TYROSINE HYDROXYLASE IMMUNOREACTIVE FIBERS IN THE UPPER LAYERS OF THE ADOLESCENT RAT MEDIAL PREFRONTAL CORTEX

BY

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THESIS

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Abstract

Adolescence is an important period of maturation for the brain. In particular, the medial prefrontal cortex continues developing throughout adolescence and is responsible for a number of executive and cognitive functions. Innervation of the medial prefrontal cortex by the dopaminergic system contributes to some of these maturing behaviors and the dopaminergic system is implicated in mental illnesses. However, much previous work on medial prefrontal cortex development over adolescence has considered only one sex or has failed to capture timing of changes. Here, the development of tyrosine hydroxylase-immunoreactive axons, to indicate dopaminergic innervation, in the upper layers of the medial prefrontal cortex was examined in both sexes of Long-Evans rats during five ages from postnatal day 25 to 90. Tyrosine hydroxylase-immunoreactive axon density was found to increase over the course of adolescence, with the most marked increases being prepubertal in both males and females. No sex differences were identified. These results suggest a similar development, and thus similar susceptibility to disruption, of the dopaminergic system in the rat medial prefrontal cortex in both sexes.

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Introduction

Adolescence is an important period of brain development for humans and other mammals. During this time, neuroanatomical changes include pruning and refinement of neurons, dendrites, synapses, and expression of receptors (Markham et al., 2007; Lewis, 1997; Huttenlocher and Dabholkar, 1997; Andersen et al., 2000). Changes during this time can show sex differences and may or may not involve sex hormones (Juraska et al., 2013). In humans, anatomical changes produce an observable volume change using MRI, with a sex difference in age of peak volume (Giedd et al., 1999). Behavioral changes are also pronounced, with changes in many executive and social behaviors, notably including decisions involving risk-taking and reward (Spear, 2000). Adolescence itself is difficult to delimit, but in humans it occurs in the second decade of life, and is often considered to be from 12 to 18 years of age, or even older; in rats, postnatal day (P)28 to P42 or P45 can be considered adolescence, although some changes continue to P60, such as increasing connections from the amygdala to the prefrontal cortex (Spear, 2000; Brenhouse and Andersen, 2011; Cunningham et al., 2002).

The prefrontal cortex, particularly the medial prefrontal cortex (mPFC), is an important brain region for executive behavior (Dalley et al., 2004), including decision making based on reward and cognitive flexibility. Many of these prefrontal behaviors mature during adolescence, with adolescents displaying increased impulsivity and lower cognitive flexibility than adults (Steinberg, 2010; Andrzejewski et al., 2011). Anatomically, the prefrontal cortex undergoes changes including cell number, synaptic density, connectivity to other brain regions, and receptor expression over the course of adolescence in both rats and humans (Markham et al., 2007;

Huttenlocher and Dabholkar, 1997; Cunningham et al., 2002; Andersen et al., 2000).

Dopaminergic innervation to the mPFC contributes to the functions of the mPFC. Tyrosine hydroxylase (TH) is the rate-limiting step in the synthesis of dopamine, an important catecholamine neurotransmitter, and is commonly used to mark dopaminergic fibers (e.g. Lewis et al., 1987; Naneix et al., 2012). Dopaminergic axons from the ventral tegmental area target the mPFC in rodents and primates, and this innervation increases over adolescence (Kalsbeek et al., 1988; Lewis et al., 1987; Naneix et al., 2012). Furthermore, dopamine receptor expression peaks during late adolescence (Andersen et al., 2000). The response of neurons to dopamine also changes during this time (Tseng and O'Donnell, 2007). Dopamine in the mPFC plays a role in adult behaviors that matured over adolescence, such as cognitive flexibility (Gruber et al., 2010).

Mental illness is also associated with adolescence. The highest rates of onset of many psychopathologies, including mood disorders and schizophrenia, occur during this time (Kessler et al., 2005). Additionally, there are sex differences in disease onset, with more adolescent males experiencing schizophrenia and females experiencing depression (Abel et al., 2010; Parker and Brotchie, 2010). Exposure to stress can expose or exacerbate these disorders (Arnsten, 2011; Monroe and Harkness, 2005). These illnesses involve the prefrontal cortex. Schizophrenia shows reductions in prefrontal cortex volume and synapses (Lewis, 1997; Giedd et al., 2008); depression also involves changes in prefrontal cortex volume and changes in activity (Palazidou, 2012). Furthermore, the dopamine system is implicated in both depression and schizophrenia (Dunlop and Nemeroff, 2007; Money and Stanwood, 2013). Combined, these data suggest that disruption of normal development of prefrontal cortex may produce or exacerbate psychopathologies (Giedd et al., 2008).

Understanding normal adolescent development is important for an appreciation of how the adult brain forms and for disease models. However, studies of adolescent development often use only one sex (e.g. Andersen et al., 2000), few timepoints (e.g. Markham et al., 2007), or methods that reveal little about the bases for observed change (e.g. Giedd et al., 1999). Previous studies of dopaminergic innervation in the mPFC have been no exception and in particular relied on male animals only (e.g. Naneix et al., 2012). The following work aims to elucidate some of the changes occurring during this important period. Here, we examine the development of TH-immunoreactive fibers in the upper layers of the adolescent rat mPFC at several ages, to identify age and sex differences from preadolescence to adulthood. These timepoints intend to capture changes over adolescence, with P25 being preadolescent and P90 adult. Female rats enter puberty before male rats, with puberty occurring by P35 in females, with males undergoing puberty after P35 but typically finishing by P45. Since development can continue after puberty, P60 is also examined as late adolescence.

