SEXUAL DIMORPHISMS AND ANDROGEN INFLUENCE IN MEDIAL POSTERODORSAL AMYGDALA ASTROCYTES

By

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ABSTRACT

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The amygdala is a highly interconnected brain region involved in fear, anxiety, social and reproductive behaviors. In humans and laboratory species the amygdala exhibits sexual dimorphisms in neuroanatomy and function both in juveniles and adults. In rodents, the medial posterodorsal amygdala (MePD) is particularly sexually dimorphic and gonadal hormone sensitive, and while neurons have been examined in this region, few reports have examined the potential influence of gonadal hormones on other cellular components of the MePD. Astrocytes are a subtype of glia involved in synapse formation and known to be plastic and dynamic cells sensitive to gonadal hormone influence in several brain regions. My dissertation reveals sexual dimorphisms in the number of astrocytes in the juvenile rat MePD and that this sexual dimorphism remains present in adult animals. I also found sex differences in the arbor complexity of astrocytes in adults that are not present prior to puberty. Astrocytes also respond to changes in circulating hormone levels in adulthood. Furthermore, while the sex difference in astrocyte numbers in juvenile animals is androgen receptor-independent, the sex differences found in adult astrocyte numbers and arbor complexity are both androgen receptor-dependent. Finally, I provide evidence that astrocytes in the MePD contain androgen receptors, suggesting that androgens may act directly on these cells. The influence of gonadal hormones on astrocytes in the MePD is likely an important

part of pubertal development and has implications for our understanding of the cellular organization of the amygdala and its function.

DEDICATION

This dissertation is dedicated to Sarah L. Johnson

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ABBREVIATIONS

AD - Alzheimer's Disease

AR – androgen receptor

BrDU - Bromodeoxyuridine

Cy2 - Cyanine 2

Cy5 - Cyanine 5

DAPI - 4',6-diamidino-2-phenylindol

DHT - dihyrotestosterone

ER- estrogen receptor

GFAP - glial fibrillary acidic protein

GFAP-ir - glial fibrillary acidic protein immunoreactive density

LTP - Long-term potentiation

MeA - medial amygdala

MeAV - medial anteroventral amygdala

MePD - medial posterodorsal amygdala

MePV - medial posteroventral amygdala

PND - postnatal day

SNP - single nucleotide polymorphism

SRC - steroid response coactivator

T - testosterone

Tfm – testicular feminization mutation

VEGF - vascular endothelial growth factor

VMH- ventromedial hypothalamus

WKY - Wister Kyoto rats

Wt – wildtype

INTRODUCTION

Although it focuses on astrocytes, this dissertation is primarily a continuation of our lab's ongoing investigation of androgen influence on medial amygdala neuroanatomy. Therefore, I begin with an introduction to the amygdala and an examination of previous work from our lab and others in this brain region. I then shift to the cell of interest in my dissertation, astrocytes, and provide a review of astrocytes in the amygdala. I then describe my work examining sex differences and plasticity of these cells in juvenile and adult animals and discuss my findings including a review of laterality in the amygdala.

The Amygdala

Located in the temporal lobes, the cluster of nuclei comprising the amygdala is involved in several important behaviors including social behavior, emotion, fear and anxiety (reviewed in LeDoux 1998; Bishop et al. 2004; Adolphs et al. 2005; Mah et al. 2007; Spezio et al. 2007; Truitt et al. 2007). In common laboratory species and in humans, the amygdala is divided into central, medial, cortical, lateral, basal and accessory basal divisions which are strongly interconnected, although the direction and strength of these connections does not appear uniform among the subregions (Price et al. 1987; Krettek and Price 1978a,b, Grove 1988a,b; Alheid et al.1995; Canteras et al. 1995; Shammah-Lagnado et al. 2000). Tract tracing work and resting-state fMRI has demonstrated that the amygdala is reciprocally connected through direct and indirect connections to important homeostatic and cognitive centers such as the hypothalamus, hippocampus, and much of the sensory cortex (Canteras et al. 1995, Stefanaci and Amaral 2002, Kilpatrick et al. 2006). Given these extensive connections, it is not

surprising that the amygdala has been implicated in many behavioral disorders and diseases including schizophrenia, drug addiction, anxiety disorders, depression, and autism.

Much of the knowledge regarding amygdala function stems from its role in fear and anxiety. A long history of conditioning experiments identified the region as crucial to the fear response (reviewed in LaDoux 2003) and additional methodologies mapped the amygdala's expanded role in emotional memory and motivation (Bechara et al. 1995; Cahill 2000; reviewed in Cardinal et al. 2002). Within these domains, it is widely acknowledged that cellular plasticity within the amygdala plays a vital role in shaping behavior (Rogan and LeDoux 1995; Ling et al. 2011; Tan et al. 2010).

Plasticity within the various amygdala sub-regions and in the amygdala as a whole has been examined through many paradigms and has been covered in numerous reviews (Sah et al. 2008; Samson et al. 2005; Cooke 2006). The human literature has examined plasticity in amygdala volume and functionality and the rodent literature has repeatedly demonstrated plasticity of amygdala neurons based on fear-related long-term potentiation (LTP) and by manipulation of circulating hormone levels (human plasticity: Killgore and Yurgelun-Todd 2004; Giedd et al. 1997; long term potentiation: reviewed in Sigurdsson et al. 2007, see also Sah et al. 2008; hormone manipulation: Cooke et al. 1999; Morris et al. 2008). While LTP and hormone mediated plasticity certainly interact within the amygdala (Kavushansky and Richter-Levin 2006; Kavushansky et al. 2006) the complex and extensive influence of gonadal hormones in the region is intriguing in itself.

Gonadal Hormones and the Amygdala

Some of the earliest investigations of the amygdala revealed the region's involvement in social/sexual behavior (Kluver and Bucy 1938). Since then neuroendocrine research has identified numerous functional and morphological sex differences in the amygdala, which may be related to the has dense concentrations of gonadal hormone receptors within this brain region (Cahill et al. 2004; Kilpatrick et al. 2006; Hines et al. 1992; Rubinow and Juraska 2009; Simerly et al. 1990; Li et al. 1993).

The medial subregion of the amygdala appears to be especially hormone sensitive and sexually dimorphic. Bubenik and Brown (1973) found a sex difference in average neuronal soma size in the medial amygdala of ovariectomized and estradiol treated female squirrel monkeys compared to normal males. Another sex difference was identified in cholinergic binding within the medial nucleus of mice (Arimatsu et al. 1981). This was followed by a string of findings from a single group demonstrating sex differences in the volume and synaptic organization of the rat medial amygdala (males greater than females) that could be reversed by treating females with estrogen (Nishizuka and Arai 1982, 1983; Mizukami et al. 1983). Another group reported sex differences in substance-P immunoreactivity in the medial amygdala of rats (Malsbury and McKay 1989), and von Ziegler and Lichtensteiger (1992) demonstrated that aromatase activity, which is responsible for the conversion of testosterone into estradiol, undergoes a pattern of lateralized sex differences in the medial amygdala during development, noting the potential such activity may have in shaping neuroanatomy (von Ziegler and Lichtensteiger 1992).

Continued investigation of the medial amygdala revealed that the medial posterodorsal portion (MePD) exhibited particularly striking sex differences (Malsbury and McKay 1989). The sex difference in regional volume reported by Mizukami and colleagues (1983) for the medial amygdala was also found for the MePD as was the ability of this sex difference to be eliminated by hormone treatment. Specifically, the greater regional volume of the MePD in males compared to females could be eliminated by castration of adult males or androgen treatment of females (Cooke et al. 1999). A sex difference in average neuronal soma size was also found in the MePD with males having larger somata compared to females and this too could be eliminated by hormone manipulation of adult animals (Cooke et al. 1999). Thus, many of the sex differences in the MePD can be reversed or eliminated by adult hormone manipulations, suggesting a profound amount of hormone-mediated plasticity in the region. Evidence demonstrating that MePD regional volume and soma size were responsive to both social cues and photoperiod in some species further emphasized the plasticity of the region (Cooke et al. 2002).

Both androgen receptors (ARs) and estrogen receptors (ERs) contribute to maintaining a fully masculinized MePD and full expression of male mating behaviors. For example, the treatment with high affinity AR ligand dihydrotestosterone (DHT) maintained penile erections, ultrasonic vocalizations, and ejaculatory behavior along with neuronal soma size in castrated male rats. In contrast, the ER ligand estradiol maintained intromission patterns, ejaculatory behavior, neuronal soma size and regional volume in castrated males, demonstrating the complexity of the hormone interactions involved in MePD neuroanatomy and associated behaviors (Cooke et al. 2003). One

caveat in these studies is that hormones were delivered systemically, so their effects on neurons in the MePD may or may not be mediated by AR and/or ER in these same neurons. Moreover, such hormone-induced changes in MePD neurons may or may not critically mediate the behavioral changes induced by these same hormones.

Testicular feminized mutant (Tfm) rats have been an important tool in isolating the contribution of the AR and androgens in the brain. Tfm rats carry a spontaneous mutation in the AR gene that causes XY males to have in many ways feminine traits (e.g., nipples and a short, feminine anogenital distance), despite having testes that produce adequate amounts of circulating testosterone (Roseli et al. 1987). Tfm rats have a single base-pair replacement mutation in the steroid binding domain of their AR gene which renders the receptor largely dysfunctional (Yarbrough et al. 1990). Because androgens and estrogens are present but only ER are functional (Olsen and Whalen 1982) research using Tfm animals has confirmed that functional AR are necessary for complete masculinization of several aspects of brain morphology and function (reviewed in Zuloaga et al. 2008). This evidence has been vital in revising the aromatization hypothesis, which attributed brain masculinization to ER activity alone (Naftolin et al. 1975).

In Tfm males, while rostral-caudal extent of the MePD was unaltered compared to wt males, the regional volume and average neuronal soma size were intermediate between wt male and female animals, suggesting functional ARs are necessary for complete masculinization of these MePD components (Morris et al. 2005). Similar results were found in a portion of the extended amygdala, the posteromedial bed nucleus of the stria terminalis (BSTMPM; Durazzo et al. 2007). Thus research using

Tfm animals provides further evidence that androgens acting via AR influence MePD structure and presumably function.

Subsequent investigations attempted to determine which cellular components of the MePD respond to androgens. Using gonadectomized male and female animals in conjunction with androgen supplementation, Morris et al. (2008) reported that males had greater numbers of MePD neurons and glia than females. However, adult androgens did not influence the number of neurons in the MePD. In contrast, glial cell numbers were affected by androgens with high androgen-treated male and female rats having more glia in the right MePD than control-treated rats (Morris et al. 2008). This result suggested that androgens may act on cells other than neurons to influence amygdala function and opens new avenues of exploration. There are several types of glia in the amygdala and I will provide background regarding the role of one type, astrocytes.

A Review of Glia in the Amygdala

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As in most of the central nervous system, several types of glia are present in the amygdala, including astrocytes, oligodendrocytes, and micro-glia. However, much of the exploration of glia in the limbic system has focused either on astrocytes specifically or on glia in general. Thus, reports examining microglia or oligodendrocytes in the amygdala are rare, but these cells are likely included in counts of total glial within the amygdala. These circumstances make attributing effects or phenomenon to specific

glial subtypes sometimes difficult, but hopefully future investigations will alleviate this confusion.

Astrocytes are perhaps the most thoroughly investigated glia subtype in the central nervous system and certainly in the amygdala. Astrocytes were first described in detail in 1895 when Cajal named them based upon their extensive star-like arbors. Cajal (1909) later suggested that astrocytes were functioning to insulate neural connections while Golgi (1885), who was also investigating glia, proposed that astrocytes provide a link between neurons and the blood supply based upon their predisposition to contact blood vessels. Unfortunately, after these initial descriptions and hypotheses, astrocytes were mostly ignored. It was not until the identification of the astrocyte inflammatory response that these cells again received attention.

Numerous reports demonstrate that astrocytes rapidly respond to all manner of brain trauma, including injury, disease, and genetic disorders with a rapid synthesis of glial fibrillary acidic protein (GFAP) at the site of injury. This reliable, complex response has been carefully examined and reviewed in detail (Eddleston and Mucke, 1993; Eng and Ghirnikar, 1994). The information derived from studies of reactive gliosis has been vital in understanding brain injury and should not be minimized, but as Cotter et al. (2001) described it, ‘‘glia were typecast, their crucial role in other cortical function overlooked”.

