Sex differences in cortical astroglia throughout postnatal development

by

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Abstract

Sexual differentiation is a robust and well characterized phenomena of the mammalian central nervous system. There are many structural and functional sex differences characterized in various regions of the brain and many of the developmental etiologies of these have been well studied. Unfortunately, less well understood is the sexually dimorphic prevalence of psychiatric, neurological, and mood and anxiety disorders in the human population. Many of these psychiatric disorders, such as schizophrenia or autism, and mood disorders, like anxiety and depression, have developmental etiologies coinciding with organization of the neocortex. Many of the functions associated with neocortical development involve the most abundant cell type in the mammalian brain, astroglial cells. Because there is a need to study the risk and resilience of sex in the development of psychiatric disorders and, due to their role in cortical development, and their known responsiveness to circulating hormones, astroglial cells are an important avenue of study. In the present study, examined sex differences in cortical astroglial cells across potnatal development. Using a transgenic mouse model expressing green fluorescent protein in astroglial cells we studied astroglial-specific proteins in male and female mice at six time points associated with developmental milestones to understand sex differences in basic functioning and neurogenic potential. As expected, the absolute number of astroglial cells and glial acidic fibrillary protein expression increased across development but surprisingly, these did not differ between sexes. However, the neurogenic potential of cortical astroglia as suggested by vimentin and Ki-67 co-expression was sexually dimorphic in the postnatal period. The functional role of astroglial cells in organizing and maintaining sex differences in neocortical architecture and communication networks remains to be determined in future studies.

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TABLE OF CONTENTS

List of Tables

List of Figures

Abbreviations

E – embryonic

P – postnatal

CNS – central nervous system

PNS – peripheral nervous system

NSC – neural stem cell

BMP – bone morphogenic protein

SRY – sex-determining region Y

SOX – SRY-related high-mobility-group box

FGF – fibroblast growth factor

RGC – radial glial cell

Cxn – connexin molecule

GABA – γ-aminobutyric acid

KCC2 – K^+/Cl^- Co-Transporter 2

NDMA – N-methyl-D-asparate

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid

JAK – Janus kinase

STAT – signal transducer and activation of transcription

NG2 – neural/glial antigen 2

NRG – neuregulin

Trk – tyrosine receptor kinase

EAAT – excitatory amino acid transporter

ATP – adenosine trisphosphate

TGF – transforming growth factor

PKC – protein kinase C

NF-κB – nuclear factor κB

AP-1 – activator protein 1

ER – estrogen receptor

BBB – blood brain barrier

AR – androgen receptor

ADHD – attention deficit hyperactive disorder

mRNA – messenger ribonucleic acid

PFA – paraformaldehyde

DHPC – 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine

PBS – phosphate buffered saline

GFP – green fluorescent protein

GFAP – glial acidic fibrillary protein

Introduction

Differences between the sexes have long been contemplated, from ancient philosophy to the study of sex differences in health and disease. Models of psychiatric disease have largely examined male subjects presumably to simplify their findings. Indeed, using females in rodent models of disease would require the consideration of cycling hormone levels, which change across the four-day estrous cycle; adding estrous cycle as a variable increases complexity and potential interactions to data interpretation. Unfortunately, this has led to an entire database of decades of health studies conducted only in male subjects. Furthermore, many, if not all psychiatric diseases show sexually dimorphic prevalence and incidence (Bao and Swaab, 2011), highlighting the need to study both sexes in these models.

Not surprisingly, given its fundamental role in emotion and cognition, cortical structure and function is implicated in nearly all psychiatric illnesses and therefore the development of the cortex is imperative to psychiatric disease. Astroglial cells have been examined and described as key mediators of cortical development, however, sex differences of cortical astroglial cells during development have not been taken into account (Cahoy et al., 2008). The purpose of this study is to understand how cortical astroglial cells change in response to developmental hormones across the postnatal period leading to risk and resilience of males or females in the development of psychiatric illness.

Development of the Central Nervous System

Human nervous system development begins early during embryogenesis and continues to remodel existing neural circuits, well into adulthood. The zygote, the fusion from two parental haploid cells consists of the primary stem cells characterized by a totipotent phenotype, giving rise to pluripotent embryonic stem cells (Osakada and Takahashi, 2010). The nervous system

begins to develop after 18 days following conception (embryonic day 18; E18) as the embryonic stem cells begin to divide and give rise to the three germ layers: the ectoderm, mesoderm, and endoderm (Purves et al., 2012). Ensuing the development of the germ layers, the primitive pit condenses and elongates into the primitive streak and eventually the notochord (Purves et al., 2012). Lower mammalian models such as mice have similar developmental mechanisms of the central nervous system and as such, it is possible to elucidate the developmental origins of the human nervous system from rodent models (Johnson et al., 2009). These rodent models are particularly useful as postnatal day 0-14 (P0-P14) have similar developmental properties as humans during the third trimester of fetal development (Clancy et al., 2007). The following temporal references will be from mouse models of brain development, unless otherwise stated.

The foundation of the central and part of the peripheral nervous system (CNS and PNS, respectively) is established as the dorsal-most section of the ectoderm matures into the neural plate and eventually folding into the neural tube through morphogenic processes (Oskakada and Takahashi, 2010). Before the foundation of the CNS and PNS, however, the first cells to populate the eventual CNS are microglia. Microglia are the only neural cells that are not born locally in the CNS. Rather, microglia are synthesized in the yolk sac beginning at E8 until late in gestation; the first microglia migrate to the CNS at E9 (Alliot et al., 1999; Chan et al., 2007). Although microglia compromise only 5-10% of the cells within the CNS, they are vital to normal brain function and development (Lyck et al., 2009). Microglia are primary regulators of the immune response within the CNS and are able to induce the production of inflammatory cytokines, regulate homeostasis through scavenging, influence synaptogenesis, and aid in the maturation of neural networks (Reemst et al., 2016). Following the migration of microglia into the developing CNS, the first neuroectodermal-derived neural stem cells (NSCs) begin to

differentiate and will give rise to the remaining cell types within the brain. NSCs have inherent capability of infinite self-reproduction and are the basic cells that are able to differentiate into both neurons and neuroglia, like astroglia and oligodendrocytes (Temple, 2001; Castrén, 2012).

Bone morphogenic proteins (BMPs) facilitate stem cell differentiation to epidermal cells whereas exposure to Noggin and Chordin antagonizes BMPs and allow stem cells to develop into NSCs (McMahon et al., 1998). NSCs are able to undergo asymmetrical division based on extracellular signaling from proximal cells within the germ layers and dictate the regional and temporal identity of developing cells via the activation of intracellular signaling cascades facilitating changes in pro-neural gene expression. Anterior neural plate NSCs are fated through BMP antagonism which allows the activation of SRY-related high-mobility-group box (Sox) proteins to influence pro-neural gene expression (Pevny et al., 1998). Similarly, posterior neural plate NSCs are formed through BMP antagonism-induced Sox expression paired with retinoic acid, Wnt, and fibroblast growth factor (FGF) signaling (Bibel et al., 2004; Haskell, 2005; Oskakada and Takahashi, 2010). Most importantly, the cerebral cortex is a highly specialized and vast brain area that is characterized by several stages of development beginning with the development of neural stem cells from the anterior lateral neural plate (Götz and Huttner, 2015).

Cortical Neurulation

Cortical neurulation is a complex and temporally staggered process that begins with the development of neuroblasts from NSCs. Primarily, the ventricular zone in the developing embryo is a neurogenic niche that persistently generates new NSCs which migrate radially to the respective layers of the cortex (Castrén, 2011). Organization of the cerebral cortex is aligned such that that the outermost layer known as layer I and the layer most proximal to the ventricular zone is known as layer VI. Neuroblast migration is temporally segregated generating an “inside-

out" mode of development, meaning that proximal layers being populated first and consequently neuroblasts in the upper layers must migrate across the already formed lower layers to reach their objective layer (Berry and Rogers, 1965). Neuroblasts synthesized in the ventricular zone express differing transcription factors that dictate which layers of the cortex the cells will migrate to. For example, cortical neuroblasts residing in deep cortical layers, like layer V and VI, express *Otx-1* and *ER81* at E10 and correlate with axonal projections to distal regions of the thalamus, whereas *Cux-1* is expressed at a later time-point in neuroblasts that will populate the upper layers of the cortex at E17 (Weimann et al., 1999; Nieto et al., 2004; Molyneaux et al., 2007). Many of the proteins involved with regional neuron identity have unclear molecular targets, but likely are used for axonal guidance to target layers of adjacent cortical and lower brain region projections (Zhang et al., 2015).

