dense. The ventral boundary of the IL is visible via a loss of laminar organization between cell layers. Within the ventral mPFC layers II/III and V/VI were parcellated for analysis. Layer I was excluded from the analysis due to a lack of neuronal cell bodies.

Parcellation was conducted by an experimenter blind to the age and sex of the animals. Frontal white matter volume and mPFC volume were calculated from every mounted section between the most anterior mounted section containing the genu of the corpus callosum and the caudal end of the IL mPFC, when the corpus callosum joins both hemispheres at the midline. Using StereoInvestigator, the experimenter defined the area of the frontal white matter, made easily distinguishable by