Fortunately, the past two decades have seen an increase in attention to glia. Much of the interest stems from findings that astrocytes exhibit rapidly propagating calcium waves, suggesting that they may form a second path of communication working in conjunction with the neuronal system (Guthrie et al., 1999). More recent work

suggests that propagating calcium waves are extremely rare in vivo (Wang et al., 2006), but new evidence reaffirms that astrocytes are active and dynamic cells.

Mature astrocytes generally have large arbors extending from their cell bodies, and in the cortex an average astrocyte connects to approximately four neurons, 300–600 dendrites, and 1,000,000 synapses (Halassa et al., 2007). Astrocyte processes are closely involved in the formation of new synapses and these processes are highly mobile and extend or retract to modulate contact between neurons (Hatton, 2002; Nishida and Okabe, 2007; Ullian et al., 2001). Interestingly, increased branch complexity in astrocytes has been associated with an increase in functional synapses in some regions (Elmariah et al., 2005; Pfrieger and Barres, 1997) but has also been associated with decreases in dendritic spines (Mong et al., 2001). Thus, it may be that astrocytes have opposing roles under different conditions, promoting and maintaining synapses in some cases, while promoting their elimination in others. In many ways, a sensitivity to varying conditions has become the hallmark of glia, and their responsiveness to various disease states, which I review next, illustrates this capacity.

Amygdala Glia and Disease

Glia are known to be important metabolic factories for normal brain functioning, responding quickly and topographically to stimuli with rapid increases in metabolic activity (Schummers et al., 2008). The nature of the glial response to disease has been puzzling, with long-standing questions about whether this response is beneficial and neuroprotective or is detrimental t

, 2008). Furthermore, in several diseases and disorders it is not at all certain if the effects seen in glia are merely a response to

disease or a contributing cause. As in most cases, that dichotomy may be an illusion, with glia likely playing both roles at various times and in various instances. What is clear is that the extreme sensitivity of glia to brain dysfunction makes them powerful indicators of abnormal brain function and has guided the examination of amygdala glia in several disease models.

Epileptic states

Investigation of patients with temporal lobe epilepsy demonstrates glial satellitosis, the accumulation of glia around neurons, in the lateral amygdala (Faber-zuschratter et al., 2009) and interesting glial phenomena have been detailed in the mouse amygdala as well. Kainic acid, an excitotoxin regularly used to investigate the brain's response to seizure-like conditions, induces several responses in rodent glia. After kainic acid injection, microglia become immunopositive for cyclin-dependent kinase 4 and cyclin D1, important regulators of apoptotic cell death (Ino and Chiba, 2001). In amygdala astrocytes, kainic acid injection results in prolonged expression of metallothioneins, a family of proteins involved in neuroprotection (Kim et al., 2003).

The apolipoproteins, a family including apolipoprotein E, are primarily expressed in astrocytes under normal conditions. However, in cases when damage induced by kainic acid extended beyond the injection site into regions such as the amygdala, apolipoprotein E expression was also found in neurons. In cases of mild damage, this effect was not seen, but increases in GFAP-immunoreactivity and microgliosis were seen. The authors conclude that in severe cases, damage to the glial network is large enough to allow accumulation of apolipoproteins from damaged glia in the surrounding

neurons, while in mild cases the reactive glia response may ameliorate this outcome (Grootendorst et al. 2000).

Amygdala-kindled seizures also promote astrocyte proliferation in the piriform cortex of rats (Vessal et al. 2004), and kindling of the olfactory bulb, a major source of input for the amygdala, results in astrocyte proliferation in the basolateral amygdala and the piriform cortex (Woldbye et al. 1996). These two studies together suggest that over stimulation of the amygdala, be it through direct kindling or though kindling of a primary input source, can have long-reaching effects on other parts of the brain. The authors suggest that astrocytes become activated in response to the formation of a new synaptic pathway brought on by the hyper-excitability of the epileptic discharge and conclude that astrocytes may support the formation of the kindling pathway (Vessal et al. 2004, 2005). Jung et al. (2009) also found amygdala astrocyte proliferation in a lithium–pilocarpine injection model, and surgical specimens from patients suffering from temporal lobe epilepsy demonstrate an inverse correlation between the extent of astrocytic-reactive gliosis and inhibitory synapses on GAD-positive projection neurons (Yilmazer-Hanke et al., 2007). Again, these results suggest that astrocyte activity may serve to promote a ‘‘rewiring’’ of seizure sensitive regions.

Astrocytes in the piriform cortex become immunoreactive for nestin, an embryonic intermediate neurofilament protein, after amygdaloid kindling, providing further evidence that astrocyte morphology is affected by seizures (Umeoka et al., 2001). Relatedly, while astrocyte reactivity in the hippocampus and amygdala after amygdala-kindled seizure was equivalent in S-100b knock outs (a type of calcium binding protein found in some astrocytes) compared to controls, the knockout animals

kindled more rapidly and exhibited more severe seizures, suggesting changes in astrocytic structural proteins are related to seizure progression (Dyck et al., 2002). However, since S-100b is expressed only in a subtype of adult astrocytes around blood vessels, only select astrocytes would be involved (Hachem et al., 2005).

Investigators have also begun to explore whether astrocytes help to preserve neurons *after* seizures. Vascular endothelial growth factor (VEGF) protects neurons from cell death and astrocytes in the amygdala up-regulate VEGF protein expression approximately 24 h after seizure induction. This VEGF up-regulation in astrocytes may benefit surrounding neurons (Newton et al., 2003; Nicoletti et al., 2008).

Finally, seizure-like states are also examined for their beneficial effects. Electroconvulsive seizures are used for the treatment of major depression, and Wennstrom et al. (2004) examined Wistar rats given five electroconvulsive treatments and injected with bromodeoxyuridine (BrdU) to monitor cell proliferation. As the seizure literature would predict, the authors report a proliferation of glial cells in the amygdala after electroconvulsive treatment. Specifically, the most dramatic increase was in oligodendrocytes, a proliferation that lasted 3 weeks after treatment and led to the establishment of new mature oligodendrocytes. Interestingly, this effect was region and cell-type specific with oligodendrocyte proliferation being found in the central, lateral, and basal subregions and microglia proliferation found in the medial subregion. The authors speculate that the formation of these new cells may play a role in regulating synaptic function (Wennstrom et al., 2004).

Depression

As the previous report suggests, glia are also implicated in major depressive and bipolar disorders. Social exploratory behavior is sometimes used as a model of depression, and Lee et al. (2007) found that blocking glutamate uptake in the amygdala resulted in a dose-dependent reduction in social exploratory behavior and altered circadian activity patterns, reminiscent of depressive states in humans. Importantly, selectively blocking glutamate uptake by astrocytes with dihydrokainic acid in the basolateral amygdala also reduced social exploratory behavior, an effect that could be reversed by simultaneous injection of the NMDA receptor antagonist AP5. These results led the authors to conclude that impaired uptake and metabolism of glutamate by astrocytes may be critically involved in depression (Lee et al., 2007).

Glial numbers in the amygdala are also affected in humans with major depressive disorder. Bowley et al. (2002) examined the amygdala from patients with major depressive and bipolar disorder postmortem and found that glia density and glia/neuron ratio were greatly reduced compared to controls. Interestingly, this reduction was mostly seen in the left hemisphere and was not due to a change in the number of neurons. This reduction in glia was also found in untreated bipolar cases but not in cases treated with either lithium or valproate, suggesting these medications may reduce the loss of glia in the amygdala (Bowley et al., 2002). Relatedly, a 4-week lithium treatment of adult males rats reduced oligodendrocyte proliferation compared to controls, but the authors note did not look for effects of lithium on the survival of cells (Orre et al., 2009).

Since the identification of reduced glial numbers in the amygdala in major depressive disorder, other reports have examined this phenomenon in more detail. One study examined the expression of glial markers in the basolateral amygdala in Wister Kyoto (WKY) rats, often used as a model for anxiety and depression due to their exaggerated responses to stressors and depressive-like performance in behavioral tests (De La Garza and Mahoney, 2004). Gosselin et al. (2009) found a significant deficit in the number of GFAP immunopositive cells (a common marker for astrocytes) in the basolateral amygdala of WKY rats compared with Sprague-Dawley control animals, a result confirmed by Western analysis of GFAP expression levels. However, the use of a second astrocyte marker, S-100b, revealed no differences in expression between controls and WKY rats, a result one might expect given that only a subpopulation of astrocytes express S-100b (Hachem et al. 2005). However, the second marker did provide an interesting finding; in control animals GFAP and S-100b were colocalized while in WKY rats a population of S-100b-positive cells was found that were devoid of GFAP immunoreactivity. This led the authors to suggest that in the WKY model GFAP expression by astrocytes is altered not the number or density of total astrocytes (Gosselin et al., 2009).

Another report found similar results using a maternal deprivation paradigm, a model of early life stress, which is a major risk factor in the etiology of depressive disorders. The maternally deprived animals exhibited a significant reduction in GFAP-immunoreactive astrocyte density in the basolateral amygdala and several other brain regions. This reduction in immunoreactivity was greater than the overall reduction in cell density, which suggests, as in the previous report, that GFAP expression, rather

than the number of astrocytes, was altered in this animal model (Leventopoulos et al., 2007).

However, it is also clear that early stress can alter cell numbers in the amygdala. Using a prenatal stress paradigm, Kraszpulski et al. (2006) found altered glial numbers in different amygdala subregions at different developmental time points. For example, the number of glia in the lateral amygdala nucleus was reduced in prenatally stressed animals at postnatal day (PND) 7 only, whereas glia numbers were reduced in the basolateral and central nuclei at PND 25. Neuron number exhibited the same pattern and the authors speculate that these reductions in cell number may underlie the enhanced anxiety seen in prenatally stressed animals in adulthood (Kraszpulski et al., 2006). Unfortunately, how reductions of GFAP expression and/or reduced glia number in the amygdala relate to depression is still unclear.

Despite several reports that astrocytes are affected in the amygdala of both human cases and in animal models of major depressive disorder, other evidence suggests that astrocytes may not be altered during depression. Hamidi et al. (2004) labeled tissue from patients diagnosed with major depressive disorder for S-100b, human leukocyte antigen, and stained for Nissl. Using S-100b, the group reported no significant difference in astrocyte density in depressed patients compared to controls. Moreover, human leukocyte antigen was used to identify microglia and revealed no difference. However, oligodendrocyte density (identified by their compact deeply stained nuclei in Nissl staining) was significantly lower in this population of depressed patients. The authors concluded that the reduction of glia previously reported in the amygdala of patients suffering from major depressive disorder was due to a reduction in

oligodendrocytes. These results are difficult to interpret for two reasons. First, as previously mentioned, S-100b is expressed only in a subset of astrocytes (Hachem et al. 2005). Furthermore, Gosselin et al. (2009) found a dissociation of S-100b and GFAP-labeled cells in their WKY rat model of depression. Perhaps, if Hamidi and colleagues had also used GFAP immunoreactivity as their astrocytic marker, they would have seen a reduction in the number of astrocytes as well. However, it is certainly possible that both astrocytes and oligodendrocytes are reduced in the amygdala of depressive disorder cases.

Adding to the complexity is an interesting report examining S-100A1, a protein closely related to S-100b that also colocalizes with GFAP. S-100A1 knockout mice appear to develop normally and show no obvious differences in brain development. However, S-100A1 knockout males exhibit reduced anxiety based on an approach-avoidance paradigm (Ackermann et al., 2006). Reduced anxiety in knockouts suggests S-100A1 may somehow facilitate fear and anxiety, two behaviors often elevated in models of major depressive disorder. Regardless of what changes occur to amygdala glia, a clear cause–effect relationship remains elusive, and the question of whether changes in glial numbers in the amygdala are a response to or a cause of depression remains unanswered (see Hercher et al., 2009).

Proteinopathies

Glia are also investigated in other diseases. In Parkinson's disease, α-synuclein fibrils aggregate and eventually lead to cellular dysfunction, and the presence of α-synuclein-immunoreactive inclusions in neurons has been an important marker in research on this disease (Mukaetova-Ladinska and McKeith, 2006). Astrocytic α-

synuclein-immunoreactive inclusions have also been described in the human amygdala (Terada et al., 2003). These astrocytic inclusions appear in the amygdala at stage 3 of the disease, after their initial appearance in neurons, and their immunoreactivity increases in the latter stages of the disease (Braak et al., 2007).