Methods

Animals

Long-Evans hooded rats (Harlan) were bred in the Psychology Department's vivarium. Following weaning at P24, same-sex littermates were group housed (2-3) in transparent plastic cages in a temperature-controlled room with 12:12h light-dark and with ad lib. access to a standard rat chow and water. Animals were checked for puberty markers between P25-48. Animal experimentation was approved by the University of Illinois' Institutional Animal Care and Use Committee.

Tissue Preparation

At P25, P35, P45, P60, or P90, 10-11 rats of each sex (total: 102 animals) were anesthetized with sodium pentobarbital (100mg/kg) and transcardially perfused with 0.1M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were removed and stored in paraformaldehyde solution for 24 hours, then transferred to a 30% sucrose solution. After 3 days, brains were cut on a freezing microtome into 40μm-thick coronal sections. Every fifth section was used for TH immunocytochemistry; other sections were Nissl stained or saved for further experiments. Sections were stored in 30% glycerol, 30% ethylene glycol, 10% PBS, and dH_2O.

Immunocytochemistry

For TH labelling, sections were rinsed in tris-buffered saline (TBS; pH 7.6), placed in a

blocking solution (1% hydrogen peroxide, 20% normal goat serum, 1% BSA in TBS) for 30 minutes, and then exposed to monoclonal mouse anti-tyrosine hydroxylase (Millipore) at 1:1000 dilution in TTG (2% normal goat serum and 0.3% triton-x-100 in TBS) for 48 hours followed by a rinse in TTG, antimouse biotinylated antibodies (Vector Labs) in TTG for 90 minutes, rinses in TTG and TBS, avidin-biotin-complexed horseradish peroxidase (Vector Labs) for 1 hour, rinsed in TBS, diaminobenzidine (Sigma Fast Tabs), and finally rinsed in TBS, and then mounted on slides, allowed to dry, and then coverslipped.

TH Analysis

To quantify TH fibers, a Zeiss Axiovert 200M (Carl Zeiss, Thornwood, NY) with a 63x oil immersion objective was used to obtain Z-stacks of layers 1, 2/3, and 5/6 from both left and right hemispheres in two sections per brain within the mPFC. The mPFC was defined as containing the prelimbic and infralimbic regions, starting rostrally with the section where white matter first occurs and ending when the genu of the corpus callosum is encountered (Chisholm et al., 2012; for cytoarchitectonic parcellation of the mPFC, Markham et al., 2007). Images in the stacks were taken every 0.275μm, resulting in around 100 images par stack. Stacks were taken approximately midway between the dorsal and ventral extent of white matter, while avoiding obvious artifacts. Layer 1 was taken as close to the medial surface of the mPFC as possible (the images contained the entire layer), layers 2/3 close to layer 1 while excluding layer 1 fibers, and layers 5/6 in the middle of 5/6. The layers have readily identifiable arrangements of fibers: layer 1 contains distinctive dorsoventally-aligned fibers, layers 2/3 contain some prominent lateral fibers, and layers 5/6 contain numerous fibers running in all directions. Z-stacks were refocused to a single image using real wavelets with the Extended Depth of Field plugin (Forster et al.,

2004) for ImageJ/FIJI (Schneider et al., 2012; Schindelin et al., 2012). Processed images were then examined for extraneous staining, which were excluded by hand with a white brush in ImageJ. Items removed were blood vessels, with membranous, weakly-staining, hollow, or segmented appearance; diffuse background staining; large amorphous or soma-like objects; dirt and tissue artifacts, which were grey, black, or blue rather than brown; and blobs or putative cells which did not appear collinear with presumptive fibers. The images were converted to binary images with black for stained fibers and the area covered in black pixels was quantified before and after processing. The binary processed image was then skeletonized, reducing contiguous black pixels to structures 1 pixel wide, and this area was also quantified. Skeletonizing the image preserves fiber length while unskeletonized images quantify differences in fiber thickness and length.

Because the mPFC changes in size across the ages examined, the percent of image stained was also multiplied by the volume of the appropriate mPFC layers to produce μm^3 of stained material. The volumes were obtained from Nissl stained sections parcellated stereologically by other experimenters as in Markham et al. (2007).

This work deals with layers 1 and 2/3; another experimenter is quantifying layers 5/6.

Statistical Analysis

Statistical analysis was performed using SPSS (IBM). ANOVAs were performed using age and sex as factors, with litter and cohort as covariates. Preplanned post hoc comparisons (t-tests) were examined between ages P25-35, P35-45, P45-60, and P60-90 when age was significant.

Results

Layer 1

The ANOVA of processed binary images (density: number of TH labeled pixels/volume) (Figure 1) revealed an effect of age ($F(4, 90)=5.630$; $p<.001$) and immunohistochemistry cohort ($F(1, 90)=34.619$; $p<.001$), but not sex ($F(1, 90)=.366$; $p=.547$) nor an age by sex interaction ($F(4, 90)=1.024$; $p=.400$). Likewise, skeletonized images (Figure 2) showed the same age ($F(4, 90)=3.951$; $p=.005$) but not sex ($F(1, 90)=.259$; $p=.612$) effect, with no interaction ($F(4, 90)=.488$; $p=.744$). The same pattern of effects was present in the unprocessed images (age: $F(4, 90)=4.949$; $p=.001$; sex: $F(1, 90)=1.111$; $p=.295$; age by sex: $F(4, 90)=.730$; $p=.574$) (Figure 3).