Although genes influence regional identity of neuroblasts, the motility of developing neuroblasts consists of three primary mechanisms. Most often, neuron migration is attributed to complex cross-talk between immature neurons and radial glial cells (RGCs) but more recent evidence indicates neurons may migrate through soma translocation and tangential migration (Nadarajah et al., 2001; Luhmann et al., 2015). Primarily, RGC-mediated neuroblast migration was first proposed by Rakic in the early 70's with evidence that RGCs extend processes to proximal migrating neuroblasts to aid in motility, the adhesion mechanisms between RGC processes and neurons are the result of connexins (Cxns) expressed in the glial cells (Rakic, 1972; Brittis et al., 1995; Elias et al., 2007). Similarly, progenitor expression of Ca^{2+} ions is essential to mediate adhesion between migrating neurons and RGC counterparts (Elias et al., 2007). RGC-assisted neuronal migration is more prominent during the "inside-out" phase of

cortical development and is mediated through the main excitatory neurotransmitter, glutamate (Nadarajah et al., 2003).

The latter modes of motility, soma translocation and tangential migration, offer a different view of neuroblast migration. Soma translocation is the movement of neurons via somatic extensions, resembling growth cones, extending between cellular structures and retracting, pulling the soma closer to the target area (Nadarajah et al., 2001). However, developing neurons are not restricted to one mode of motility and exhibit both radial migration and soma translocation to reach distal targets (Nadarajah et al., 2001). As a consequence of the deep laying neurogenic niche in the subventricular zone, most developing neurons use soma translocation to reach the ventricular zone and preplate rapidly, allowing RGC-assisted mechanisms to guide the developing neurons into their respective upper cortical layers (Luhmann et al., 2015). Developing neuroblasts express tyrosine receptor kinases allowing the cell to autophosphorylate to extend radial processes through microtubule construction, facilitating somatic translocation in a Ca^{2+}-mediated manner (Marín & Rubenstein, 2003). As such, most of the glutamatergic neurons in the cortex migrate in a purely radial manner via RGCs (Luhmann et al., 2015).

A Shift in Excitability

The cortex is the master regulator of higher order cognitive function based on intricate communication between excitatory and inhibitory signals. Primarily, glutamate is the main excitatory neurotransmitter in the adult brain whereas γ-aminobutryic acid (GABA) acts as the negative regulator of glutamatergic signals. During development however, immature neurons express high concentrations of intracellular Cl^- anions, allowing GABAergic to act as excitatory cells during neuronal migration and synapse development (Ben-Ari et al., 2007). The formation of functional GABAergic and glutamatergic circuits is sequential. During cortical development,

GABAergic synapses are established before glutamatergic counterparts (Ben-Ari, 2002). It is however notable that both layer-specific interneuron and projection neuron migration occurs simultaneously, establishing microcircuits using the developmental properties of both GABA and glutamate (Molyneaux et al., 2007; Lodato et al., 2011). The excitatory GABAergic neurotransmission is essential for regular migratory patterns of developing neurons through activity of the ionotropic $GABA_A$ whereas the trophic effects of GABA are mediated via metabolic $GABA_B$ receptor subtypes (Behar et al., 2000; Represa, 2007). Similarly, GABA receptors are essential for the formation of mature neuronal networks through spontaneous and coincidental massive depolarizing potentials which initiate oscillatory waves of Ca^{2+} to facilitate cell-to-cell communication (Garaschuk et al., 2000; Ben-Ari, 2002). GABAergic transmission assumes a canonical inhibitory role in cortical networks around P14 following a shift in K^+/Cl^- Co-Transporter Channel 2 (KCC2) expression on the maturing neurons (Ben-Ari, 2002). GABAergic interneuron migration cessation occurs coincidentally with the upregulation of KCC2 at P7 that reverses the intercellular Cl^- concentration responsible for the excitatory actions of GABA during development (Bortone and Polleux, 2009). The migration facilitated by GABA is regulated through glutamatergic activity via canonical glutamatergic receptor signalling. The two main glutamatergic targets during migration are the Ca^{2+}-dependent N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) receptors. However, the exact mechanisms of NMDA- and AMPA-mediated migration are yet to be elucidated (Maskos and McKay, 2003; Jansson et al., 2013). Migration requires both AMPA and NMDA receptor activity by modulating Ca^{2+} concentration whereas ionic GABA receptors are required for giant depolarizing potentials and the maintenance of cell motility (Bortone and Polleux, 2009). NMDA and AMPA receptors are not only relevant in migration, but serve as

regulators in cortical synaptogenesis in the postmigratory period. Cortical synaptogenesis becomes prevalent only after the first 19-23 weeks of human gestation (Molliver et al., 1973). The extensive period of synaptogenesis is regulated via glial cells that are generated after neurogenesis during gliogenesis. Astroglial cells are essential in synapse formation, modulation, and removal, and are the most abundant cell type in the mammalian brain comprising 50% of cells in the adult human brain (Cahoy et al., 2008). Cxns, those molecules responsible for adhering migrating neurons to RGCs, are one of the main mediators in network formation throughout the neocortex (Nadarajah et al., 1997). Interestingly, astroglial cells express and mediate Cxns, critical to their role in establishing and modulating synaptic connections.

Gliogenesis

Simultaneously occurring with the end of neurogenesis, extracellular cues initiate a switch from neurogenesis to gliogenesis. The switch between neurogenesis and gliogenesis occurs during the third trimester in humans and within the first 10 postnatal days in rodents (Salmaso et al., 2014). During this time, molecular cues enhance Notch and Janus kinase/ signal transducer and activator of transcription (JAK/STAT) signaling swinging progenitor cell pro-neuronal gene expression to pro-glia gene expression (Avet-Rochex et al., 2012). The glial cells being generated following the period of neurogenesis include astroglia and oligodendrocytes. FGFs are astroglial specific growth factors that are found in the epidermis but are vital in the CNS for the switch to pro-glia gene expression. FGF receptor signaling acts upstream of *olig2* and *Sox9* in progenitors, initiating a switch to oligodendrocyte differentiation and oligodendrocyte or astroglia differentiation, respectively (Esain et al., 2009). Consequently, as oligodendrocytes and astroglia mature, the stem cell potential of the glia diminishes, marked by an increase in glutamate synthetase in astroglia and myelin basic protein in oligodendrocytes,

coinciding with decreased expression of Sox2, vimentin, and neural/glial antigen 2 (NG2) (Salmaso et al., 2014).

Astroglial cells display heterogeneity in morphology that is related to different astroglial cell functions. Classically, there are three main subtypes of astroglial cells. The two mature subtypes are characterized as protoplasmic; canonical astroglia with large domains of many fine processes and fibrous; astroglial cells with many long fibrous extensions (Sofroniew and Vinters, 2010). Both the mature subtypes of astroglial cells interact with blood vessels however protoplasmic astroglial cells modulate synaptic activity whereas fibrous astroglial cells envelope and modulate activity at the nodes of Ranvier along axonal projections (Sofroniew and Vinters, 2010). The third main subtype of astroglial cells are the immature RGCs that populate the brain early in development. Aforementioned, RGCs are required for neuron migration but also act as neural progenitor cells in all regions of the developing brain (Anthony et al., 2004).

Interestingly, mature astroglia are derived from RGCs that have already populated the brain during cortical neurulation through neuregulin (NRG) signaling. Specifically NRG-1 signaling through tyrosine receptor kinases (Trk) drives RGC transformation to astroglia (Schmid et al., 2003). Like migrating neurons, astroglia migrate through soma translocation but their migration is influenced in an FGF-mediated manner (Smith et al., 2006). Individual astroglia have large and distinct spatial domains allowing them to interact independently from other astroglial cells with thousands of synapses (Kacerovsky and Murai, 2016). Primarily, astroglial cells were thought of as support cells to influence a healthy milieu for neurons to survive and thrive, however the role of astroglia regulating microenvironments, and neural circuit development, maturation, and synapse modification in the CNS is becoming more apparent.