Astrocytes are not thought to produce α-synuclein themselves and it's suspected they take up altered α-synuclein from afflicted neurons, likely explaining why glia are also eventually affected by disease (Braak et al. 2007). Whether these α-synuclein-immunoreactive glia influence the progression of disease symptoms is unclear, but based upon how astrocytes respond to trauma in the healthy brain, afflicted astrocytes in Parkinson's disease may cause a loss of neuronal connections, effectively severing the amygdala from other brain regions in the latter stages of the disease (Braak et al., 2007). Given that α-synuclein-immunoreactive astrocytes and astrocytes positive for other disease markers (tau and ubiquitin inclusion bodies) have been found in the amygdala of non-Parkinson's patients who were diagnosed with diffuse neurofibrillary tangles with calcification dementia, this disease may share pathophysiological mechanisms with Parkinson's and other proteinopathies (Hashimoto et al., 2003; Yokota et al., 2002).

Glia also appear to be affected in Alzheimer's disease (AD), and astro-gliosis in the amygdala has been reported in many AD cases (Corsellis, 1970). Scott et al. (1992) investigated the cortical and basal amygdala subregions in brains of AD patients postmortem. They counted glia and divided them into two categories: large, which the authors presumed to be astrocytes, and small, presumed to be oligodendrocytes and microglia, based on size. When examining glial cell density (cells per mm^2), they found

that the overall glia density was greater in AD cases in both the basal and cortical regions. However, this was attributable mostly to increased density of large glia. When the data were corrected for structural atrophy, total numbers of glia were reduced in AD cases in both size categories in the basal amygdala. In the cortical region, only the number of small glia was reduced (Scott et al., 1992).

Additional reports found an increase in the number of astrocytes expressing peroxiredoxin 6, an antioxidant enzyme, in the amygdala of AD cases compared to controls. Peroxiredoxin 6 staining, which is not found in oligodendrocytes or microglia and only at very low levels in neurons, was found mostly in astrocytes associated with amyloid-β plaques, suggesting the plaques produce reactive oxygen species via astrocytes (Power et al., 2008). Furthermore, Kaku et al. (2003) found that a mutant mouse (osteopetrotic mice), known to have reduced number of microglia, also have greater fibrillary plaques in the amygdala and other regions compared to controls, suggesting microglia regulate plaque formation and/or clearance in the region. Additional reports confirm that activated microglia follow plaque formation in the amygdala, and activated astrocytes form later around these plaques (Dudal et al., 2004).

One difficulty in measuring astrocyte proliferation in any age-related disease such as AD is the well-documented increase in GFAP production as the brain ages (Kohama et al., 1995; Linnemann and Skarsfelt, 1994). Interestingly, this increase in GFAP can be attenuated in several brain regions, including the amygdala, by systemic administration of the steroid pregnenolone in aging animals (Legrand and Alonso, 1998). This implies that reduced hormone levels during aging may cause widespread

increases in GFAP production and may contribute to the increased incidence of many neural disorders with age.

Other pathologies

Along with their roles in epilepsy, depression, and various proteinopathies, glia also figure into other areas of disease and disorder research. For example, myo-inositol, a glial marker and second messenger in intracellular calcium regulation, is reduced in the amygdala of narcoleptic patients compared to controls, suggesting glia involvement in sleep disorders (Poryazova et al., 2009). Additionally, Yokota et al. (2008) found several interesting glial abnormalities in the amygdala during their investigation of cases of neurofibromatosis type 1, a disease in which tumors develop from nervous system tissues. They conclude that the glial clusters and satellitosis seen in the disease may be due to altered astrocyte growth regulation.

Although currently isolated in their fields, these two reports suggest that glia in the amygdala may be involved in, or at least be an indicator of, a broad range diseases and disorders. Due to their sensitivity and prominent role in maintaining brain homeostasis, the use of glia as indicators of neurological trauma has great potential for disease research. Furthermore, given their role in the formation of synapses and facilitation of neuronal communication, exploration of glia in disease models may lead to novel treatments focusing on restoring damaged communication pathways.

Amygdala Glia and Hormones

As described above, the amygdala contains a number of sexual dimorphisms (reviewed in Hamann, 2005; Stefanova and Ovtscharoff, 2000). These sex differences have important implications beyond sexual behavior as most of the diseases associated

with the amygdala such as depression, anxiety, schizophrenia and autism exhibit strong sex biases in the population (American Psychiatric Association, 2000). While it is clear that gonadal hormones are key in the early establishment of sex differences and in CNS plasticity, the mechanisms behind hormone-induced alterations in neuroanatomy are still not clear. Are glia involved in the ontogeny of sex differences? What role due glia play in hormone-induced plasticity in adulthood? This line of questioning is beginning to provide a detailed and rich picture of the amygdala's cellular components.

The evidence for gonadal hormone influence on neurons is well established (Hamson et al. 2010; Cooke et al. 1999; Woolley, 2007; Forger 2006). However, glia also respond to gonadal hormones signals. ER are up-regulated in reactive astrocytes after injury and estrogen influences astrocyte morphology in the hypothalamus, hippocampus and in primary culture (Blurton-Jones and Tuszynski, 2001; Milner et al. 2000; Garcia-Segura et al. 1989, 1994). Estrogen appears to influence glia in the amygdala as well. The scaffold protein, MNAR/PELP1, is important coactivator in estrogen's nongenomic activity and has been found in glia in the amygdala suggesting glia may respond directly to estrogen through nongenomic means (Khana et al., 2006; Raza et al. 2008).

Gonadal hormones and amygdala glia during developmental

Evidence suggests that gonadal hormones may influence glia in the amygdala early in development. For example, a single dose of estrogen during neonatal development increased the number of proliferating glia in the basolateral nucleus 3 days later in male rats but not in females (Dmitar et al. 1995). A slightly more complex pattern of results was found in the medial, cortical, and central nuclei. In estrogen-

treated male rat pups, the percentage of BrdU-labeled glia was increased in all three nuclei, based upon light microscopy identification, whereas in females, estrogen increased the number of labeled glia only in the medial nucleus, while decreasing their numbers in the cortical nucleus and having no effect in the central nucleus (Drekic et al.1995).

Glia also appear responsive to hormones during puberty, another critical period in development. Using BrdU labeling, Ahmed et al. (2008) demonstrated that new cells, including GFAP-positive cells, are added to the medial amygdala during puberty in rats. This pubertal addition was sexually dimorphic with males having more BrdU-positive cells in the medial amygdala than females. Interestingly, the addition of BrdU-positive cells in males was eliminated by prepubertal castration, strongly suggesting that the addition of new cells in the amygdala during puberty, including GFAP-positive cells, is modulated by gonadal hormones. Thus, while gliogenesis in the amygdala appears to be regulated by gonadal hormones during development, the level of responsiveness is both sex- and subregion-specific. This specificity is also found in the adult amygdala in response to gonadal hormones.

Gonadal hormones and amygdala glia during adulthood

Several reports have documented glial sensitivity to fluctuations in hormone levels within the amygdala of adult animals. For example, Blutstein et al. (2006) reported that estradiol treatment of mice produces an increase in glutamine synthase gene expression in the medial amygdala of adults, an interesting effect given that neurons are incapable of synthesizing glutamate and are dependent upon glia to replenish their glutamine supply. Furthermore, treatment of meadow voles with either

testosterone propionate or estradiol benzoate, but not DHT, resulted in a significant increase in BrdU-labeled cells, 35% of which were glia, in the amygdala but not in the dentate gyrus or ventromedial hypothalamus (VMH; Fowler et al. 2003). Interestingly, estradiol benzoate seems to have this effect in meadow voles but not in prairie voles (Fowler et al. 2005). The presence of an effect on glia in the amygdala, a region closely linked to sexual behavior, in a polygamous rodent species (meadow voles) but not in the monogamous rodent species (prairie voles) suggests hormone-dependent changes in the number of amygdala glia may be involved in reproductive behaviors.

The medial amygdala is involved in reproductive behavior in several species and glia within this region seem particularly responsive to changes in adult hormone levels. For example, multiparous females given postpartum contact with pups exhibit reduced numbers of GFAP-positive cells in the medial amygdala compared to pup-exposed primiparous females (Featherstone et al. 2000), a difference which may be due to greater estrogen exposure in postpartum dams. In addition, the density of GFAP-immunoreactivity is higher in the medial subregion during the proestrus phase compared to the other phases of the estrous cycle in rats (Martinez et al., 2006), and in ovariectomized females, injection of estradiol, alone or with progesterone, increased GFAP-immunoreactive density in portions of the medial amygdala (Martinez et al., 2006).

Similarly, testosterone increases glial cell numbers (based on their appearance in Nissl staining) in the MePD of ovariectomized adult females compared to ovariectomized controls (Morris et al., 2008), a pattern of results strongly suggesting estrogenic metabolites of testosterone may lead to glial proliferation in some parts of the

adult medial amygdala. In the anteroventral medial amygdala (MeAV), by contrast, Carrillo et al. (2007) found no changes in glial number across the estrous cycle, despite changes in volume and other elements. Thus, even within subregions of the medial amygdala (MePD compared to MeAV) glia may vary in their responsiveness to changes in steroid hormone levels.

In addition to hormone mediated plasticity in adulthood, dramatic sex differences can be found in adult amygdala glia. As mentioned above, total glia numbers based on Nissl staining are sexually dimorphic in the MePD of adult rats with males having more glia than females (Morris et al. 2008). GFAP-ir density measures also suggest a sex difference in astrocytes within the MePD and MePV regions with females having greater GFAP-ir density than males (Rasia-Filho et al. 2002).

Of course, gonadal hormones can influence astrocytes through several possible pathways. In the portions of the hypothalamus estrogens influence the complexity of astrocyte arbors through indirect actions through neurons (Mong et al. 2002). Neurons in the amygdala, especially the medial regions, are rich in steroid hormone receptors, suggesting that a similar indirect pathway may occur in the MePD (Simerly et al. 1990; Sholl and Kim 1990; Donahue et al. 2005). Alternatively, steroid hormones may act directly on glial cells.

Astrocytes in several species are known to express ARs and ER although this is highly region specific (guinea pig: Langub and Watson 1992; rat: Azcoitia et al. 1999; DonCarlos et al. 2006; primates: Finley and Kritzer, 1999; Blurton-Jones and Tuszynski 2001). However, as far as I am aware, no report has confirmed the presence of either ER or ARs in amygdala astrocytes in vivo, leaving open the possibility that hormone

effects on astrocytes in the amygdala are through indirect pathways—that steroids directly affect neurons, which then affect astrocytes.

Amygdala Glia: Conclusions

The available evidence makes a single cohesive report of glia function in the amygdala difficult to produce. Clearly glia have offered a wealth of intriguing and informative findings within the past several decades, and several excellent reviews have summarized glia-based findings in various brain regions and in the brain as a whole (Barres, 2008; Eng, Ghirnikar and Lee, 2000; Freeman and Doherty, 2006; Garcia-Segura and Melcangi, 2006). Glia throughout the brain, not just the amygdala, are involved in several disorders and diseases, but the amygdala's prominent sexual dimorphisms suggest glial sex differences in this region may be linked to sex differences in vulnerability to disorders such as depression, schizophrenia, anxiety disorders, and autism.

Unfortunately, little work has quantified the effects of gonadal hormones on astrocytes in the amygdala. While measures of GFAP-ir and classification systems for astrocyte complexity are important early steps, they provide no information about astrocyte numbers and fews details about the arbors of these highly plastic cells. Are the numbers of astrocytes in the MePD different in male and female animals? If so, when does the sex difference arise? Furthermore, GFAP-ir evidence suggests that medial amygdala astrocytes are plastic and hormonally responsive in adulthood (Martinez et al. 2006). Which class of gonadal hormones are amygdala astrocytes responding to in adulthood? Answering these questions will certainly provide a more

complete picture of amygdala neuroanatomy and may provide important clues to the regions function.

Overview

Astrocytes are both important in brain functioning but poorly understood. Their role in the formation, maintenance and function of synapses makes understanding astrocytes particularly important in brain regions known to be plastic throughout the lifespan, such as the amygdala. While examination of hypothalamic astrocytes has produced many intriguing findings, I now present the first detailed analysis of astrocytes in the amygdala.

In Chapter 1 I describe the number and arbor complexity of MePD astrocytes in unmanipulated adult animals. These results represent the first actual quantification of astrocyte arbor complexity to date and reveal complex sex and hemisphere differences. I also include Tfm male rats in this analysis which establishes that the sex differences found depend upon functional ARs, leading us to question whether these differences are 1) influenced by circulating hormones in adulthood and/or 2) are present earlier in development.

In Chapter 2 I examine the former question by gonadectomizing adult male and female rats and giving some testosterone. I demonstrate that both astrocyte numbers and arbor complexity are influenced by circulating adult hormone levels but in a hemisphere specific manner, further suggesting that the two amygdalae respond differently to circulating hormone levels. Furthermore, I provide evidence that astrocytes in the MePD, like astrocytes in the hippocampus and hypothalamus, contain

AR, which suggests the possibility of a cell-autonomous response of astrocytes to androgens.