When percent of image stained was multiplied by volume of layer 1 to produce the amount (μm^3) of stained axons, a main effect of age persisted for processed images in males ($F(4, 44)=3.375$; $p=.017$) and females ($F(4, 44)=2.723$; $p=.041$) (Figure 4). Post hoc comparisons of the amount of stained axons showed a significant difference between P25 and P35 in females ($p=.030$) and a trend in males ($p=.060$); all other post hocs were not significant. Only trends in skeletonized images for males ($F(4, 44)=2.390$; $p=.065$) and females ($F(4, 44)=2.092$; $p=.098$) were found (Figure 5).

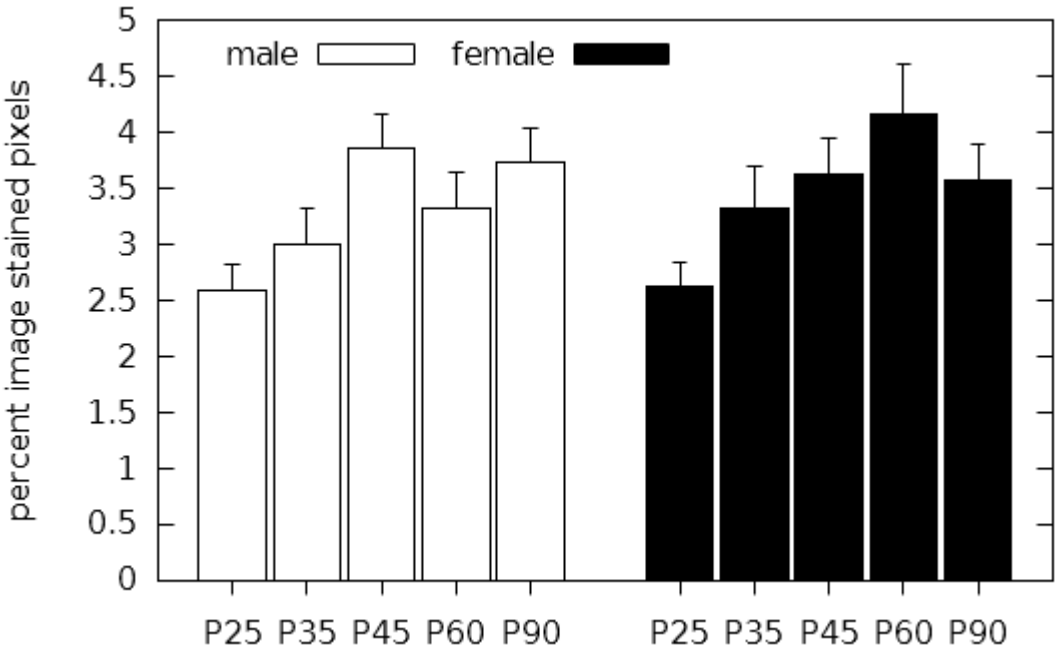


Figure 1: Percent stained of binary processed layer 1 images. Significant effect of age: F(4, 90)=5.630; p<.001. Means + SEM.

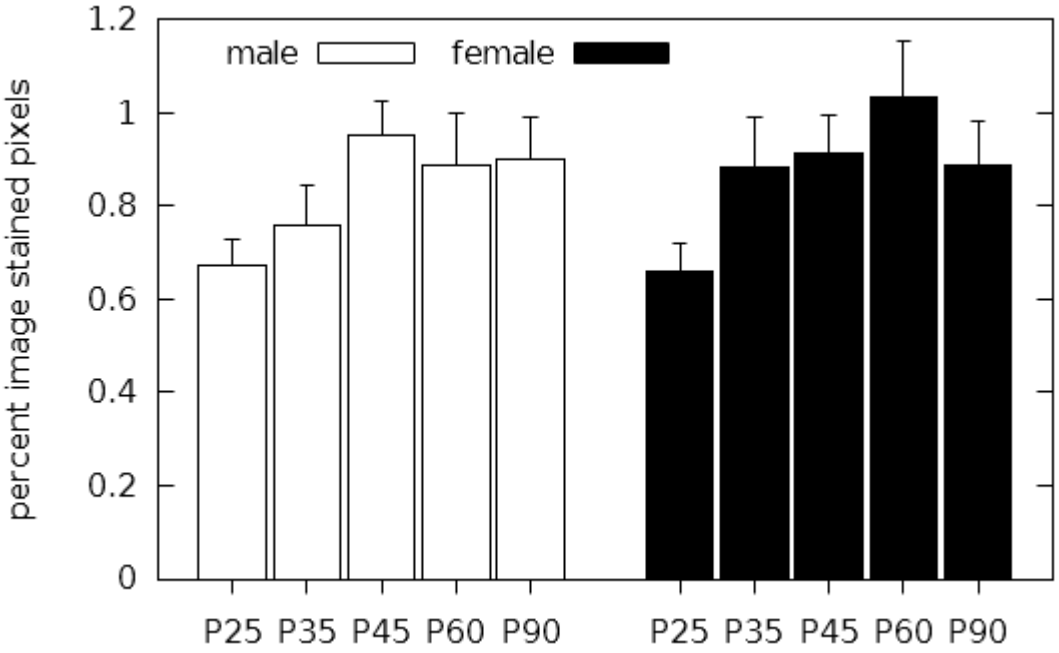


Figure 2: Percent stained of skeletonized layer 1 images. Significant effect of age: F(4, 90)=3.951; p=.005. Means + SEM.