Synaptogenesis

Mature astroglia are a unique subset of glia within the CNS that have been attributed with the formation, maintenance, modulation, and removal of synapses (Clarke and Barres, 2013). Broadly, one of the main tenants of astroglia is their ability for glutamate uptake through excitatory amino acid transporters (EAAT) to buffer the excitotoxic effects of excess glutamate activity at the synapse and also modulate intracellular Ca^{2+}signalling (Sutherland et al., 1996; Gittis and Braiser, 2015). Intracellular Ca^{2+} signaling in astroglia is sufficient to alter gene expression and induce the release gliotransmitters like glutamate and adenosine trisphosphate (ATP), and other processes with the ability to modulate synaptic characteristics (Di Garbo et al., 2007). The adhesive properties of Cxns during migration extend into the post-migratory period while astroglia are creating and modulating synapses and highlight the importance of glia-neuron communication in the formation of synapses and functional neuronal networks.

Astroglial cells expressing Cxn-43 modulate activity of glutamatergic circuits in the hippocampus through neuron-astroglia communication (Chever et al., 2014). The action of Cxns in the hippocampus may be present in other glutamate-rich areas of the brain, specifically in the cortex. Cortical astroglia also express transforming growth factor β-1 (TGF-β1), which has been implicated in the development of both excitatory and inhibitory synapses (Diniz et al., 2012; Clarke and Barres, 2013). In culture, neuronal clusters that do not have proximal contact with astroglia fail to form synapses, elucidating that cell-to-astroglia contact is vital for the induction of Ca^{2+} signaling in neurons (Hama et al., 2004). Although astroglia are clearly implicated in the development of excitatory synapses that mediate glutamate activity in the cortex and hippocampus, inhibitory synapses are equally important and are similarly influenced by astroglia (Clarke and Barres, 2013). Correspondingly, TGF-β1 signaling in astroglia has been shown to

mediate excitatory synaptogenesis, and recently, emerging evidence reveals that it may also induce inhibitory synaptogenesis through D-serine modulation (Diniz et al., 2012). While there are undoubtedly many other cellular mechanisms involved in inhibitory synapse formation, the nature of these and the extent of astroglial involvement remains to be determined.

Excitatory synapses require astroglia not only for synaptogenesis, but also for synapse maturation and maintenance through contact signals. Excitatory neuron terminals with proximal astroglia contact increase protein kinase C (PKC) expression in the pre- and postsynaptic membranes, ultimately facilitating the up regulation of AMPA gluR1 subunit expression (Hama et al., 2004). Astrocytes also mediate synaptogenesis through chemical messengers like BMPs, such as TGF-β1, which is regulated through astrocytic release of D-serine (Cahoy et al., 2008). TGF-β1 signaling up regulates NDMA receptor expression in neurons and increase CaMKII expression leading to the subsequent release of glutamate from astroglia and the strengthening of inhibitory synapses in the developing CNS (Diniz et al., 2014). Interestingly, D-serine is the co-agonist to NDMA receptors and is released by astroglia to regulate neuronal signaling and strengthen synapses (Diniz et al., 2012).

Importantly, many of these synaptic modulatory processes are regulated by transcription factors. EAAT2 expression on astroglia is influenced through the neuron -activated transcription factor nuclear factor (NF)-κB. Specifically, the canonical p50/p65 activator protein-1 (AP-1) transcription factor heterodimer is the most abundant NF-κB complex in humans and is expressed by neurons. The AP-1 complex translocates to the nucleus of astroglial cells and binds to the EAAT2 promoter to facilitates a self-mediated upregulation of transcription (Ghosh et al., 2011). Similarly, EAAT2 expression is increased in the cortex of individuals with schizophrenia (Matute et al., 2005), a psychiatric disorder that affects more males than females (Bao and

Swaab, 2011). Adding to the allure of this developmental anomaly, endogenous estrogens can suppress the NF-κB AP-1 complex activation through estrogen receptor alpha (ERα), alluding to potential mechanisms for the prevalence of some sexually dimorphic neuropsychiatric disorders (Cerillo et al., 1998; McCarthy, 2010). Understanding the various molecular signals in sexual differentiation of the brain will illuminate an understanding of the sexually dimorphic prevalence of neuropsychiatric diseases and mood disorders.

Sexual Differentiation

The fundamental basis of sexual differentiation in mammals relies of the presence of the Y chromosome, which encodes the sex-determining region (SRY) gene (Swaab, 2007). Sox genes are highly conserved across eukaryotic organisms and influence increased DNA-affinity to transcription factors (Lefebvre et al., 2007). The SRY gene influences the expression of hormones vital for defeminization and masculinization of sex organs in the developing embryo. Testosterone secreted by the testes of a developing embryo migrates to the brain and, being a steroid hormone, readily crosses the blood brain barrier (BBB) to exert its effects centrally (Raskin et al., 2009). Testosterone transported to the CNS is readily aromatized by cytochrome p-450 into estradiol, which then binds to ERs to induce masculinization of nervous tissues through various mechanisms (Bodo and Rissman, 2008). Estradiol interacts with ER-α or ER-β on both neurons and astroglia to facilitate cellular functions such as genomic regulation in neurons enabling apoptosis and neural branching, and in astroglia to stimulate synthesis of neuroprogesterone (Kudwa et al., 2005; Michevych et al., 2007; Wright et al., 2010).

Although most sexual dimorphism research focuses on the hypothalamus, there are marked sex differences in the telencephalon, which comprises of the neocortex, hippocampus, amygdala, and basal ganglia. The amygdala (Johnson et al., 2013) and hippocampus (Woolley

and McEwen, 1990) have well established sexually dimorphic properties dependent on the expression of androgen receptors (ARs) and circulating endogenous hormones. As such, it is not impossible to conceive the idea of the cortex exhibiting sexually dimorphic features in a similar manner to the amygdala and hippocampus.

A Delicate Dance Between Development, Sex, and Astroglia

It is evident that the development and maturation of NSCs into fully functional cortical circuits is temporally sensitive and relies on a complex and wide range of activating molecules, signals, and cell communication. Further adding to the complexity of telencephalic development is the influence of peptide hormones on any or all of these developmental processes and their ultimate contribution to the formation of a mature, sexually differentiated brain. While substantial work has been done to understand hypothalamic sexual differentiation (Gorski and Wagner, 1965; MacLusky and Naftolin, 1981; Swaab and Hofman, 1988; Swaab and Hofman, 1995), less research has examined sexual differentiation of the telencephalon. Yet, as aforementioned, almost all psychiatric disorders in which the hippocampus and cortex are implicated show sexual dimorphism including schizophrenia, autism, and ADHD being more common in males; in females, depression and anxiety disorders are more than in males (Bao and Swaab, 2011). It is therefore difficult to ignore the possibility that sexual differentiation of the telencephalon may play a role in mediating the sexually dimorphic nature of these psychiatric disorders. Understanding that astroglia are implicated in every stage of neural development leading to the expression of sexually dimorphic neuropsychiatric conditions begs the question as to whether and how astroglial processes may be influenced by circulating hormones.

As we gain greater understanding of CNS development, it is now vital to consider the sexually dimorphic nature of the organizational processes mediated by astroglial cells including,

but not limited to, migration and synaptogenesis, that underlie mammalian nervous system development. It is clear that almost all psychiatric illnesses are sexually dimorphic and yet we rarely consider sexual differentiation when studying the pathophysiology of these disorders. This is not surprising given the sheer number of processes involved and the temporal sensitivity of many of these. When studying cortical development, including sexual dimorphism as a variable therefore increases the level of complexity of the results exponentially, and it is a challenge to know which processes of cortical development to examine first. However, whatever processes we consider, they should at minimum, show sexual dimorphism and involve mechanisms that can be mediated by hormonal status. Beyond the sexually dimorphic hypothalamus, which is less likely to be directly involved in the higher cognitive processes involved in psychiatric illnesses, the cortex does show sexual dimorphism. There is a vital role for astroglia in the migration and maturation of NSCs into functional neurons and development and integration of neurons into new and existing cortical networks. In addition, astroglial cell function and activity are clearly responsive to and modulated by circulating hormones, therefore, consideration of these cells as mediators of cortical sexual differentiation may be warranted in future studies.