In Chapter 3 I investigate the developmental timing of the sex differences in astrocyte number and complexity by examining astrocyte in the MePD of juvenile wt and Tfm male rats. The results demonstrate that while a sex difference in astrocyte number exists before puberty, no sex differences in astrocyte complexity are found in juvenile animals. The use of Tfms is again informative, revealing that Tfm males have a are masculine number of astrocytes before the important influence gonadal hormones during puberty.

Due to the striking laterality found in almost every aspect of MePD astrocytes that I examined, I review lateralized sex differences in the amygdala in Chapter 4. While baffling and lacking a clear explanation, the laterality in the amygdala is potentially of great importance since effects previously identified may be eliminated, or new ones revealed, if the hemispheres are investigated separately. Finally, I briefly discuss my findings and provide possible avenues for future exploration to expand the research, both microscopically in terms of synapse formation within the MePD, and macroscopically in terms of intra-amygdala connectivity.

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CHAPTER 1: SEX DIFFERENCES AND LATERALITY IN ASTROCYTE NUMBERS AND COMPLEXITY IN THE ADULT RAT MEDIAL AMYGDALA

Abstract

The posterodorsal portion of the medial amygdala (MePD) is sexually dimorphic in several rodent species. In several other brain nuclei, astrocytes change morphology in response to steroid hormones. I visualized MePD astrocytes using glial-fibrillary acidic protein (GFAP) immunocytochemistry. I compared the number and process complexity of MePD astrocytes in adult wildtype male and female rats, and testicular feminized mutant (Tfm) male rats that lack functional androgen receptors (ARs), to determine whether MePD astrocytes are sexually differentiated and ARs have a role. Unbiased stereological methods revealed laterality and sex differences in MePD astrocyte number and complexity. The right MePD contained more astrocytes than the left in all three genotypes, and the number of astrocytes was also sexually differentiated in the right MePD, with males having more astrocytes than females. In contrast, the left MePD contained more complex astrocytes than did the right MePD in all three genotypes, and males had more complex astrocytes than females in this hemisphere. Tfm males were comparable to wildtype females, having fewer astrocytes on the right and simpler astrocytes on the left than do wildtype males. Taken together, these results demonstrate that astrocytes are sexually dimorphic in the adult MePD and that the nature of the sex difference is hemisphere-dependent: a sex difference in astrocyte number in the right MePD and a sex difference in astrocyte complexity in the left MePD. Moreover, functional ARs appear to be critical in establishing these sex differences in MePD astrocyte morphology.

Introduction

The amygdala is implicated in the control of many social behaviors, including fear, anxiety, and social interactions (Adolphs et al. 2005; Bishop et al. 2004; LeDoux et al. 1998; Mah et al. 2007; Spezio et al. 2007; Truit et al. 2007; Williams et al. 2005). Interestingly, many behavioral disorders in which the amygdala has been implicated have clear sex-biases in the human population. For example, schizophrenia, autism, and drug-addiction are all more common in males, while anxiety disorders and depression are more common in females (American Psychiatric Association, 2000). Many aspects of the amygdala are sexually dimorphic in a variety of mammalian species, lending support to the idea that structural differences in the brain may underlie biases in behavior and disease susceptibility, and that the amygdala may be a key player in the control of such behaviors.

In rats, the medial amygdala is composed of several subnuclei that are extensively interconnected (Alheid et al. 1995; Grove 1988a, 1988b; Shammah-Lagnado et al. 2000). The posterodorsal medial amygdala (MePD) in particular receives input from chemosensory and hypothalamic regions and projects to the preoptic area and ventral medial hypothalamic nucleus, both of which play important roles in reproductive behaviors. The MePD normally contains a high concentration of both androgen receptors (ARs) and estrogen receptors (ER; Simerly et al. 1990), and is highly sexually dimorphic in adults. The MePD in male rats has a larger overall volume (Hines et al. 1992), contains larger neuronal somata, more neurons (Morris et al. 2008), and greater dendritic spine density than the MePD in female rats (Rasia-Filho et al. 2004). The adult MePD is also highly plastic. Castration of adult male rats eliminates

the sex difference in regional volume and neuronal soma size (Cooke et al. 1999), and causes deficits in noncontact erections and ultrasonic vocalizations, while treatment with androgens and/or estrogens prevents these changes following castration (Cooke et al. 2003).

Typically, estrogen acting on ER is regarded as the pathway responsible for hormone-induced masculinization in the rodent brain. However, recent results indicate that AR also contributes to sexual differentiation of many brain regions, including the MePD. Testicular feminized mutant (Tfm) male rats lack functional AR and are insensitive to androgens (Yarbrough et al. 1990) and thus represent an important tool for assessing the role of androgens in the origin of neural sex differences. I have found that several aspects of MePD morphology are only partially masculinized in Tfm males, indicating that ARs are necessary for complete masculinization of this nucleus (Morris et al. 2005), but where androgens act to masculinize the MePD has not been established.

Gonadal hormones not only influence neuronal morphology, but also modulate the morphology of non-neuronal cells such as astrocytes, which are thought to contribute critically to some forms of neural plasticity. For example, estrogens increase the complexity of astrocyte arbors in the arcuate nucleus and preoptic area of the hypothalamus (reviewed in Garcia-Segura et al. 1994 and in Garcia-Segura and McCarthy 2006). Moreover, androgens increase the number of astrocytes in hippocampal cultures (Gatson and Singh 2007). Astrocytes are also known to express steroid hormone receptors, including both ARs and ERs (Doncarlos et al. 2005; Lorenz et al. 2005). Thus, steroid hormones may act directly on astrocytes to regulate their morphology and function. Given the implicated role of astrocytes in adult neural

plasticity, their steroid responsiveness in some brain regions, and the highly plastic nature of the adult MePD, I speculated that astrocytes might exhibit sexually dimorphic morphologies in the MePD. Indeed, reports of sex differences in the MePD in both the density of glial fibrillary acidic protein (GFAP) immunoreactivity and in the number of Nissl-stained glial cells (Martinez et al. 2006; Morris et al. 2008) suggest that MePD astrocytes may be sexually dimorphic in both number and process complexity. I now report sex differences in both measures, and that Tfm males are like females in having fewer and less complex MePD astrocytes than wildtype males, indicating that both the number and complexity of MePD astrocytes in rats are normally masculinized through the activation of ARs.

Methods

Ninety to 120 day-old wildtype male, wildtype female and Tfm male rats were obtained from our Tfm colony at Michigan State University. Female carriers of this spontaneous mutation of the AR gene have been bred with Long Evans male rats from Charles Rivers for over 10 generations. Estrous cycle was not monitored for the females used in this study, thus differences in ovarian hormones were not accounted for. Animals were housed three per cage in a single colony room in standard rat cages with food and water available ad libitum. Lights were on at 0700 and off at 1900 hours. Animals were cared for in accordance with the guidelines set forth by the National Institute of Health and all procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Animals were given an overdose of sodium pentobarbital (120mg/kg, ip). Once animals were deeply anesthetized (showing no reflex response to either tail or foot

pinch) they were intracardially perfused with 0.9% saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4, ~250 mL/animal). The brains were then removed and postfixed for five hours at 4^0 C in the same fixative solution and stored in 30% phosphate-buffered sucrose at 4^0 C for 48 hrs. The left cortex of each brain was scored with a shallow cut to mark that hemisphere. Brains were sectioned coronally on a freezing microtome at 40µm through the region of interest. Sections were collected into cryoprotectant (de Olmos et al. 1978) and stored at -20^0 C. Every third section through the rostrocaudal extent of the MePD was processed for immunocytochemistry.

Histology

For immunocytochemistry, 0.1 M phospate buffered saline with 0.3% Triton X-100 (PBS-T; 140 mM NaCl, 10.7 mM KCl, 1 mM KH2PO4, 10 mM Na2HPO4, pH 7.4) served as a rinsing agent between all incubations and as dilutant. Upon removal from cryoprotectant, tissue was placed into Netwell plates (Corning Life Sciences) and thoroughly rinsed. Sections were then incubated for 20 min in 1% sodium borohydride followed by a 25 min incubation in 1% H2O2, one hr incubation in 10% normal horse serum (Chemicon, Temecula, CA), and a 24 hr incubation at 40 C in mouse anti-GFAP monoclonal antiserum (1:50,000, Chemicon, MAB360). The antiserum was prepared against purified glial fibrillary acidic protein from porcine spinal cord. In western blots, the antiserum recognizes a single band at approximately 51 kDA (manufacturer's technical information). Staining of sections through the amygdala and hypothalamus produced a pattern of GFAP immunoreactivity that was identical to other descriptions (Mong et al. 1999; Rasia-Filho et al. 1999; Martinez et al. 2006).

Following incubation in primary antiserum, tissue was incubated in biotinylated rat-absorbed horse anti-mouse secondary antiserum (1:500, Vector Laboratories) for one hour at room temperature. This was followed by one hr incubation in peroxidase avidin-bitotin complex solution at half recommended strength (Elite ABC kit, Vector). HRP was visualized using NiCl-enhanced diaminobenzidine (DAB, Sigma) in a 0.05 M in Tris Buffer, pH 7.2. After rinsing to quench the peroxidase reaction, tissue was mounted on gel-subbed slides, dried, dehydrated in alcohol rinses, and counter-stained using Harris hematoxylin solution (Sigma), with 10% lithium carbonate used as the bluing agent. Slides were then coverslipped with Permount.

Stereological Analysis

A Zeiss Axioplan II microscope with the visual field captured by an Optronics MicroFire digital video camera and displayed on a monitor was used to quantify the number and complexity of astrocytes within the region of interest. Using StereoInvestigator software (v. 7.0, MBF Bioscience; Williston, VT), the perimeter of the MePD was traced in serial sections at low magnification. MePD boundaries were identified using the standard rat atlas (Paxinos and Watson, 2005), in conjunction with previously established standards within our laboratory (Morris et al. 2005, 2007) and others (Canteras et al. 1995; Hines et al. 1992). Several consistent morphological features were used to accurately identify and trace the MePD, including the angle and length of the optic tracts, the overlap of the optic tract with the cerebral peduncle, the descent of the stria terminalis into adjacent regions, and the dense collection of neurons forming the posteroventral medial amygdala. After tracing the boundaries of the MePD at low magnification, astrocytes were counted and traced using a 100X Plan-NeoFluar,

1.3 N.A oil-immersion objective. Slides were coded to ensure that all measures were performed by an observer who was "blind" to the group membership of each animal. Images of astrocytes were captured using the image capture option in StereoInvesitgator. Images were resized and adjusted using Adobe Photoshop (v 7.0 San Jose, CA). All images received a +5 increase in brightness through a brightness/contrast adjustment layer available in the software.

Astrocyte number: An optical fractionator probe (West et al. 1991) was used to generate an unbiased estimate of astrocyte numbers within the boundaries of the MePD. The optical fractionator estimates the total number of objects within a space based on a sample representing a known fraction of that space. The method uses a 3 dimensional probe with a fixed, specified volume inserted into the tissue. Initial probe insertion is random within the region of interest but then proceeds at fixed intervals allowing for an unbiased and accurate estimate of the number of objects within the entire region of interest. Probe dimensions were 45 by 45 μm with a height of 6-9 μm. The coefficient of error (CE; Gunderson m=1) for each hemisphere was at or below 0.10.

Criteria for identifying an astrocyte included a distinct and recognizable nucleus in a plane of focus within the sampling probes' Z-value, and from which at least 2 GFAP labeled fibers extended. Such nuclei were often, although not always, smaller than surrounding (presumably neuronal) nuclei, often elliptical in shape, and lacking a clear nucleolus. At times, especially around vascular pathways and at cortical boundaries, GFAP immunoreactivity was dense enough to obscure the nucleus of origin and in these cases, no cells were marked or traced. These methods produced estimates of

overall MePD volume, number of astrocytes, and average area of astrocyte nuclei per hemisphere for each subject. The number of cells counted per hemisphere ranged from approximately 250-400 depending on the sex and hemisphere of the subject.

Astrocyte process complexity: Neurolucida software (v. 7.0, MBF Bioscience; Williston, VT) was used to trace and measure the entire visible arbor of 24 randomly selected astrocytes per subject. The MePD was traced via the criteria and methodology described above and a fractionator probe was used to define random tracing sites throughout the MePD, with 12 sites per hemisphere. At each of these sites, the astrocyte nearest the randomly placed marker, but at least 30 μm from the MePD boundary, was traced in its entirety. The average number of primary processes, average number of branch points, average number of branch endings, and average branch length were calculated for each animal.