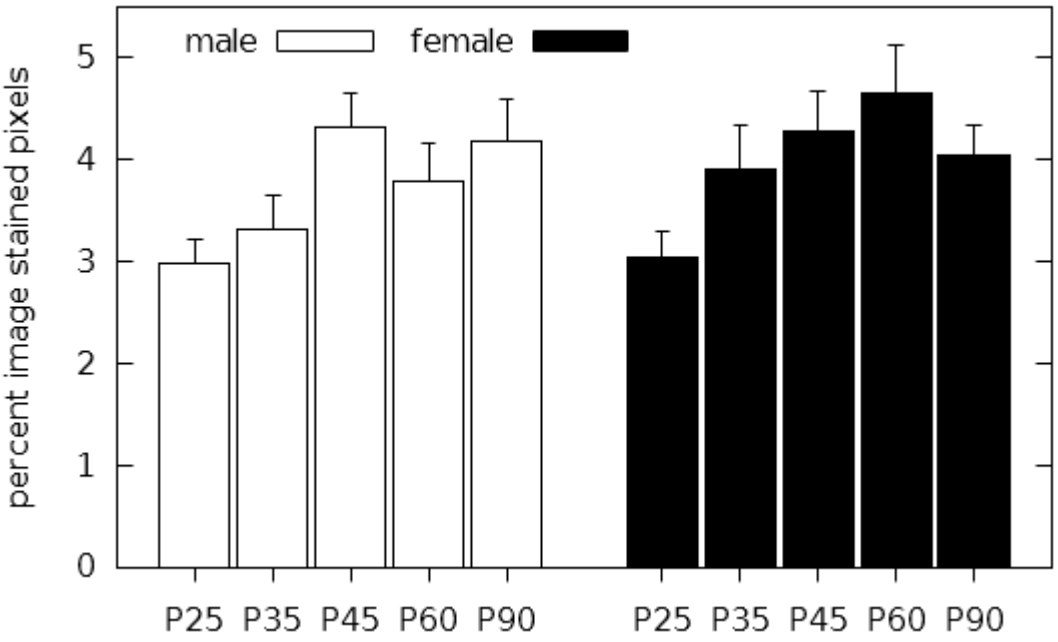


Figure 3: Percent stained of unprocessed layer 1 images. Significant effect of age: F(4, 90)=4.949; p=.001. Means + SEM.

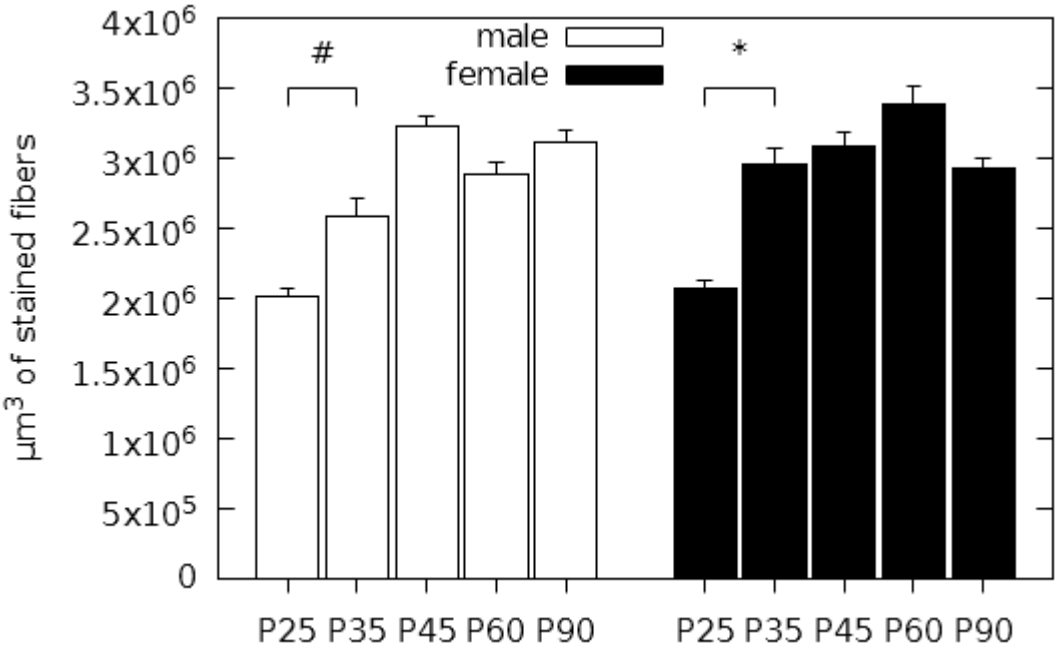


*Figure 4: Volume (μm^3) of stained axons from binary processed layer 1 images. Effect of age in males: F(4, 44)=3.375, p=.017; and females: F(4, 44)=2.723, p=.041. *: p<.05; #: p<.10. Means + SEM.*