Using a mouse model, we outlined the developmental changes in astroglial morphology and protein expression in the cortex between males and females at time points relating to critical developmental processes. Understanding the differences and dynamic properties of astroglial cell morphology and protein expression throughout postnatal development in both males and females may elucidate mechanisms for the sexually dimorphic discrepancies in mental illness and psychiatric disease prevalence in the population. For example, critical shifts in astroglial cell function influencing the “male brain” to be developmentally biased towards the development of neuropsychiatric illnesses, such as autism, schizophrenia and substance abuse disorder, whereas

the “female brain” may be developmentally biased towards mood and generalized anxiety disorders, eating disorders, and neurodegenerative diseases; in addition to gender dysphoria (Bao and Swaab, 2011). Since the default mode of sexual brain development is female (Phoenix et al., 1959) and females typically begin and end puberty earlier than males (Kail, 2010), we hypothesized that females may exhibit a mature astroglial profile sooner in development than male counterparts. Additionally, we hypothesized that (a) the cortex will be sexually dimorphic in terms of volume; and specifically pertaining to astroglial cells, (b) the number and morphological characteristics of astroglial cells and (c) the proteins expressed by astroglial cells will be sexually dimorphic across development. To understand the developmental time-shift between males and females we examined the expression of astroglia-specific proteins highlighting basic astroglial cell function and the neurogenic potential of astroglial cells in the developing mouse brain. We examined astroglia in mice at P1 coinciding with the end of neurogenesis, P4 coinciding with the end of neuron migration, P7 coinciding with synaptogenesis and peak gliogenesis, P14 coinciding with peak synaptogenesis and myelination, P35 coinciding with sustained myelination and synaptic elimination/pruning, and in adulthood coinciding with a mature state (see figure 1 below). Previous reports have developed genetic assays of astroglia and other neural tissue in the developing brain (Cahoy et al., 2008), however, these reports did not consider the role of sex and circulating hormones in their analysis and offer an avenue to understand the complex underpinnings of the role astroglial cells play in the development of a sexually dimorphic cortex.

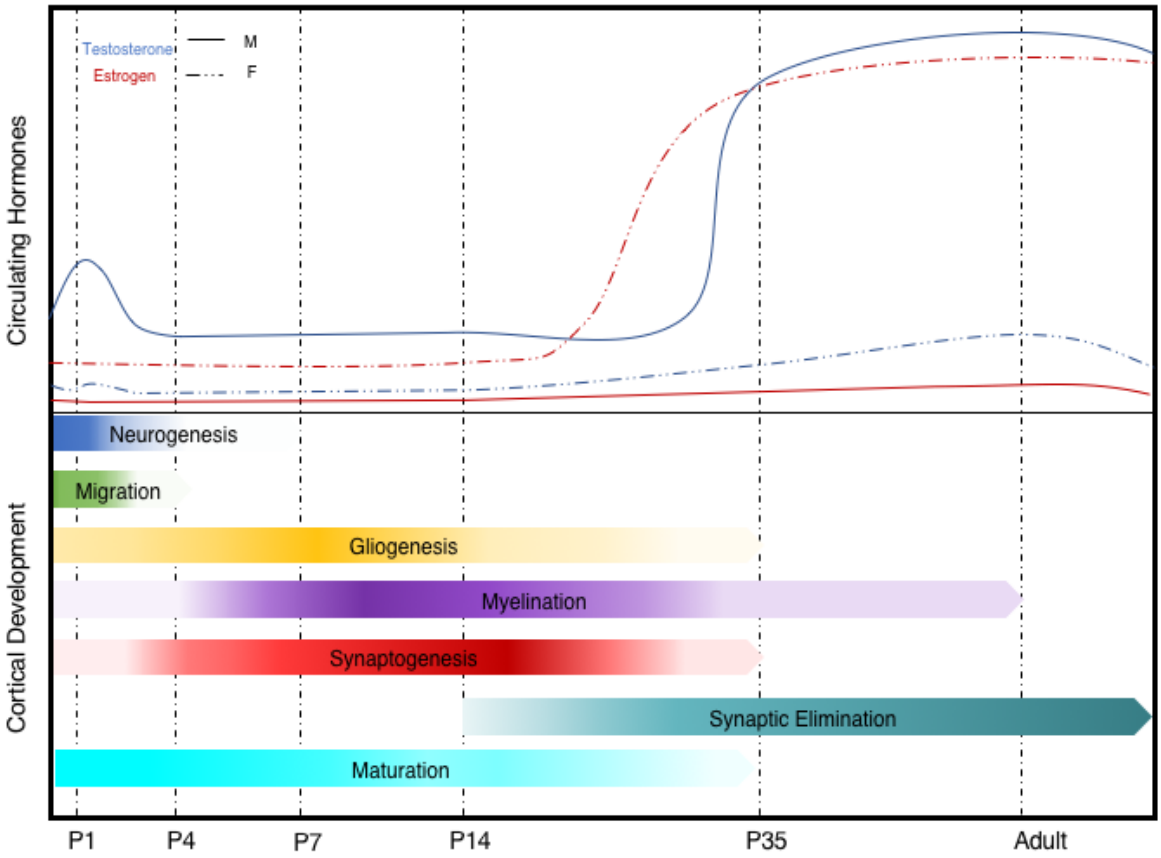

Figure 1. Circulating hormone levels and important cortical events during normal mouse development. The top panel represents circulating testosterone (blue) and estrogen (red) levels across development in males (solid lines) and females (broken lines). Adapted from (Ellis 2004; Gillies and McArthur, 2010). The lower panel represents major events during cortical development. The event lines indicate when each stage occurs. The lighter the colour is indicates when the event is less active and the darker the colour is indicates when the event is most active. The time points on the bottom axis represent postnatal days used for the present study. Adapted from (Anderson, 2003; Estes and McAllister, 2016).

Methods

Animals

Subjects used were male and female C57/Bl6-AldHl1-L10-GFP transgenic mice generated from our breeding colony maintained at Carleton University. Mice were sacrificed from postnatal day 1* (P1) (defined as 24-36 hours postnatal) to adulthood (defined as 7-9 weeks of age) at the same time of day. All animals were group-housed until appropriate sacrifice date in standard (27cm x 21cm x 14cm), fully transparent polypropylene cages with chew block, bedding, plastic house and *ad libitum* access to standard lab chow (2014 Teklad Global 14% protein®) and water. Animals were raised in the standard environment with no outside manipulation except for standard care and to monitor estrous cycle stage. The mice were maintained on a 12-hour light/dark cycle in a temperature controlled (21 degrees) facility. All animal use procedures have been approved by the Carleton University Committee for Animal Care, according to the guidelines set by the Canadian Council for the Use and Care of Animals in Research. In total, there are 6 time points at which both males and female mice were sacrificed for tissue analysis for a total of 12 experimental groups. Developmental time points and animal use is summarized in the table below.

Age/Sex	P1*	P4	P7	P14	P35	Adult
M	5 (2)	5 (3)	3 (3)	5 (2)	4 (2)	5 (3)
F	4 (2)	4 (3)	4 (3)	5 (3)	5 (3)	5 (5)**
Total	9	9	7	10	9	10

Table 1. Animal Use Summary Table. Number of animals used and in parenthesis the number of litters mice originated from. *indicates animals in P1 time point are to be used between 24 and 36 hours postnatally. ** indicates females being used at metestrus stage of estrous cycle.

Estrous Cycle Monitoring

All adult group female animals were monitored daily after P35 to identify stage of estrous cycle using a saline lubricated swab inserted into the opening of the uterus to collect cells from the vaginal wall. Samples were smeared on a glass microscope slide and examined under 10x upright light microscope (VistaVision^{TM}). As we were interested in the baseline organizational sex differences in astroglia, we considered the point at which circulating hormones have the least immediate effect on astroglial morphology and protein expression: metestrus. After the stage of estrous cycle (Metestrus, Diestrus, Proestrus and Estrus) had been established for a least two full cycles, females were sacrificed during the metestrus stage.

Animal Sacrifice

Rapid Decapitation

Animals in the P1 immunohistochemical group were rapidly decapitated and brains immediately placed in a 4% paraformaldehyde (Fisher Scientific) (PFA) solution at 4 ºC for twenty-four hour period after which the brains were switched to 30% sucrose at 4 ºC for another twenty-four hour period. Following this period, brains were flash frozen until slicing.

Cardiac Perfusion for Immunohistochemical Analysis

Animals were sacrificed at their respective time point for tissue collection at the same time of day during the light cycle. All animals in the immunohistochemical group underwent cardiac perfusion aside from those in group P1 (see above). Animals in P4, P7, P14, P35 and adult groups were given an overdose of 44mg/kg sodium pentobarbital (CDMV Canada),

followed by intra-cardiac perfusion upon all spinal reflex cessation. Due to their relative small size, animals from the P4 and P7 groups underwent manual cardiac perfusion wherein the circulatory system, through left atrium, was flushed with 1mL of saline solution before receiving 1mL of 4% PFA via syringe. For animals in P14, P35, and adult groups blood was flushed using 10mL of saline through the left atrium followed by 20mL of 4% PFA to fix the tissue. Brains were extracted and placed into vials containing 4% PFA and put on ice until being properly stored at 4 ºC. Following a twenty-four-hour period, the brains were transferred to a 30% sucrose (Fisher Scientific) solution and placed at 4 ºC.