Additionally, each astrocyte was classified into one of four groups based on overall morphological complexity following a classification scheme developed by Mong and McCarthy (1999). In brief, Group I astrocytes exhibit few and short primary processes (Fig 1A). Group II astrocytes possess more and longer primary processes but few secondary processes (Fig 1B). Group III possess more secondary processes and finally Group IV astrocytes have large and complex arbors with numerous secondary processes (Mong et al. 1996; Mong et al. 1999, Figs 1C and 1D). The percentage of astrocytes within the MePD that fall into each of the categories was calculated for each of the 3 experimental groups (male, female and Tfm male).

Statistical Analysis

Separate two-way mixed-design analyses of variance (ANOVA) were conducted for each dependent variable (MePD volume, astrocyte number, number of primary processes, number of branch points, number of branch endings, and branch length). The left and right hemispheres served as a repeated measure and genotype (male, female, Tfm) as a between group measure. This was followed by one-way ANOVAs for each individual hemisphere and LSD post hoc analysis to determine differences between genotypes on each side. For all analyses, results are expressed as mean ± SEM (standard error of the mean), and a p value of 0.05 was considered statistically significant, with number of animals per group reported in figures.

Results

Regional Volume

Mixed design 2-way ANOVA (left and right hemisphere as repeated measures) revealed a main effect of sex on MePD volume ($p < 0.01$) and a main effect of hemisphere ($p < 0.01$) and a sex by hemisphere interaction ($p < 0.001$). In all three groups, the right hemisphere had greater regional volume than the left (males: $p < 0.01$; TFMs: $p < 0.05$; females: $p < 0.05$, Fig 2A). Posthoc analysis revealed that volume of the MePD varied significantly across genotypes only in the right hemisphere ($p < 0.05$). Thus, the overall main effect of genotype is due to differences in the right but not the left hemisphere. Additional post-hoc analyses revealed that the right MePD of males has a greater volume than that of either Tfm males or females (ps <0.04, Fig 2A) and that volume of the right MePD is not different in Tfm males and females ($p = 0.323$). These data agree well with estimates of MePD volume based on Nissl stained material (Morris

et al. 2005, 2008), suggesting that the MePD boundaries revealed by GFAP and hematoxylin is comparable to that shown by Nissl stain.

Astrocyte Numbers

There was a main effect of hemisphere ($p < 0.001$) on the number of MePD astrocytes indicating that astrocytes are more numerous on the right than on the left side in all three groups (males: $p < 0.001$; TFMs: $p < 0.01$; females: $p < 0.001$, Fig 2B). There was no significant main effect of genotype on the number of astrocytes but there was a significant hemisphere by sex interaction ($p = 0.01$, Fig 2B), which led to separate one-way ANOVAs for each hemisphere. Although astrocyte number does not differ significantly by genotype in the left hemisphere ($p = 0.49$), it does in the right hemisphere ($p < 0.05$). Additional post-hoc analyses reveal that wildtype males have more astrocytes on the right than do Tfm males ($p < 0.01$) and marginally significantly more do females ($p = 0.058$). Thus, in the left hemisphere, astrocyte numbers are not sexually dimorphic whereas in the right hemisphere, astrocyte numbers are sexually dimorphic, with wildtype males having more than wildtype females and Tfm males. Since Tfm male rats lack functional ARs but have slightly higher than normal male levels of androgen (Roselli et al. 1987), the difference between wildtype and Tfm males in astrocyte number suggests that functional ARs are necessary to reach normal masculine levels.

Astrocyte Complexity

Astrocyte complexity was assessed using several measures: the number of primary branches, the number of branch points, the number of branch endings, and the average length of branches. In all complexity measures, I found a significant main

effect of laterality (*p*s < 0.005) with the left hemisphere consistently exhibiting more complex astrocytes than the right (Fig 3). I also found significant main effects of genotype on each measure of astrocyte complexity (*p*s< 0.05) except for number of primary branches where there was no main effect of genotype. Despite finding no significant interactions of genotype with hemisphere on any measure of astrocyte complexity, posthoc analyses revealed that astrocyte complexity varied significantly by genotype on three of the four measures (number of branch points, number of branch endings, and average branch length), but only in the left hemisphere (*p*s< 0.01). Further analyses of astrocyte measures indicate that left MePD astrocytes of wildtype males are more complex than those of females on all four measures, having on average more primary branches as well as total number of branches, more branch points and longer branches than do astrocytes in the left MePD of females (*p*s < 0.5, Fig 3). Except for average number of primary branches, males also showed more complex astrocytes than did Tfm males on the left MePD (*p*s < 0.01, Fig 3). Thus, although the number of astrocytes in the left hemisphere was not sexually differentiated, astrocyte complexity was. This is in contrast to the right hemisphere, which exhibits an AR-dependent sexual dimorphism in total astrocyte numbers but exhibits no sex differences in astrocyte complexity. Furthermore, the sex difference in astrocyte complexity in the left MePD also appears to be AR-dependent, as Tfm males did not significantly differ from females in any measure of astrocyte complexity.

A principle components analysis for each hemisphere revealed that all four measures of astrocytes arbor complexity loaded very strongly onto a single factor, suggesting that the four measures used are closely related and could be viewed as a

single measure of overall arbor complexity in that hemisphere. One-way ANOVAs examining this single factor in each hemisphere confirmed a significant effect of genotype on overall arbor complexity in the left MePD ($p < 0.01$) but no effect in the right MePD. Post hoc analysis of the left MePD revealed that males have greater overall arbor complexity than either Tfm males or females (ps < 0.01).

Morphology Classification

Astrocyte arbor complexity was measured and analyzed using methodology established by Mong and McCarthy (1999). Chi-square analysis indicates that distribution of the four classes of astrocytes is different in males compared to females or Tfm males (ps < 0.01), in the left MePD, but not the right, with males having more class IV astrocytes in the left hemisphere than do females or TFMs. Furthermore, Tfm males did not differ from females ($p = 0.611$). Thus, this nominal method of assessing astrocytes arbor complexity agrees well with the quantitative measures of complexity. Both indicate that males exhibit more complex astrocytes than females or TFMs, but only in the left hemisphere, and that Tfm males are feminine in their astrocyte complexity. GFAP labeling may not reveal ultra fine astrocyte processes, so these data should not be taken as absolute measures of astrocytes complexity, but as relative measures across the three groups.

Astrocyte Nuclear Size

Analysis of the average area of individual astrocyte nuclei revealed no main effects of sex or hemisphere. However, there were several correlations; in the left MePD, average astrocytic nuclear size across all genotypes correlated positively with the number of primary branches and the average branch length (Pearson correlations:

0.72 and 0.53 respectively). No measure of astrocyte complexity correlated with astrocyte nuclear size in the right MePD. These correlations suggest that nuclear size may become critical when astrocytic branching becomes extensive as in the left MePD.

Discussion

Prior investigations demonstrated that the adult MePD is highly plastic and responds to steroid hormones (Cooke et al. 1999, 2003; Morris 2005, 2008). Because astrocytes are implicated in regulating neuronal plasticity and are known to be steroid-responsive in some brain regions (Mong et al. 1999; Mong and McCarthy 2002; Garcia-Segura et al. 1994), I asked whether astrocytes in the adult MePD are sexually dimorphic in number and/or complexity and if so, whether such differences are sensitive to hormone action via ARs. I found sex differences in, and dramatic laterality of, astrocyte number and complexity in this nucleus, and by utilizing Tfm males that lack functioning AR, I also determined that masculinization of astrocyte number and complexity in the MePD requires functional AR.

MePD Laterality

Laterality in adult MePD anatomy is not unexpected and has been reported previously in the literature. Briefly, the adult right MePD is larger in volume than the left based on Nissl stained material (Morris et al. 2005). Surprisingly, although the left MePD is smaller in volume, it contains more neurons and is sometimes reported to contain neurons that have larger cell bodies than those in the right MePD (Morris et al. 2008). Although reports have detailed MePD connectivity in the rat (Canteras et al. 1992; Coolen et al. 1998), hemisphere differences in these parameters are either absent or unreported.

The current results report a new and dramatic lateralization in the anatomy of the adult rat MePD, with the right having more astrocytes than the left (Fig 2), while the left has more complex astrocytes than the right (Fig 3). The increased number of astrocytes in the right MePD may not be particularly surprising, given its larger volume compared to the left. However, data on neuronal number makes it clear that larger regional volume need not predict greater cell number (Morris et al, 2008). The increased complexity of astrocytes in the left MePD, as revealed in several morphological measurements, was unexpected. The use of a previously established astrocyte complexity classification scheme (Mong and McCarthy 1999) also suggests that the left hemisphere has more complex astrocytes than the right (Table 1).

Thus, it appears that astrocytes follow different strategies to populate each side of the MePD: fewer but more complex astrocytes on the left versus more but simpler astrocytes on the right. Understanding the significance of this dramatic laterality is a challenge. The left MePD contains more neurons than the right (Morris et al. 2008) and prior to puberty contains more dendritic endings and more excitatory synapses than the right MePD (Cooke et al. 2007; Cooke and Wooley 2005). Given that astrocytes ensheath neurons and synapses, the increased number of neurons and synapses on the left may explain why astrocytic processes are more extensive on this side. On the other hand, astrocytes in the right MePD are more numerous but significantly less complex. The simpler morphology of right MePD astrocytes is reminiscent of less mature glia (Sancho-Tello et al. 1995), suggesting that there may be a higher turnover of astrocytes in the right MePD than the left. This would lead to a greater proportion of young astrocytes with less complex arbors. Because adult treatment with testosterone

can increase the number of apparent glia (based on counts in Nissl) in the right but not the left MePD of adult females (Morris et al., 2008), androgens may influence ongoing turnover of astrocytes in the right MePD by regulating their genesis and/or survival. Indeed, finding more newly generated glia in the medial amygdala of male than female rats supports this view (Ahmed et al., 2008). In contrast, the left MePD may contain a more stable, and hence older and more complex, population of astrocytes. Combining cell birth and/or cell death markers with astrocytic markers such as GFAP could help to address this possibility in future experiments.

How this laterality in astrocyte morphology relates to behavior will be more challenging to understand. Unfortunately, reports describing the functional consequences of unilateral medial amygdala lesions in rodent models are rare. The few reports available suggest that the right medial amygdala may exert a stronger influence on the hypothalamic-pituitary-gonadal axis than the left in both sexes (Sanchez and Dominguez 1995; Gerendai et al. 1995). It is also possible that hemispheric and sex differences exist in the communication between the MePD and other brain regions.

Reports of functional laterality in the amygdala of humans are more common and offer a consistent portrait (Canli et al. 2002; Hamann et al. 2004; Cahill et al. 2004; LaBar et al. 1998). For example, Cahill et al. (2001) found that in males, activity of the right amygdala, but not the left, was related to recall of emotional memory. In females, activity of the left amygdala, but not the right, was related to such processing. Furthermore, by examining patients in a resting state using fMRI, Kilpatrick et al. (2006) found that the right amygdala exhibited a more widespread distribution of functional connectivity at rest in men than in women, and that the left amygdala exhibited a more

widespread distribution of functional connectivity at rest in women than in men. Interestingly, the connectivity pattern was also different for males and females. Kirkpatrick and colleagues (2006) concluded that during resting states there are dramatic differences in the functional networks formed by the amygdala in men and women, and that there is evidence for a female-left, male-right pattern of lateralization.

Perhaps, as is suggested in the human literature, sex differences and hemisphere differences in the rodent MePD reflect differences in functional connectivity with other regions. It is clear that several behaviors are influenced by the medial amygdala and it is likely that astrocytes in this region are involved in restructuring of neural connections in response to gonadal hormones. Recent evidence suggests that astrocytes play a critical role in regulating neuronal responses and cerebral blood flow (Schummers, Yu and Sur, 2008), raising the possibility that sex differences in asymmetrical activity of the human amygdala may be related to sex differences in the asymmetry of astrocyte number or morphology. However, the importance of greater astrocyte numbers in the right hemisphere and greater astrocyte complexity in the left hemisphere for brain function and behavior requires further study.

Astrocytes and MePD Anatomy

Dissociations between neuron number and sexually dimorphic regional volume are not common in the literature. Because the MePD is one such rare example (Cooke et al. 2003; Morris et al. 2008), I expected that astrocytes might be a large determinant of regional volume. Although significant correlations existed between regional volume and astrocyte number in the right hemisphere, astrocyte density (regional volume/astrocyte number) was not equivalent across sexes or hemispheres (data not

shown), which one would expect if astrocyte number and regional volume were closely linked. In fact, significant differences were found in astrocyte density, with females having greater density compared to males, a result is consistent with a previous reports of se differences in GFAP immunoreactivity in the MePD based on optical density (Rasia-Filho et al. 2002). Accordingly, in our multiple regression analysis examining which measures significantly contribute to right regional volume, total astrocyte number emerged as a significant positive predictor ($p < 0.05$).