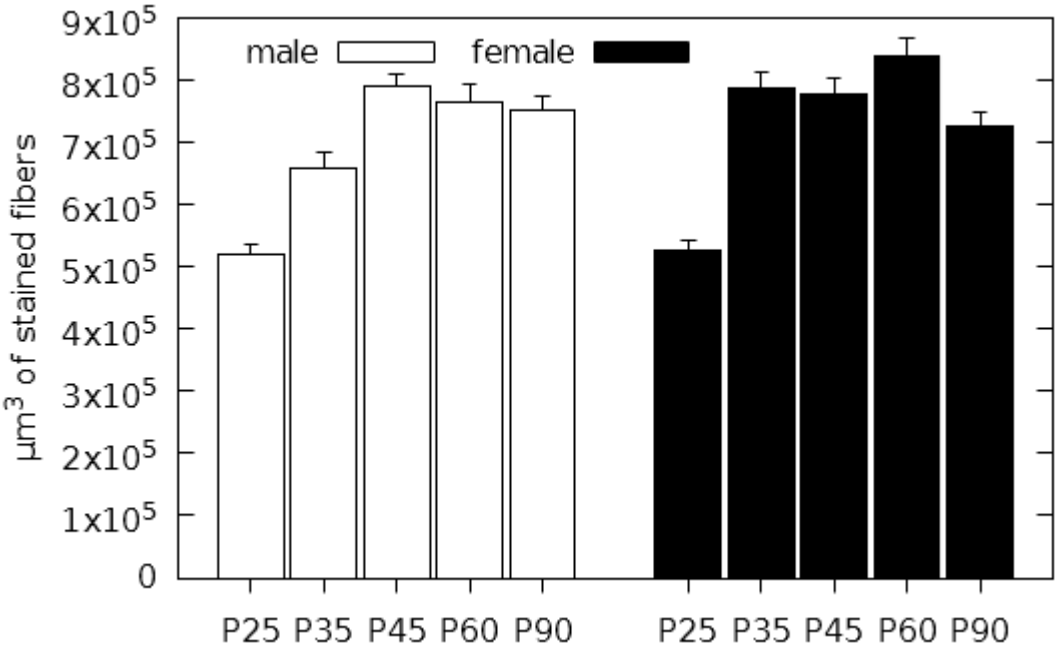


Figure 5: Volume (µm³) of stained axons from layer 1 skeletonized images. Trend of age for males: F(4, 44)=2.390, p=.065; and females: F(4, 44)=2.092, p=.098. Means + SEM.

Layer 2/3

There was an effect of age (F(4, 90)=3.103; p=.019) in the processed binary images of density (Figure 6) and an effect of immunohistochemistry cohort (F(1, 90)=13.484; p<.001) but not sex (F(1, 90)=.315; p=.576) nor age by sex (F(4, 90)=.683; p=.606). Similarly, skeletonized images (Figure 7) showed age (F(4, 90)=3.237; p=.016) but not sex (F(1, 90)=.045; p=.832) nor age by sex (F(4, 90)=.547; p=.702). Unprocessed images (Figure 8) showed similar results (age: F(4, 90)=2.596; p=.042; sex: F(1, 90)=.477; p=.492; age by sex: F(4, 90)=.579; p=.679).

When the volume (µm³) of stained axons was considered, binary processed images (Figure 9) showed an effect of age in males (F(4, 44)=2.939; p=.031) and females (F(4, 42)=2.890; p=.034). Post hoc tests again showed a significant difference only between P25 and P35 in males (p=.006) and only a trend in binary images in females between P25 to P35

(p=.062). Skeletonized (Figure 10) also showed an effect of age in males (F(4, 44)=3.268; p=.020) but a trend in females (F(4, 42)=2.292; p=.075). Post hocs revealed a significant difference between P25 and P35 in males (p=.002).