Tissue sectioning

Following sucrose treatment, all brains were then flash frozen at -80 ºC until sectioning on a Leica (Leica™ CM1900) cryostat (30µm thick). 15 sets of sagittal sister sections were adhered to electrostatic slides (Fisher Scientific™) in rotating order, each slide therefore contained a full representation of the brain for stereological analysis as per previous studies (Salmaso et al., 2012; Komitova et al., 2013). Following slicing, representative slides for each mouse was used for immunohistochemistry for analysis of astroglial cells (e.g., changes in morphology) and of astroglial mediated protein expression with emphasis on the cortex.

Immunohistochemistry

All immunohistochemical processes take place at room temperature (~21 ºC) as per our previous studies (Bi et al., 2011; Salmaso et al., 2012, Salmaso et al., 2015). One representative slide was taken from every subject and all subjects were processed simultaneously. Brain tissue from all groups were prepared for immunohistochemistry using a 10% horse serum (Gibco™)

PBS-T (0.3% Triton (Fisher Scientific)) pre-block solution for 1 hour before being incubated with the respective primary antibody solution (see table 2 below). Primary antibodies were diluted in 10% horse serum PBS-T (0.3% Triton). Following approximately twenty-four hours of primary antibody incubation, slices were washed in a 1x PBS solution 3 times to remove unbound antibodies before being incubated with the species-appropriate fluorescein conjugated secondary antibody for visualization. Secondary antibodies were diluted in 10% horse serum PBS-T (0.3% Triton) and incubated for 2 hours before slides were submerged in a 1x PBS wash 3 times to remove unbound antibodies. Slides were then coated with a nuclear stain, DAPI with hard setting mounting medium (Vector), to fix glass cover slips (Fisher Scientific) and allowed to set before analysis. See table below for antibodies used for analysis:

Antibody	Supplier	Species	Dilution
GFP	Abcam	Chicken	1:1000
GFAP	Sigma	Mouse	1:500
Vimentin	Sigma	Mouse	1:500
Ki67	Abcam	Mouse	1:500
Donkey Anti-Chicken	Invitrogen Alexa Fluor 488	n/a	1:1000
Donkey Anti-Mouse	Invitrogen Alexa Fluor 546	n/a	1:500

Table 2. List of Antibodies used for Immunohistochemistry

Stereological Analysis

Unbiased estimates of cell numbers are obtained through use of Zeiss AxioImager M2 with ApoTome motorized fluorescent microscope (Carl Zeiss, Thornwood, NY, USA) in conjunction with a motorized stage and a computer running on Windows 7 using the program

StereoInvestigator™ (MicroBrightfield, Colchester, VT, USA). Serial sagittal sections of the right hemisphere obtained through cryosectioning at 30µm on 15 sister sections were used for stereological analysis. From the right hemisphere brain sections, contours encompassing the whole right hemisphere cortex on each section were drawn as boundaries in StereoInvestigator™ as accurate counting areas. Cells were counted for expression of individual and/or co-expressed proteins using the optical fractionator probe at 40x. Sampling grids were optimized for cortical contours to include at minimum 3 sampling sites per contour to allow for a systematic and unbiased method to estimate cell density and cell quantification for right hemisphere of the cortex regardless of cell shape, size, orientation, spatial distribution, or post-mortem brain shrinkage (Schmitz and Hof, 2005). Sampling boxes automatically placed by StereoInvestigator™ are three-dimensional within the sampling frame measuring 150µm x 150µm x 30µm with 3 of 6 exclusion borders. Total number of cells per count are reported via StereoInvestigator™ output. For analysis of astroglial morphology, StereoInvestigator™ (MBF, Vermont ™) software on a Zeiss Observer with Apotome (Zeiss™) was employed. The observer identified and distinguished between radial and non-radial astroglial cells, counting these using unbiased sampling via the StereoInvestigator (MBF, Vermont ™) at 40x.

Representative confocal images used for photomicrographs were taken using ZEN software (Zeiss™) with the Airyscan 800 microscope (Zeiss™).

Statistical Analysis

All data was analyzed using a 2 (male vs. female) x 6 (P1 vs. P4 vs. P7 vs. P14 vs. P35 vs. Adult) between-subject analyses of variance (ANOVA) design using IBM SPSS Statistics (Version 20.0). If interactions observed are $p \leq 0.05$ they are considered significant and post-hoc

analysis using Bonferroni pairwise comparisons were conducted. When warranted, two-tailed t-tests to compare males and females in the same age group were completed and reported as significant when $p \leq 0.05$. In addition, we re-ran all of the statistics including litter composition (male vs. female) as a covariate and found no significant effects of litter on any of the variables measured (data not shown).

Results

Cortical Volume

Using unbiased stereology, we assessed neocortical volume for changes across age and sex. Neocortical volume did not exhibit sexual dimorphism ($p>0.05$). Not surprisingly, we observed a significant increase of brain volume due to age ($F=115.70$; $p<0.01$) (Figure 2 below).

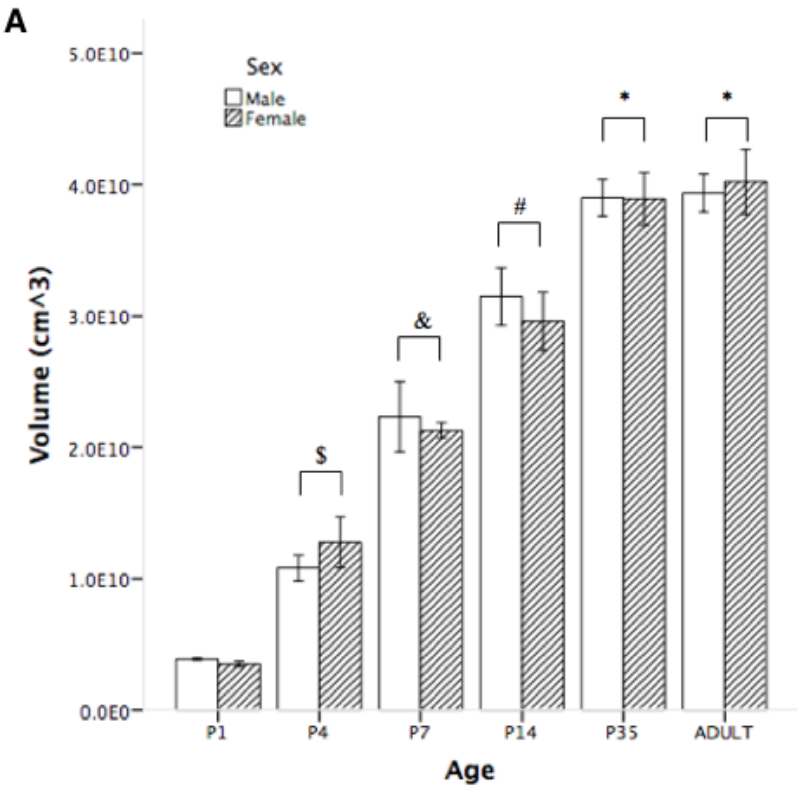

Figure 2. Volume of the neocortex across development. Panel A- Graphical representation of the volume of the neocortex in males and females across the postnatal period. Volume increases with age in both males and females. * denotes males and females at Adult and P35 time points have significant statistical differences in neocortical volume from males and females at all other time points ($p<0.01$). # denotes males and females at P14 have significant statistical differences in neocortical volume from males and females at all other time points ($p<0.01$). & denotes males and females at P7 have significant statistical differences in neocortical volume from males and females at all other time points ($p<0.01$). $ denotes males and females at P4 have significant statistical differences in neocortical volume from males and females at all other time points ($p<0.01$). Data are displayed with error bars as +/- SEM.

Total Astroglia & GFAP Expression Across Development

To quantify total numbers of neocortical astroglial cells across development, we used unbiased stereology to quantify GFP+ cells (eGFP (endogenous GFP) was expressed under the control of the pan-astroglial promoter, AldH-L1), herein referred to as astroglial cell number. No sex differences in total astroglial number were observed ($p>0.05$) (See figure 3A below), however the number of astroglial cells increased until postnatal day 14, when the total number of astroglial cells reached a plateau through to adulthood ($F=51.911$, $p<0.01$) (See figure 3A).