In the left hemisphere, astrocytic primary branches, branch endings, and branch length all demonstrated significant positive correlations with left hemisphere volume. Interestingly, left hemisphere volume also significantly correlated with average astrocyte nuclear size. Thus, right MePD volume appears loosely linked to astrocyte numbers and left MePD volume may be linked to overall astrocyte complexity and average astrocyte nuclear size. Future experiments, which manipulate androgens and measure astrocyte morphology, may clarify which components change with androgen-induced changes in regional volume and neuronal soma size.

Since astrocytes appear to regulate the formation and stabilization of dendritic spines (Ullian et al. 2001; Nishida and Okabe 2007), one might expect a relationship between astrocytes and dendritic complexity in the MePD. In prepubescent rats, the volume occupied by glia and the number of dendritic branch endings are greater in males than in females in the left hemisphere (Cooke et al. 2007). In adults, males have greater dendritic spine density than females in the MePD, but hemispheric information is not available (Rasia-Filho et al. 2004). Depending on which hemisphere is considered, adult sex differences in spine density would depict two very different pictures of

astrocyte-neuron interactions. Increased branch complexity in astrocytes has been associated with an increase in functional synapses in other brain regions (Pfrieger and Barres 1997; Elmariah et al. 2005) but has also been associated with decreases in dendritic spines (Mong et al. 2001). Currently, it is not clear how various aspects of astrocyte morphology (number versus complexity) relate to neurons, or whether this relationship varies in a region-specific manner.

However, it is also possible that the male bias in astrocyte number in the right MePD is related to synaptic connectivity. MePD volume occupied by glia has been reported to be sexually monomorphic in the right hemisphere prior to puberty in mice (Cooke et al. 2007), suggesting that sex differences in glial number emerge during puberty. Given that neuron-astrocyte communication is important in normal pubertal development in the hypothalamus (Dziedzic et al. 2003; Prevot et al. 2003), perhaps part of the rewiring of the MePD during puberty is accomplished through gliogenesis, with glia leading the way in establishing sex differences in connectivity, similar to the role glia may play in establishing sex differences in neuron number in the bird song system (Nordeen and Nordeen 1996). These new astrocytes may aid in the formation and maintenance of crucial neuronal connections that ultimately contribute to the expression of sex-specific behaviors. Future studies examining the interaction between MePD astrocytes and local dendrites in each hemisphere are needed to understand the role of astrocytes in modulating MePD structure and function.

Influence of AR

I found striking sex differences in astrocyte morphology in the MePD, with adult wildtype males having more astrocytes than females on the right and more complex

astrocytes than females on the left. Moreover, MePD astrocytes in Tfm males were like those in females and not like those in wildtype males. The number of astrocytes in the right MePD of Tfm males was equivalent to that of females and significantly less than that of males. All measures of astrocyte complexity except the number of primary branches were also reduced in Tfm males compared to wildtype males. Also based on the classification method, Tfm males exhibited a distribution of astrocytes complexities that matched that of females, but differed from males (Table 1). Finally, Tfm males did not significantly differ from females for any astrocyte measure, in either hemisphere. These results clearly indicate that the sex differences in the number of astrocytes in the right MePD and astrocyte complexity in the left MePD of adult rats are dependent upon functional AR. ARs may act to promote the extension and elaboration of branches of already established astrocytes on the left side, whereas ARs may promote gliogenesis on the right. Either of these actions may be due to organizational effects of androgens during development or activational effects during adulthood. How ARs exert these different actions on the two sides is not clear but perhaps lateralization of expression of ARs or critical cofactors are involved.

AR may influence astrocyte morphology through direct or indirect pathways. If AR is found in MePD astrocytes, then androgens could act directly on these cells to produce changes. Testosterone treatment increases the number of astrocytes in the female rodent hippocampus (Conejo et al. 2003), and cultured astrocytes taken from the optic nerve, which contain AR, respond to androgens with increases in GFAP and/or proliferation (Aqapova et al. 2006). Furthermore, in cultured cortical astrocytes, activation of membrane bound AR leads to proliferation and an increase in GFAP

(Gatson and Singh 2007) further suggesting that direct androgen-induced modulation of astrocytes might occur in the MePD. However, the majority of AR containing cells in the adult MePD are neurons (Greco et al. 1998; Lorenz et al. 2005), despite reports of astrocytic AR in other brain regions (DonCarlos et al. 2005).

Alternatively, androgens may be acting on neuronal AR in the MePD or elsewhere to affect MePD astrocytes indirectly. Androgens may stimulate neurons to communicate with astrocytes via a chemical messenger or may induce morphological changes in neurons, which astrocytes respond to. In the arcuate nucleus, evidence suggests that estrogens induce GABA synthesis and release in local neurons, which then act on GABA receptors in surrounding astrocytes to induce growth of astrocyte processes (Mong et al. 2002). A similar, but AR-dependent, mechanism may occur in the MePD. Because AR protein is defective throughout life in Tfm males, our data do not address the stage at which AR promotes masculinization of MePD astrocytes. Nor do they address whether adult androgen manipulations affect astrocyte morphology in the MePD of either hemisphere. Such information will add greatly to our understanding of how gonadal hormones influence the MePD to regulate behavior.

APPENDIX

Table 1 - Percent of Astrocytes Within Each of the Four Categories of Complexity[1], by Genotype and Hemisphere.

	Left Hemisphere				Right Hemisphere			
Astrocyte Group	1	2	3	4	1	2	3	4
Male	0.0	3.3	23.3	73.3	23.3	20.0	25.0	31.6
Tfm males	5.0	13.3	31.6	50.0	8.3	21.6	36.6	33.3
Female	1.6	11.7	36.7	50.0	15.0	18.3	28.3	38.3

[1] Group 1 astrocytes are the least complex; group 4 are the most complex.

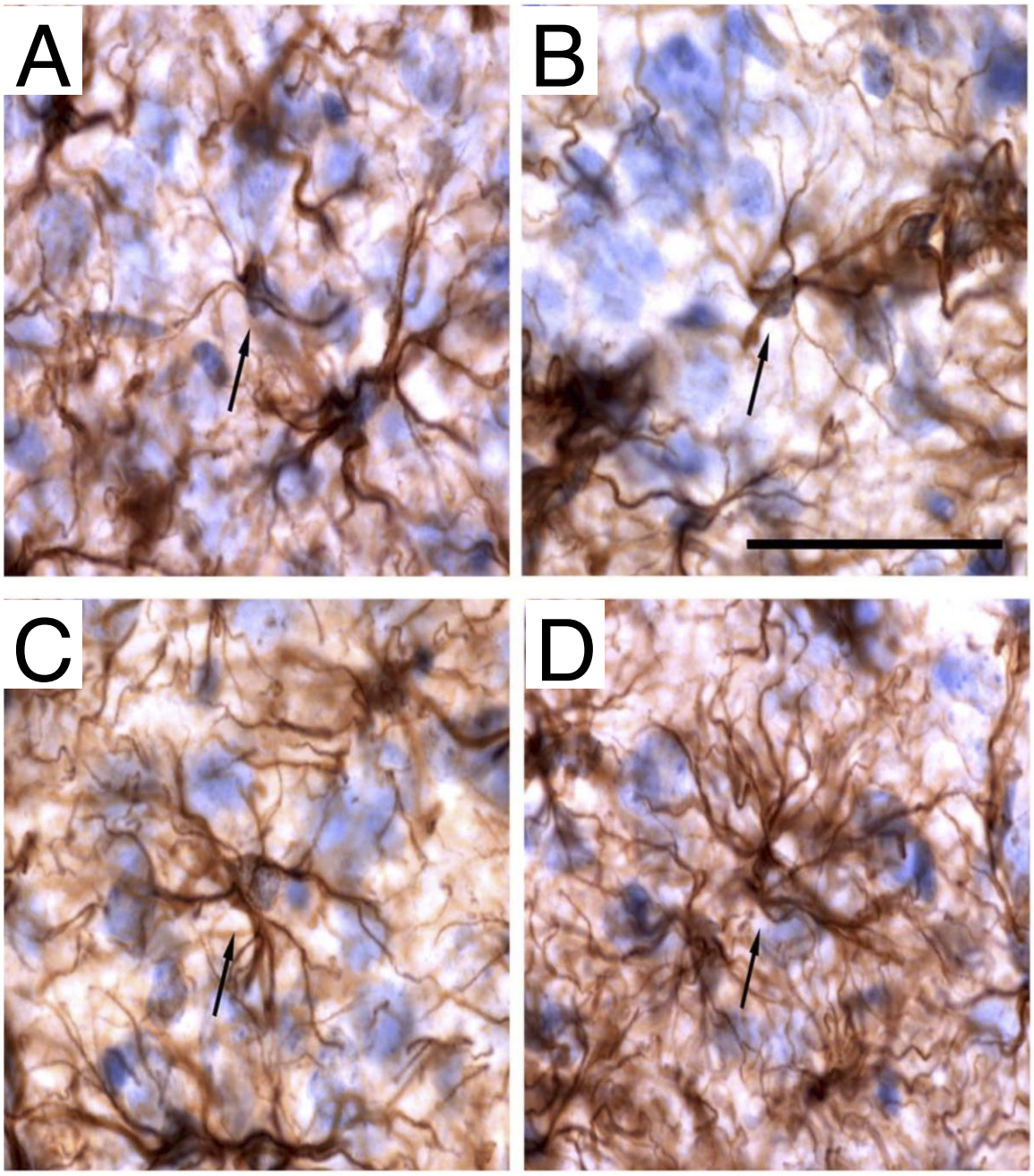

Figure 1 - For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation. Photomicrographs depicting astrocytes of varying complexity. Panel A depicts a Group 1 astrocyte, which has few and short processes. Panel B depicts a Group 2 astrocyte, which has several processes but branches remain unelaborated. Panel C depicts a Group 3 astrocyte, which has multiple elaborated processes and Panel D depicts a Group 4 astrocyte, which is highly elaborated. Because of the single plane of focus depicted, only a portion of the full extent of arbor complexity is visible here. Complex astrocytes frequently demonstrated multiple branches that emerged as the plane of focus was moved through the tissue. Scale bar = 50 microns