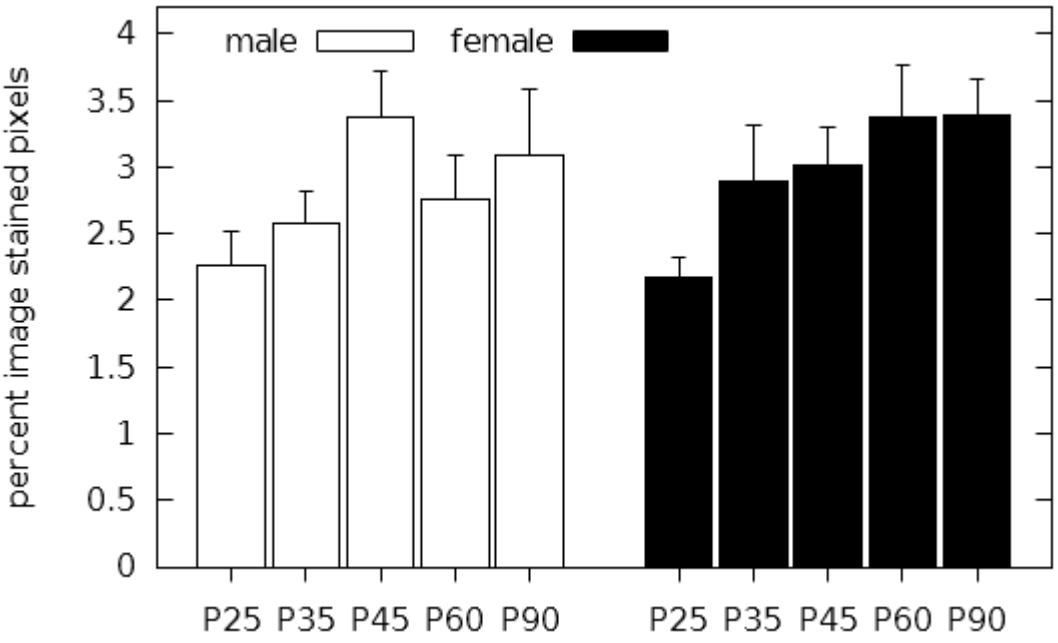


Figure 6: Percent stained of binary processed layer 2/3 images. Significant effect of age: F(4, 90)=3.103; p=.019. Means + SEM.

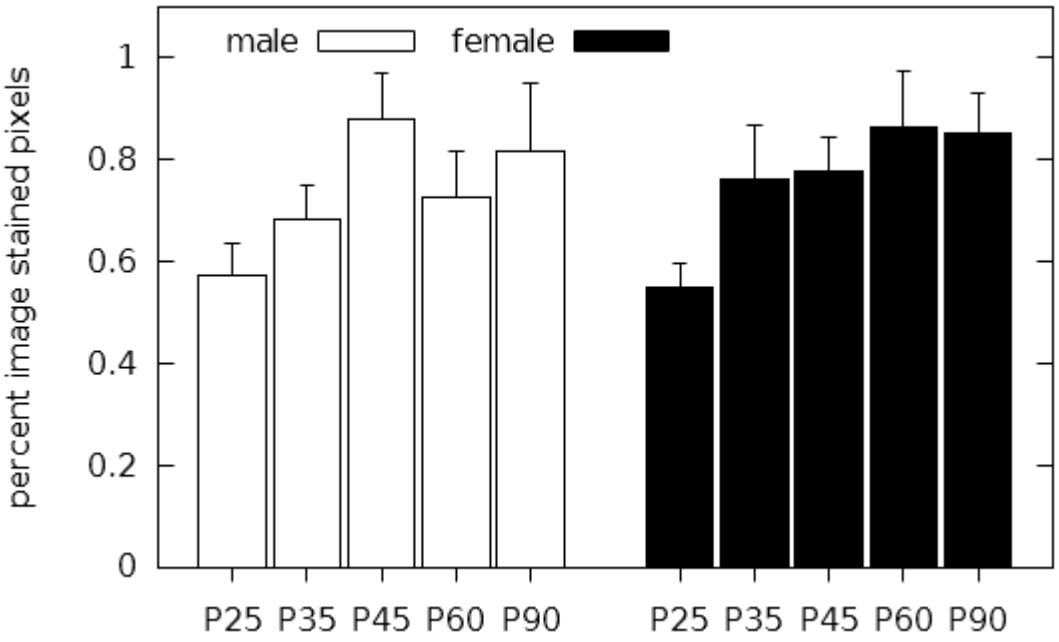


Figure 7: Percent stained of skeletonized layer 2/3 images. Significant effect of age: F(4, 90)=3.237; p=.016. Means + SEM.

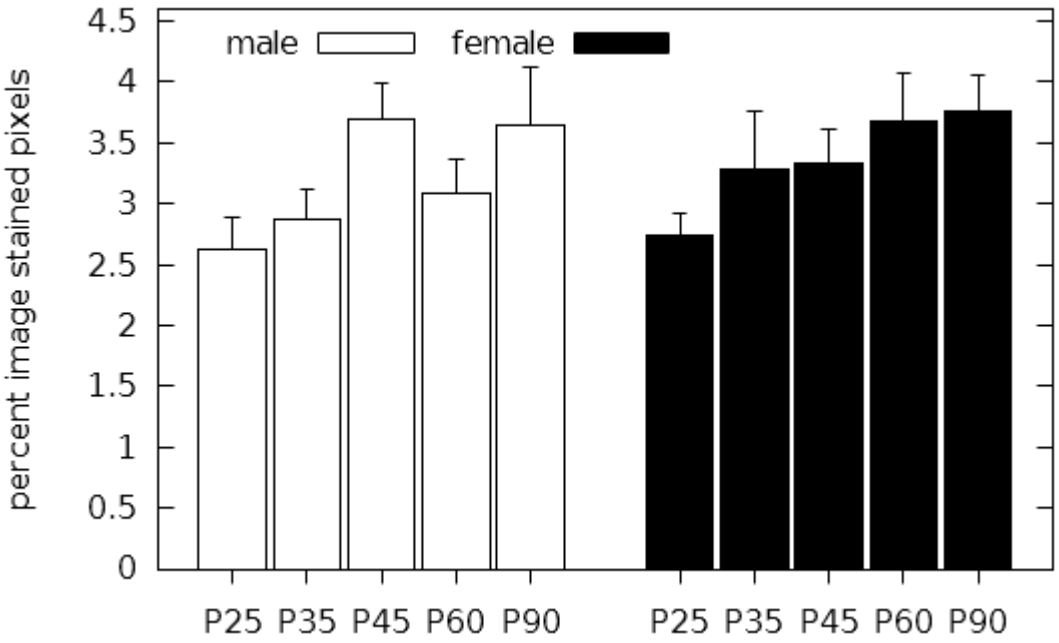


Figure 8: Percent stained of unprocessed layer 2/3 images. Significant effect of age: F(4, 90)=2.596; p=.042. Means + SEM.