Overall GFAP expression changed significantly with age, however no sex differences were observed ($F=27.614$, $p<0.01$; $p>0.05$, respectively) (Figure 3C). There were no changes in GFAP expression between males and females from P14 to adulthood (Figure 3B).

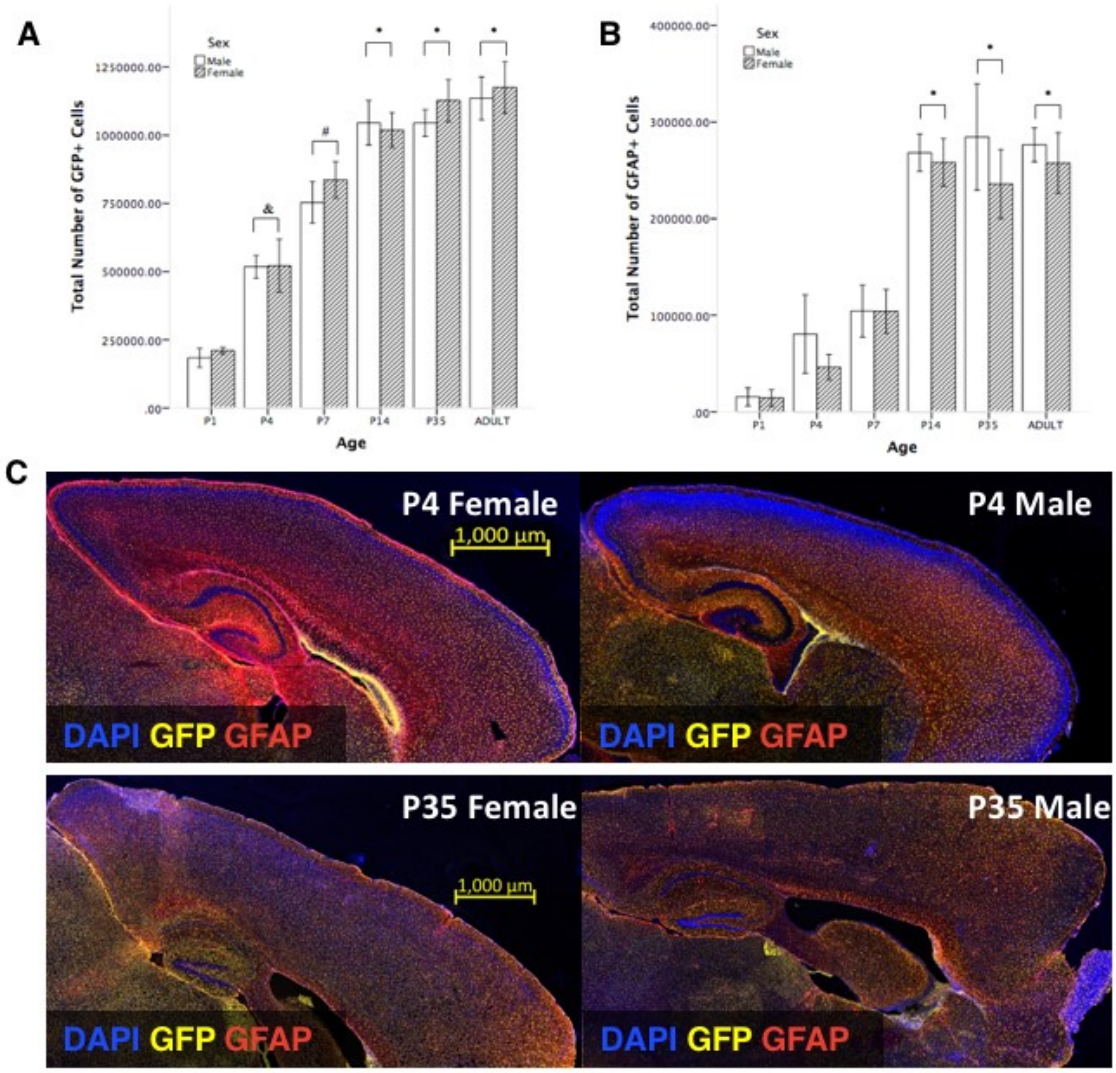

Figure 3. Developmental changes in total number of astroglia and GFAP expression. Panel A- graphical representation of total number of astroglial cells in the neocortex of males and females across the postnatal period observed using GFP under control of the pan-astroglial marker, Aldh-L1. There are no sex differences in total number of astroglia but there is a significant effect of age ($p<0.05$; $F= 51.91$, $p<0.01$, respectively). *, #, & denotes differences from all other time points ($p<0.05$). Panel B- graphical representation of the total number of GFAP immunoreactive astroglia in the neocortex of males and females across the postnatal period ($F= 27.614$, $p<0.05$). There are no sex differences but there is an effect of age. * denotes differences from all other time points ($p<0.01$). Panel C shows a representative mosaic of confocal pictomicrographs of a P4 female, P4 male, P35 female, and P35 male at 10x. Note the change in size of the neocortex. There is an increase in overall number of astroglial cells (in yellow) and GFAP immunoreactive astroglial cells (in red) in both males and females from P4 to P35.

Astroglial Morphology Across Development

Astroglial cells show morphological heterogeneity that are often associated with functionality. In order to assess changes in morphology across age and/or sex, astroglial cells were characterized as radial (See figure 4B' below) and non-radial (Figure 4C') based on number of processes and presence of a leading, radial process in the neocortex. Again, no significant sex effects were found ($p>0.05$), however there was a significant effect of age ($F=3819.157$, $p<0.01$) (Figure 4D). Nearly all (~95% in both males and females) astroglial cells in the neocortex at P1 exhibited radial morphology (Figure 4D). At P4, there was a marked decrease in the proportion of radial astroglial cells and most cells shifted to a non-radial morphology with no marked sex differences (Figure 4D). By P7, only 5% of astroglial cells in males and 4% of astroglial cells in females exhibited a radial morphology and by P14 there was virtually no radial astroglial cells remaining in the neocortex (<1%) (Figure 4D). Similarly, at P35 and in adulthood less than 0.5% of astroglial cells exhibited a radial morphology in both males and females.