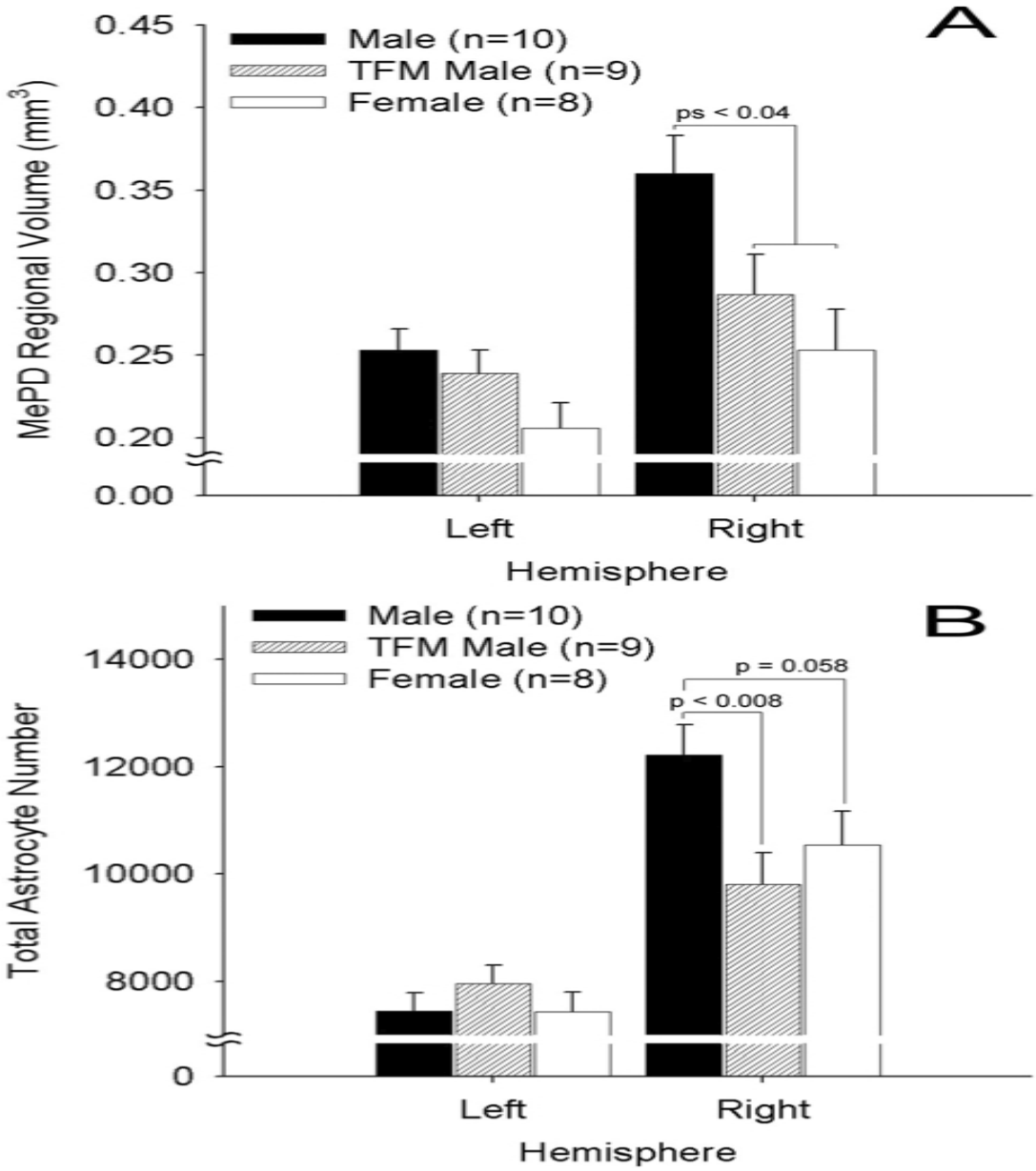

Figure 2 - Estimated regional volume (A) and total astrocyte number (B) in the posterodorsal medial amygdala (MePD) of adult wildtype male and female rats and testicular feminized mutant (Tfm) male rats, which lack functional AR. The MePD volume is significantly larger on the right than the left in all three groups, but this laterality was most dramatic in males. The right hemisphere is also significantly larger in males than in females and Tfm males. The right MePD contained significantly more astrocytes than the left in all three groups (B). This laterality was most prominent in males, leading to a sex difference in astrocyte number only on the right side. Note that Tfm males have fewer astrocytes than wildtype males but no more than do females in the right MePD, indicating that the sex difference in astrocyte number depends on AR. In contrast, the left hemisphere showed no effect of genotype. Values are means of N = 8 – 10 rats/group (+ standard error of the mean, SEM).

Figure 3

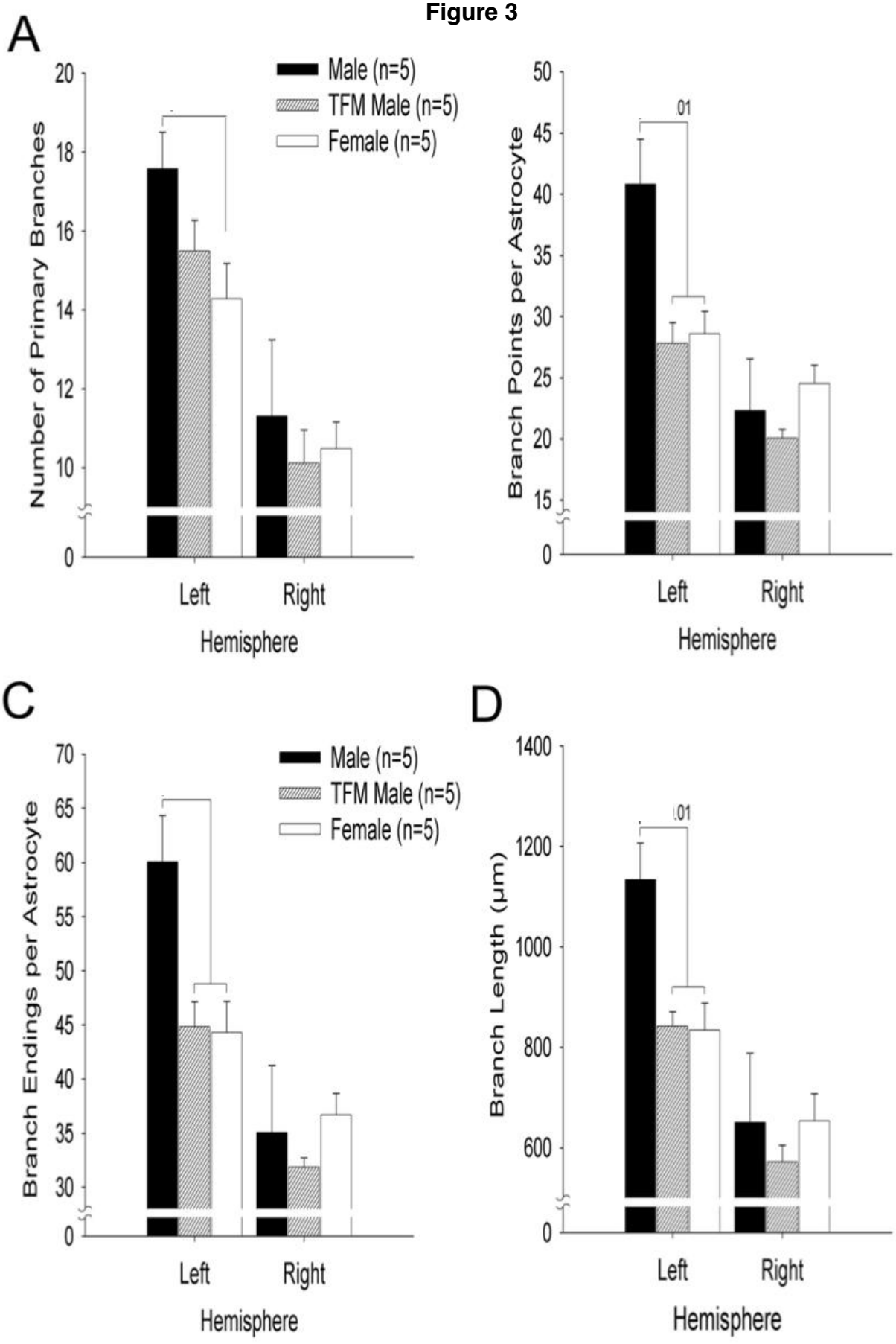
A
Male (n=5)
TFM Male (n=5)
Female (n=5)
Number of Primary Branches
20
18
16
14
12
10
0
Left
Right
Hemisphere
Branch Points per Astrocyte
50
45
40
35
30
25
20
15
0
Left
Right
Hemisphere
C
Male (n=5)
TFM Male (n=5)
Female (n=5)
Branch Endings per Astrocyte
70
65
60
55
50
45
40
35
30
0
Left
Right
Hemisphere
D
Branch Length (µm)
1400
1200
1000
800
600
0
Left
Right
Hemisphere

Figure 3 - Estimated number of primary branches (A), branch points (B), branch endings (C), and average branch length (D) in the MePD of adult wildtype male, Tfm male and female rats. In all three groups, astrocytes in the left MePD had more primary branches, more branch points, more branch endings and a larger average branch length than those on the right, indicating that astrocytes in the left MePD have more complex morphologies than astrocytes in the right. Moreover, there was a significant sex difference in astrocyte complexity in the left MePD, with all measures indicating that males have more complex astrocytes than females on the left side. Tfm males are not significantly different from females for any measure of astrocyte complexity. Data from Tfm males demonstrates that masculinization of astrocyte complexity in the left MePD depends on AR. Values are means of N = 5 rats/ group (+ SEM).

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CHAPTER 2: ASTROCYTES IN THE RAT MEDIAL AMYGDALA ARE RESPONSIVE TO ADULT ANDROGENS

Abstract

The posterodorsal medial amygdala (MePD) exhibits numerous sex differences, including differences in volume, and in the number and morphology of neurons and astroctyes. Gonadal hormones in adulthood have been shown to play a role in determining some of these sex differences but whether adult circulating hormones affect the morphology of MePD astrocytes is unknown. I examined astrocytes in the MePD of male and female Long Evans rats that were gonadectomized and treated for 30 days with either testosterone or control treatment. At the end of treatment, brains were collected and immunostained for glial fibrillary acidic protein (GFAP). I also co-stained for GFAP and the androgen receptor (AR) in normal adult, gonadally intact male rats to determine whether MePD astrocytes express ARs. Stereological analysis revealed that both the number and complexity of astrocytes in the MePD were influenced by adult androgens although many of the previously reported sex and laterality differences persisted, suggesting that androgens affect the morphology of MePD astrocytes both before and during adulthood. Furthermore, using confocal microscopy, I found robust AR expression in MePD astrocytes. This finding suggests testosterone may act directly on MePD astrocytes to influence their structure and function and raises the possibility that androgens work via astrocytes to influence MePD function.

Introduction

The amygdala, a highly conserved brain region, is primarily associated with two realms of behaviors: fear/anxiety behaviors (Rogan et al. 1997; LaBar et al. 1998; Nagy

et al. 1979; Adamec 1990), and social/mating behaviors (Sanders and Shekhar 1995; Adolphs et al. 2002; Adolphs and Tranel 2003; Faber-Zuschratter et al. 2009; Lehman and Winans 1982; Baum and Everitt 1992; Kondo and Arai 1995). The amygdala as a whole is also implicated in several diseases and disorders related to these behaviors, including autism, depression and anxiety disorder. Interestingly, all of these diseases and disorders exhibit sex biases in prevalence rates (American Psychological Association 2000).

The posterodorsal division of the medial amygdala (MePD) shows marked adult plasticity controlled by gonadal hormones (Morris et al. 2005; 2008a, b). For example, the volume of the MePD and size of neuronal somata in this region is normally greater in adult male than in adult female rats, but castration of adult males eliminates these sex differences and causes deficits in social/sexual behaviors (Cooke et al. 1999; 2003). Treatment with androgens and/or estrogens prevents these morphological responses to castration (Cooke et al. 2003), indicating that gonadal hormones play a critical role in regulating adult MePD anatomy and function. While the influence of adult androgens on MePD neurons has been demonstrated, there are few reports on the influence of circulating androgens on other cellular components of the MePD such as glia.

I previously reported sex differences in the number and complexity of astrocytes in the adult rat MePD, which showed striking lateralization (Johnson et al. 2008). Moreover, I found that the sex differences in astrocyte morphology depended on androgen receptors (ARs), because astrocytes were entirely feminine in both number and complexity in genetic (XY) male rats with a dysfunctional AR, due to the testicular

feminization mutation (Tfm) (Johnson et al. 2008). The highly plastic nature of astrocytes in other systems (Garcia-Segura et al. 1989; Mong 1996; Emamian et al. 2010), together with the AR-dependent sexual dimorphism of MePD astrocytes suggests that they may also exhibit androgen-dependent adult plasticity. To test this hypothesis, I examined the number and morphology of MePD astrocytes in male and female rats after adult castration and testosterone (T) replacement. I now report that MePD astrocytes are indeed hormone-sensitive in adulthood, with both the number of astrocytes and the complexity of the astrocytic arbor responding to adult hormone manipulations. Intriguingly, these responses are highly lateralized, echoing hemisphere-specific responses of MePD regional volume, and providing further evidence that the two amygdalae respond differentially to circulating hormones.

Methods

Animals and Surgeries

Ninety-day-old male and female Long Evans rats were obtained from Charles River (Charles River Laboratories, Wilmington, Massachusetts). Same-sex animals were housed three per cage in a single colony room in standard rat cages with food and water available ad libitum. Lights were on at 0700 and off at 1900 hrs. Animals were cared for in accordance with the guidelines set forth by the National Institute of Health and all procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University.

After 3 weeks of acclimation, animals were randomly assigned to treatment groups with N = 9 animals/group. Under isoflurane anesthesia, male and female rats were gonadectomized and implanted s.c. with two Silastic capsules (each 20 mm effective

release length and 30 mm in total length, inner diameter 0.062 inches, outer diameter 0.125 inches) containing either crystalline testosterone (T, Sigma) or nothing (blank). All capsules were incubated in phosphate-buffered saline (pH 7.4) for 48 hrs at room temperature (RT) prior to implantation. All surgeries were performed using aseptic procedures, and animals were provided postoperative analgesia (0.51 ⌈g Buprenorphine/g of body weight).