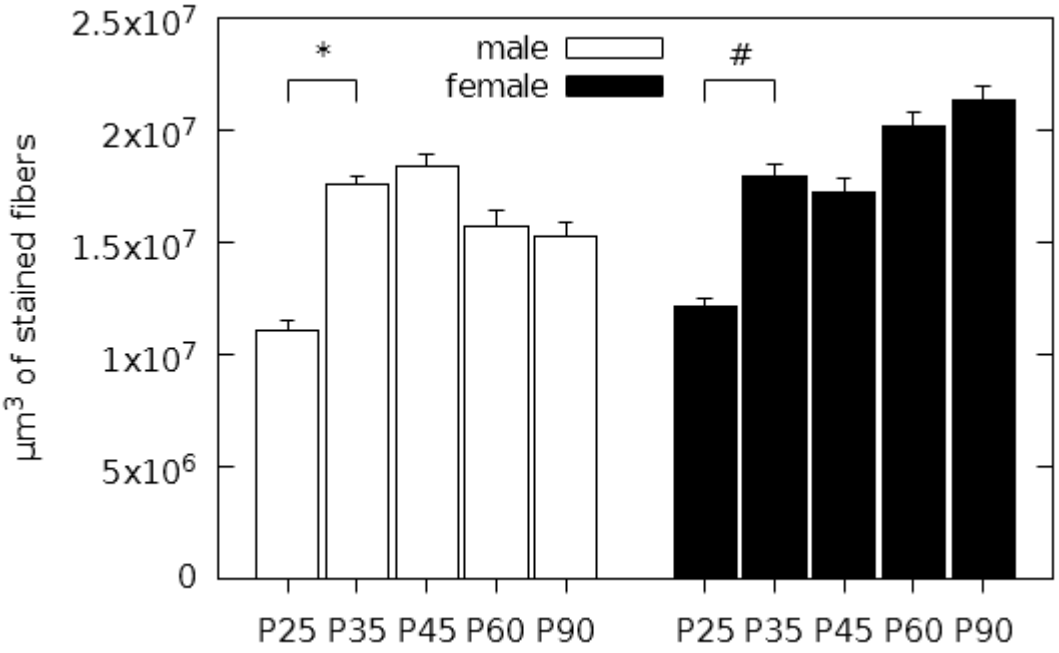


*Figure 9: Volume (µm³) of stained axons from binary processed layer 2/3 images. Significant effect of age in males: F(4, 44)=2.939, p=.031; and females: F(4, 42)=2.890, p=.034. *: p<.05; #: p<.10. Means + SEM.*

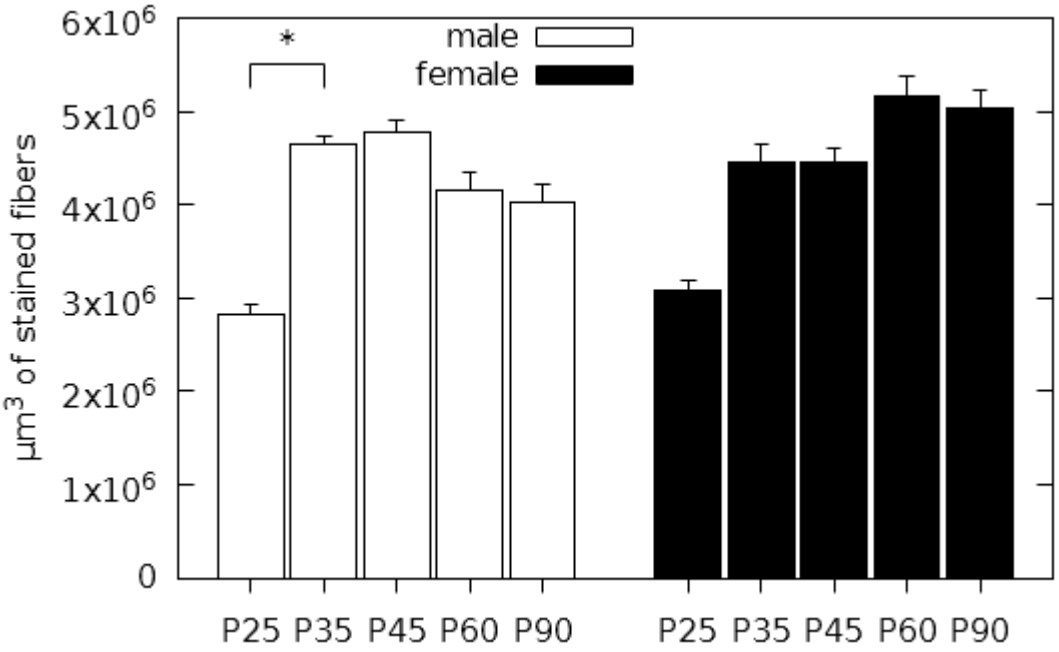


*Figure 10: Volume (µm³) of stained axons from skeletonized layer 2/3 images. Effect of age in males: F(4, 44)=3.268; p=.020. Trend of age in females: F(4, 42)=2.292, p=.075. *: p<.05. Means + SEM.*

Discussion

The work presented here finds no significant sex differences in the increase of TH immunoreactive axons in the rat mPFC, and with much of the increase seeming to occur prepubertally in both sexes.