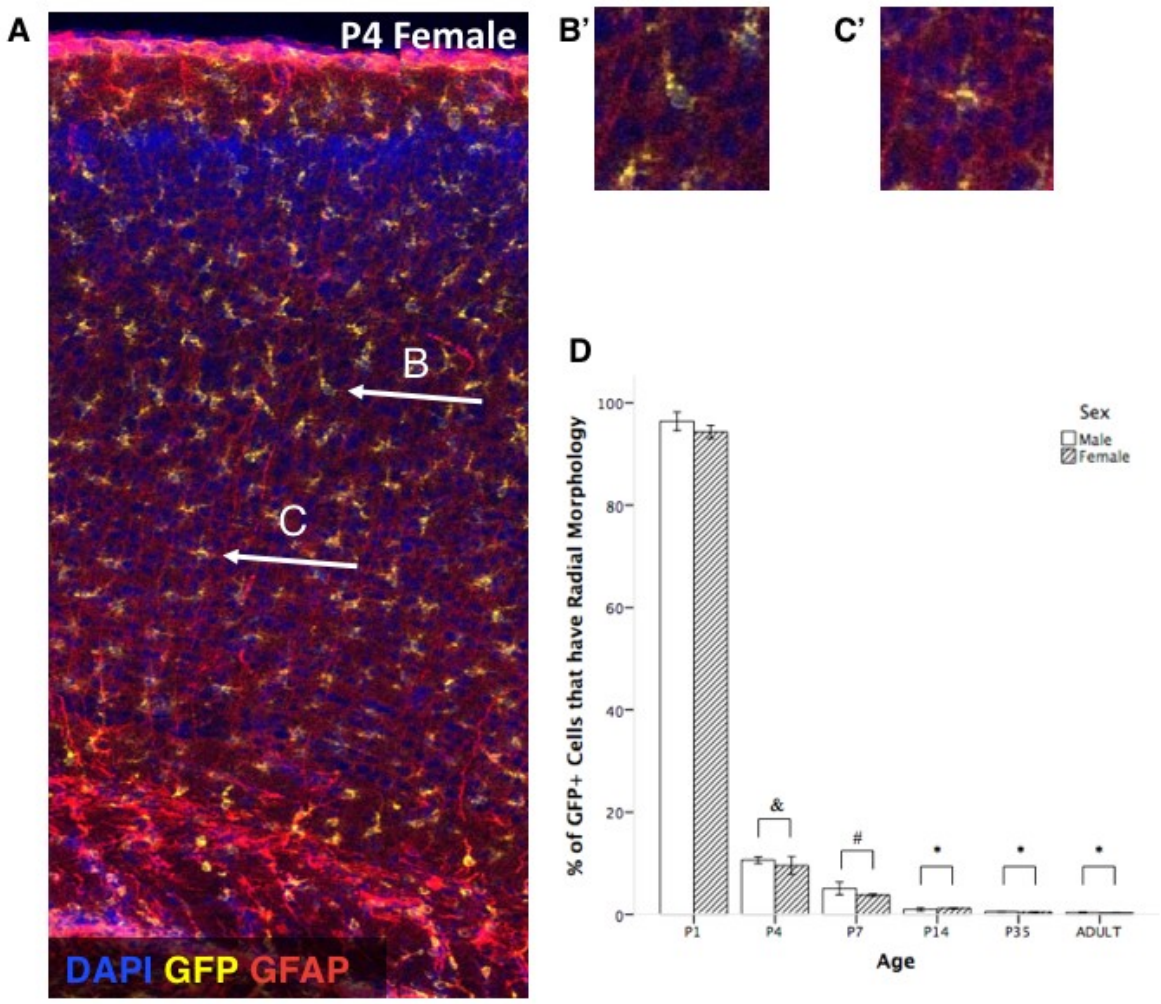

Figure 4. Developmental changes in proportion of radial astroglial cells in the neocortex. Panel A shows a confocal photomicrograph mosaic representation of a cross section of the neocortex of a P4 female from layer VI to the molecular layer at 10x. Arrows to indicate visual morphological differences in radial and non-radial astroglial cells in the cortex. B- arrow pointing to a radial astroglial cell in the developing cortex. B'- zoom on the radial astroglial cell. Note the small circular cell body with only 1 process being filled with GFP. C- arrow pointing to a non-radial astroglial cell in the cortex. C'- zoom on the non-radial astroglial cell. Note multiple processes sprouting from the cell body filled with GFP. Panel D- Graphical representation of the total percentage of radial astroglial cells across development. Total percentage of radial astroglial cells decreases over development but is not sexually dimorphic ($F= 3819.157$, $p<0.01$; $p>0.05$, respectively). * denotes differences from all other time points ($p<0.01$), # denotes difference from all other time points ($p<0.05$), & denotes difference from all other time points ($p<0.05$). Data are displayed with error bars as +/- SEM.

Vimentin & Ki67: Astroglial Cell Neurogenic Potential

To further phenotype neocortical astroglial cells, we quantified the number of cells that expressed vimentin, an intermediate filament protein associated with a sub-type of astroglial cells that show neural stem cell properties. A significant interaction of sex by age was found ($F=3.452$, $p=0.01$) (See figure 5A below), such that females showed significantly more vimentin positive cells compared with males at P4 and P7; although there were no sec differences at P14 or P35, females had significantly less vimentin positive than males in adulthood (Figure 5A, C).

Because vimentin expression is associated with astroglial cell neurogenic and stem cell potential and because we observed sex differences in vimentin expression, we examined whether the proportion of actively dividing astroglial cells across the postnatal period was sexually dimorphic. Using a marker of active s-phase cell division, Ki67, we quantified the percent of Ki67+ astroglial cells.

Ki67 was present in a small proportion (less than 2%) of the astroglial cell population throughout the postnatal period in both males and females (See figure 5B below). Peak proportion of Ki67+ astroglial cells occurred at P1 and declined to P35. In adulthood, there were no actively dividing astroglial cells in the neocortex. At P7, females show a higher proportion of actively dividing astroglial cells compared to males (Figure 5B).

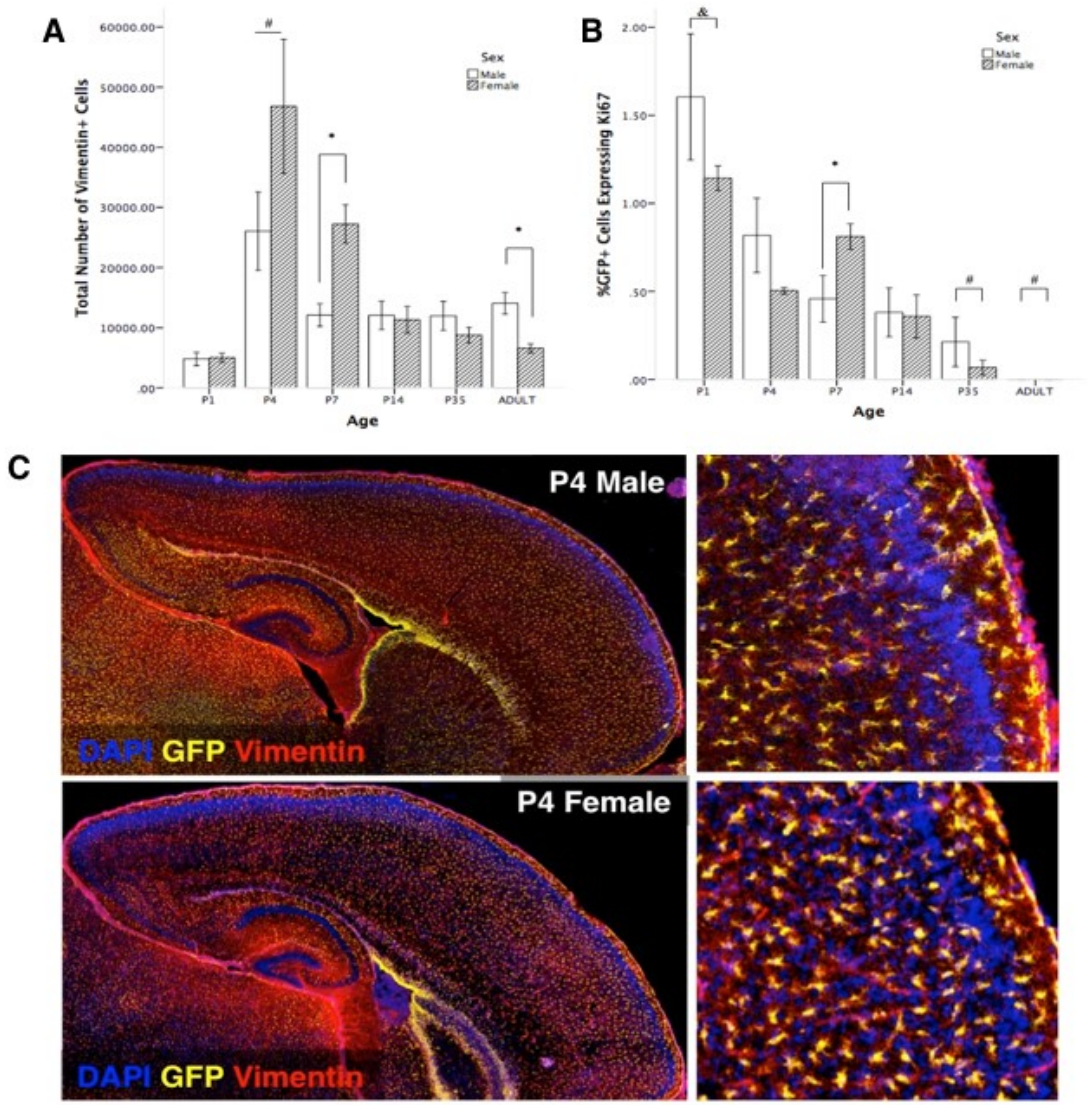

Figure 5. Sex differences in stem cell potential of astroglia. Panel A- graphical representation of the total number of Vimentin immunoreactive cells in the neocortex of males and females across the postnatal period. * denotes significant difference between males and females (t=3.7146, p=0.01 at P7; t=3.8954, p<0.01 in adulthood), # denotes significant difference from all other time points (p<0.05). Panel B- graphical representation of the percentage of GFP+ cells expressing the cell division marker Ki67 in the neocortex of males and females across the postnatal period. * denotes significant difference between males and females (t=2.5338, p=0.05), # denotes difference from all other time points (p<0.05), & denotes difference from all other time points (p<0.01). In both panel A and panel B data are displayed with error bars as +/- SEM Panel C shows a representative mosaic of confocal photomicrographs of a P4 male and P4 female at 10x. To the right is a zoom to allow closer examination of vimentin (in red) expression, peak vimentin expression in both males and females is at P4.