Previous work has shown an increase in TH density, and in the dopaminergic system, in the mPFC over the course of adolescence in male rats in particular. Kalsbeek et al. (2008) qualitatively described mPFC dopamine-immunoreactive fibers from embryonic development to P60 and P90, but did not report potential sex differences from their mixed-sex subjects. They found an increase in dopamine-containing axons between P20 and P60, but no changes from P60 to P90, and they report the increase as being obvious in the upper layers of prelimbic mPFC. Naneix et al. (2012) examined a number of dopaminergic features in the mPFC, but used only male rats. They found a gradual increase in TH-immunoreactive fibers in the mPFC from P25 to P70, along with an increase in cortical dopamine between P45 and P70 and a peak in the expression of several dopamine receptor mRNAs around P45. Other studies report overproduction of dopamine receptors in late adolescence in the male rat mPFC (Andersen et al., 2000). However, the possibility of sex differences in the development of this system has not been well-studied.

The timing of TH increases observed here suggests that these changes are not mediated by pubertal hormones in male rats. Androgens surge around P45 with the onset of puberty in males. Since no hormonal quantification or manipulation was performed, whether pubertal hormones in females ramping up around P35 occurred early enough to affect TH cannot be ruled

out. That males and females may have different mechanisms underlying development is not unheard of. Changes in neuron number in the rat mPFC over adolescence have been associated with ovarian but not testicular hormones (Koss et al., 2015) Adult animals show effects of gonadal hormones on TH. Adult male rats show increased TH density in prefrontal cortex following orchiectomy which is reduced with testosterone but not estrogen (Kritzer et al., 2007). A decrease in TH density caused by androgens during adolescence might be overwhelmed and hidden by stronger coincident growth of fibers resulting in a net increase in density, or the neurons may respond differently at this stage of development so that androgens do not negatively influence density. In adult rats, some ventral tegmental dopaminergic neurons that project to the mPFC express androgen receptors and a smaller number express estrogen receptor β (Kritzer and Creutz, 2008), and counts of TH positive neurons in the ventral tegmental area can change by P90 following orchiectomy on P60 (Johnson et al., 2010), suggesting androgens can influence the dopaminergic system before the final age examined here. Whether pubertal hormones affect the adolescent development of TH-immunoreactive projections to the mPFC directly, indirectly, or not at all, the dopaminergic system displays sex differences and may respond to perinatal and adult hormonal milieux (Gillies et al., 2014).

The present work considers only the upper layers of the cortex. However, TH immunoreactive fiber density is greater in layer 5/6 in the mPFC (Descarries et al., 1987). It is not improbable that the deeper layers may show more pronounced changes. Position within the layers of the cortex might not be the only influence on degree of observed change. When extremely rostral or caudal sections of the male rat mPFC are considered, an effect of age is seen in rostral, but not caudal, sections over adolescence (Naneix et al., 2012). The present work used

sections from about the same rostrocaudal level in each animal, about midway through the mPFC; the effects seen here may be muted or amplified compared to more extreme sections.

TH is used as a marker for dopaminergic neurons, but other cells may be immunoreactive. Besides background staining, noradrenergic axons project throughout the cortex and may be responsible for much cortical dopamine (Devoto and Flore, 2006). These fibers, however, are generally weakly staining for TH, depending on the antibody used (Verney et al., 1982; Verney et al., 1993; Lewis et al., 1987; Miner et al., 2003; Zhang et al., 2011). The rat mPFC is notable for being richly innervated by dopaminergic fibers but is not especially enriched in noradrenergic fibers compared to the rest of the neocortex (Devoto and Flore, 2006). Other studies which stained for dopamine β-hydroxylase, which converts dopamine to norepinephine, did not find an increase in mPFC noradrenergic fibers in male rats over adolescence (Naneix et al., 2012), or found distinct populations of TH fibers in primates where TH fibers survived lesioning of noradrenergic pathways (Lewis et al., 1987). Lesions of noradrenergic pathways do not significantly lower TH levels in medial prefrontal cortex (Emson and Koob, 1978). Tritiated norepinephrine reveals adult levels of noradrenergic fiber density in non-mPFC cortex prepubertally (Levitt and Moore, 1979). Thus, it is not thought that incidental staining of noradrenergic fibers has significantly influenced the present study. Additionally, a few interneurons in the cerebral cortex express TH but do not produce catecholamines and lose immunoreactivity with age (Asmus et al., 2008; Asmus et al., 2011). The location, and even presence, of these cells varies by species but in rats they tend to reside in layer 2/3 (Satoh and Suzuki, 1990; Benavides-Piccione and DeFelipe, 2007; Asmus et al., 2008) and are most common in a postnatal window from P16 ending around P40 (Asmus et al., 2008). It is not

expected that these cells affect the present results because they peak prepubertally and decrease rapidly.

The development of the TH innervation of the mPFC continues to pose questions. Since the increases in TH-immunoreactive fibers did not show sex differences and had the most significant gains prepubertally, insults to this system may be most disruptive when presented early. Barring hidden sex differences, male and female rats would be expected to display similar disruption to TH innervation and similar deficits in dopamine-related mPFC behaviors. How the development of the mPFC and sex differences in pathological outcomes are related remains to be clarified.

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