Discussion

In the current study, we examined astroglial cells across cortical development in both male and female mice. In particular, we examined overall volumetric changes in the cortex, phenotypic changes in astroglial cells in relation to their morphology, basic functional properties, and stem cell potential. Currently, the nature of sexually dimorphic changes in the brain throughout development are well documented (Swaab, 2007), however there is little understanding of the role sex hormones and consequently sexually dimorphic brain development have on the phenotype of astroglial cells. Alternatively, the role astroglial cells play in the development of sexually dimorphic brain regions, circuits, and developmental processes is unknown and remains to be elucidated. The following table summarizes the findings from the present study.

Analysis	Early postnatal	Adult
Volume	M = F	M = F
Total Astroglia (GFP)	M = F	M = F
GFAP	M = F	M = F
Vimentin	F > M	M > F
Ki67+ Astroglia	F > M	M = F

Table 3. Summary of findings. A comparison between males and females of astroglial cell phenotype in early and adult time points.

Cortical Volume

Although the mammalian brain is considered a sexually dimorphic structure, there are discrepancies in the literature regarding the degree and regional variability in volumetric sex differences. Classically, males are considered to have larger brain volumes than females across

mammalian species (Carne et al., 2006; Ruigrok et al., 2013), and in particular, sex differences in cortical volume have been shown across species. Surprisingly, in the current study, we observed no significant differences in cortical volume between males and females. This lack of sexual dimorphism may the result of our methodology. We only included neocortical regions due to its association with psychiatric disease, whereas many studies include both neo and archicortical regions. Further studies examining archicortex would be needed to determine whether this is the case. In addition, it is possible that mice, or sub-strains of mice that are inbred do not show similar volume-related sexual dimorphism to higher mammals such as in rats and humans. In the current study, we employed an inbred transgenic mouse model and as such many of the animals have the same maternal or paternal X-chromosome lineage. Previously reported, outbred mice with X-chromosome diversity exhibit more sexually dimorphic variations in the brain and behaviour (Gatewood et al., 2006). As many genes found on sex chromosomes are expressed in the brain, the lack of diversity in X-chromosome inheritance in our study may facilitate the lack of overall sex differences observed in neocrotical volume. In addition, it should be noted that female mice were examined prior to estrus and during the metestrus stage, when female hormone profiles are most physiologically similar to males, and that astroglial volume (Klintsova et al., 1995), neuronal somatic volume (Rocha et al., 2007), and dendrite spine density (Woolley and McEwen, 1993) change across the estrus cycle.

There is a distinction to be made that although overall cortical volume may not differ, there are additional regional (Ruigrok et al., 2013) and asymmetrical (Raz et al., 2004) sex differences in cortical volumes. Interestingly, many of the regional and asymmetric sex differences in cortical volume lay in areas that are implicated with neuropsychiatric and mood disorders that are distributed in a sexually dimorphic pattern in human populations (Bao and

Swaab, 2011; Ruigrok et al., 2013). Therefore, further analysis of regional or asymmetric sex differences in astroglial phenotype is warranted. Although astroglial cells may play a role in the phenotypic changes in brain volume through synapse modulation and spatial occupation, the astroglial cells may play a more important role in typical functional changes associated with sex differences in astroglial phenotype.

Phenotypic Changes in Cortical Astroglial Cells across Development

Unsurprisingly, the total number of astroglial cells in the mouse brain increases steadily until the brain reaches a "mature" and stable state. Within the first 4 days postnatally, the total number of astroglial cells in the mouse cortex doubles and then doubles again within the next 10 days, as seen from P1-P4 and from P4-P14 (Figure 3A). It is apparent that the first 4 days postnatally is a critical window of astroglial proliferation as well as a shift in morphology of astroglial cells from radial states to non-radial states (Figure 4D). It is expected that most astroglial cells shift to non-radial subtypes as the end of radial neuron migration, using radial glial cells as scaffolding occurs between P1 and P4 (Clancy et al., 2001; de Graffe-Peters and Hadders-Algra, 2006). GFAP, the intermediate filament protein expressed by astroglial cells in the CNS and typically associated with a reactive state or with recent morphological changes, showed a similar pattern as the total number of astroglial cells with a key window of upregulation occurring between P7 and P14 (Figure 3B).

Astroglial Neurogenic Potential is Sexually Dimorphic

Vimentin is a dynamic intermediate filament protein in astroglial cells required for radial extensions during development and is associated with a stem cell-like state in a subtype of astroglia (Lowery et al., 2015). Interestingly, there are sex differences in the total number of vimentin positive cells at multiple time points (Figure 5A) and the number of actively dividing

cortical astroglia in the perinatal period (Figure 5B). At P7 females exhibited both higher numbers of vimentin positive cells and a higher proportion of actively dividing astroglial cells in the cortex. Increased plasticity exhibited by astroglial cells during this critical period of cortical organization may buffer against some adverse effects of disrupted cortical organization.

Schizophrenia and autism are more prominent in the male population (Bao and Swaab, 2011) and have important postnatal developmental etiologies (Matute et al., 2005; Galvez-Contreas et al., 2017). There may be a relationship between astroglial neurogenic potential and the terminal phases of neuronal migration and construction and maturation of functional cortical networks that buffers females against developmental anomalies which, if left unchecked, may lead to schizophrenia or autism later in life. In-line with this hypothesis, females show augmented recovery when exposed to a hypoxic environment (Mayoral et al., 2009). Chronic postnatal hypoxia in rodents is used as a model of premature birth and induces loss of brain volume, particularly in the cortex and hippocampus, and leads to motor and cognitive impairments later in life (Mayoral et al., 2009; van der Kooji et al., 2010). The loss of volume in the hypoxic model is less pronounced in females (Mayoral et al., 2009) and interestingly, estradiol treatment improves white matter damage recovery (Gerstner et al., 2009). It is also known that chronic postnatal hypoxia increases astroglial stem cell capacity (Bi et al., 2011). This process may be enhanced in females allowing augmented recovery from the aversive effects of hypoxia, but may also elucidate to the nature of enhanced early postnatal plasticity in females compared to males. It is possible that females experience an enhanced or shifted neurogenic period early postnatally when we have observed an increase in Ki67+ astroglia that may translate to increased neurogenic potential of astroglial cells in the cortex, although whether this occurs in the developing brain remains to be determined.

The allure of astroglial plasticity persists into adulthood. In adulthood, males had higher vimentin expression compared to females (Figure 5A) indicating adult male astroglial cells have enhanced neurogenic potential than in females at adulthood. In contrast to females at P7, the adult males do not exhibit a larger proportion of astroglial cells that are actively dividing (Figure 5B) in conjunction with increased vimentin expression, indicating there is a *hypothetical* increase in neurogenic potential of astroglial cells compared to females. This potential for increased astroglial plasticity may also translate to sexual dimorphisms found in the prevalence of mood and anxiety disorders in human populations (Bao and Swaab, 2011).

Although it is known that estrogen can facilitate many changes to neuronal and glial populations, the effects of estrogen are dynamic and depend on the ratio of ER-α to ER-β (Kuiper et al., 1997). The ratio of ER-α to ER-β is different across brain regions (Shughrue et al., 1997) and changes across development (Schaub et al., 2008). A limitation to our present study is that the dynamic properties of the ER and its isoforms are unknown and are not currently studied in astroglial cells. Therefore, it is imperative to study to the expression of ERs on astroglial cells throughout the neocortex and across development to understand how circulating hormones effect the astroglial cells during different time points.

Finally, another limitation to our study is that there is a low number of animals being studied. This study aimed to understand the baseline sex differences in cortical astroglial cells however, this study is still in its infancy and there is more analysis, including the addition of more animals, to be conducted before we can draw definitive conclusions.

The current analysis of cortical sexual differentiation yielded mixed results; no gross sex differences in volume or total number of astroglia were noted, however changes in functional morphology and protein expression patterns were observed in response to sex and age. Further

characterization of the functional implications of these changes need to be explored in future studies. The enormous sex differences in prevalence for psychiatric disorders related to cortical function implore further understanding of the mechanisms by which the cortex may modulate risk and resilience between the sexes.

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