Thirty days later, animals (age 141-142 days) were given an overdose of sodium pentobarbital (150mg/kg, ip). Once animals were deeply anesthetized, showing no reflex response to either tail or foot pinch, they were intracardially perfused with 0.9% saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4, ~250 mL/animal). Before perfusion, blood was collected via cardiac puncture to measure T titers. After perfusion, hormone implants were confirmed and seminal vesicles in males were removed, trimmed and weighed. Brains were removed, weighed and postfixed for five hrs at 4^0 C in the same fixative solution before immersion in 30% phosphate-buffered sucrose at 4^0 C for at least 48 hrs. Brains were then sectioned coronally on a freezing microtome at 40µm through the region of interest after the left cortex of each brain was scored to mark that hemisphere. Sections were collected into cryoprotectant (de Olmos et al., 1978) and stored at -20^0 C. Every third section through the rostrocaudal extent of the MePD was processed for immunocytochemistry (ICC).

For colocalization of ARs within the MePD, a separate cohort of gonadally intact 100-110 day-old Long Evans male rats (N = 3) were obtained from our breeding colony at MSU and housed as described above. Animals were sacrificed and tissue collected and stored as described above.

Immunostaining for the Astrocytic Marker GFAP

Archived sections were transferred from cryoprotectant to Netwell plates (Corning Life Sciences) and thoroughly rinsed in 0.1 M phospate buffered saline (PBS; 140 mM NaCl, 10.7 mM KCl, 1 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.4) containing 0.3% Triton X-100 and 0.1% Knox gelatin (PBS-GT). PBS-GT was used throughout as the vehicle for ICC reagents and for rinsing. Sections were first incubated for 15 min in 0.5% sodium borohydride before blocking in 10% normal horse serum containing free avidin (Avidin Biotin blocking kit, Vector Laboratories), rinsed and then incubated one hr at RT in mouse anti-GFAP monoclonal antiserum (1:50,000, Chemicon, MAB360) containing free biotin (Avidin/Biotin blocking kit, Vector Laboratories). Tissue was then incubated overnight in GFAP primary antiserum at 4^0 C in PBS-GT containing. The GFAP primary antiserum was prepared against purified glial fibrillary acidic protein from porcine spinal cord. In western blots, the antiserum recognizes a single band at approximately 51 kDA (manufacturer's technical information).

Following incubation in primary antiserum, tissue was incubated in biotinylated rat-adsorbed horse anti-mouse secondary antiserum (1:500, Vector Laboratories), followed by incubation in peroxidase avidin-biotin complex solution (*Elite* ABC kit, Vector at half recommended concentration). HRP was visualized using diaminobenzidine (DAB, Sigma) in a 0.05 M tris buffer (pH 7.2). After rinsing to quench the peroxidase reaction, tissue was mounted on gel-subbed slides, dried, and counter-stained using Harris hematoxylin solution (Sigma), using 10% lithium carbonate as the bluing agent. Slides were then rinsed, dehydrated, cleared and coverslipped with Permount.

Stereological Analysis

A Zeiss Axioplan II microscope with the visual field captured by an Optronics MicroFire digital video camera was used to quantify astrocyte number and the complexity of astrocyte arbors within the region of interest. Using StereoInvestigator software (v. 8.0, MBF Bioscience; Williston, VT), the perimeter of the MePD was traced in serial sections at low magnification. MePD boundaries were identified using a standard rat atlas (Paxinos and Watson 2005), in conjunction with previously established standards within our laboratory (Morris et al., 2005; 2008a) and others (Hines et al., 1992). After tracing the boundaries of the MePD at low magnification, astrocytes were first counted and then traced using a 100X Plan-NeoFluar, 1.3 N.A. oil-immersion objective as described below. Slides were coded to ensure that all measures were performed "blind" to group membership.

Astrocyte number**:** An optical fractionator probe was used to generate an unbiased estimate of astrocyte numbers within the boundaries of the MePD. Initial probe insertion is random within the region of interest but then proceeds at fixed intervals allowing for an unbiased estimate of the number of objects within the entire region of interest. Probe dimensions were 35 by 35 μm with a height of 11 μm. The coefficient of error (CE; Gunderson m=1) for each hemisphere was at or below 0.10.

Criteria for identifying an astrocyte included a distinct and recognizable nucleus in a plane of focus within the sampling probe and from which at least 2 GFAP-labeled fibers extended. Such nuclei were often, although not always, smaller than other nearby (presumably neuronal) nuclei, often elliptical in shape, and lacking a clear nucleolus. These methods produced estimates of overall MePD volume and number of astrocytes

per hemisphere for each subject. The number of cells counted to produce these estimates ranged from approximately 250-500 per hemisphere depending on the hemisphere and the sex of the subject.

Astrocyte process complexity: Following previously described methods (Johnson et al., 2008), I used Neurolucida software (v. 7.0, MBF Bioscience; Williston, VT) to trace and measure the entire visible arbor of 20 randomly selected astrocytes per subject (10 astrocytes per hemisphere) with 5 subjects per treatment group. A fractionator probe was used to define random tracing sites throughout the MePD, with 10 sites per hemisphere. At each of these sites, the astrocyte nearest the randomly placed marker was traced in its entirety using a Wacom Cintiq 12WX tracing display (Wacom Co.; Saitama, Japan). The average number of primary processes, average number of branch points, average number of branch endings, and average branch length per astrocyte were calculated for each hemisphere of each animal.

Double ICC for AR and GFAP

Sections were stained first for AR followed by staining for GFAP using well described methods (Xiao and Jordan, 2002; Johnson et al., 2008). Briefly, cryoprotected sections were rinsed in PBS-GT, blocked in 10% normal goat serum (Vector Laboratories) containing free streptavidin (Streptavidin/Biotin blocking kit, Vector Laboratories), followed by 72 hrs of incubation at 4^0 C in rabbit anti-AR monoclonal antiserum (1:2000; Epitomics #1852-1 directed against the C-terminus of the human androgen receptor, Burlingame, CA) containing free biotin. Following incubation in AR primary antiserum, tissue was incubated in biotinylated goat anti-rabbit secondary antiserum (1:2000, Vector Laboratories) followed by Cy2-conjugated streptavidin

(1:48000, Jackson Immunoresearch, West Grove, PA). Following AR immunofluorescence, GFAP was visualized incubating tissue overnight at 4^0 C in primary antiserum (1:2000) using the same mouse anti-GFAP monoclonal as described above, but blocking for nonspecific binding of streptavidin in this case (Streptavidin/Biotin blocking kit; Vector Laboratories). Tissue was incubated for 2 hr in biotinylated goat anti-mouse 2° antibody (1:500, Vector Laboratories) followed by incubation in Cy5-conjugated streptavidin (1:32000). Tissue was thoroughly rinsed and mounted in the dark on gel-subbed slides and allowed to dry in a dark environment. Cell nuclei were counterstained with DAPI (1 µg DAPI / 1 ml distilled H_2O) for 5 mins, before dehydrating, clearing in Citrisolv and cover slipping with DPX (Sigma-Aldrich, St. Louis, MO), which we find preserves fluorescent signals for more than a year when slides are stored at -20º.

AR Immunohistochemisty Controls

Multiple controls were run to test the specificity of the AR primary antibody. First, adjacent sections from the same subjects were processed with the anti-AR primary antibody omitted. Omission of the primary antibody eliminated immunostaining (Fig 4). Furthermore, preadsorbing the AR primary antibody with the immunizing peptide (Epitomics P-1852, diluted 1:80 or 1/25 less than the 1:2000 dilution used for the AR primary) eliminated AR labeling in the MePD (Fig 5) and other AR-containing brain regions of normal adult male rats.

Third, we have developed an AR-knock out (KO) mouse using the cre/lox system. Along with confirming AR gene recombination based on PCR, we find that such AR-KO male mice faithfully recapitulate the Tfm male phenotype, including the

expected feminized ano-genital distance and nipples (Breedlove, personal observation), like spontaneous Tfm males (Dugger et al. 2007). This observation demonstrates the efficacy of the genetic manipulation in disabling AR function using the cre/lox system. Using the same AR staining protocol, I see no immunolabeling in the brains of such AR-KO male mice (Fig 6).

Confocal Microscopy: AR/GFAP Colocalization

DAPI-labeled astrocyte nuclei within the MePD were examined for AR immunoreactivity. Analysis was conducted on an Olympus Fluoview 1000 confocal scanning laser microscope equipped with argon (458, 488 and 514 nm), HeNeG (543 nm), HeNeR (633 nm), and LD405 lasers. The MePD was identified and defined at low magnification (10x objective) using well-established criteria (Paxinos and Watson 2005; Morris et al. 2008a; Hines et al. 1992).

Twenty to thirty sites per animal were visited within the MePD using a 60X Plan-Apo, 1.42 N.A. oil-immersion objective. Sites were chosen semi-randomly throughout the MePD. At each site a sequential scan with 2 µm spacing between scans and a depth of at least 18 µm was run and saved as image stacks using Olympus Basic software (v 5.0).

To estimate the number of AR expressing astrocytes in the MePD, all DAPI-stained cell nuclei fulfilling the criteria for astrocytic nuclei (described above) in the image stacks were examined and judged as immunopositive for AR if immunoreactivity was found clustered in the nucleus above background levels. The software's z-stack orthogonal viewer was used to confirm colocalization of markers for a sub-sample of nuclei. From this analysis, I obtained estimates of the proportion of astrocytes that are

AR+ in the MePD in each hemisphere. All confocal images were saved as 24-bit TIFF files and opened in Adobe Photoshop 7.0 for production of figures. Images were resized using "nearest neighbor" resampling when necessary. Images were otherwise unaltered in Photoshop other than adding appropriate labels.

Hormone Assay

Plasma T concentrations were measured in duplicate from 50 µl samples using the Coat-a-Count Total Testosterone Kit (Diagnostic Products Corp., Los Angeles, CA) by the Diagnostic Center for Population and Animal Health at Michigan State University. The lower limit for detection was 0.1 ng/ml, and the intra-assay coefficient of variation was 9.0%.

Statistical Analysis

Treating left and right hemispheres as a repeated measure with sex (male, female) and treatment (T, blank) as between group measures, separate three-way mixed-design analyses of variance (ANOVA) were conducted for each dependent variable (MePD volume, astrocyte number, number of primary processes, number of branch points, number of branch endings, and branch length). When appropriate, these were followed by two-way ANOVAs for each hemisphere and LSD post-hoc analysis to assess differences between groups. For all analyses, results are expressed as mean±SEM (standard error of the mean), with N= the number of animals per group, and statistical significance set at $p \leq 0.05$.

Results

Serum Testosterone Levels

As expected, serum T levels were significantly different between T-treated (Means±SEMS; 11.18±0.14 ng/ml) and blank-treated animals (0.00±0.00 ng/ml; $p < 0.001$). This result, in conjunction with a significant difference in seminal vesicle weight between T-treated (1.62±0.14g) and blank-treated males (0.18±0.01g; $p < 0.001$), confirms that the hormone manipulations were successful.

Regional Volume

Effects of Hemisphere and Sex: MePD volume overall is larger in males than in females (males: 476.14±20.45 versus 353.03±21.34 mm^3x10^{-3}, $p < 0.001$) and is greater in the right hemisphere than the left for both sexes ($ps < 0.001$, Fig 7A). I also find main effects of sex within each hemisphere, with males having a greater MePD volume than females for both ($ps < 0.005$, Fig 7C, D). These sex and hemisphere differences in MePD regional volume replicate previous findings (Morris et al., 2005; Johnson et al., 2008).

Effects of Testosterone Treatment: I also confirmed that T-treated rats have a larger MePD volume than blank-treated animals (T-treated: 468.93±19.49, blank-treated: 360.24±24.33 in mm^3x10^{-3}, $p < 0.001$) and found a significant treatment by hemisphere interaction ($p < 0.05$), leading us to analyze the effect of treatment on MePD volume separately for each hemisphere. I found that T treatment significantly affected MePD volume in both hemispheres ($ps < 0.01$; Fig 7B). Individual planned comparisons revealed that T-treated females had greater volume compared to blank-treated females in the left hemisphere ($p < 0.01$, Fig 7C), but T did not affect left MePD volume in males. However, in the right MePD, T treatment increased MePD volume in

both sexes (*p*s < 0.01, Fig 7D). The greater MePD volume in T-treated animals conforms to prior results based on similar hormone manipulations (Morris et al., 2008a) and confirms the robust adult plasticity of this sexually dimorphic region (Cooke et al., 1999).

Astrocyte Numbers

Effects of Hemisphere and Sex: GFAP staining in the amygdala and hypothalamus produced a pattern of GFAP immunoreactivity matching that reported previously (Johnson et al., 2008; Mong et al., 1999; Martinez et al., 2006). I replicated our previous finding that there are more MePD astrocytes overall in the right hemisphere than the left (number of astrocytes in left hemisphere: 6379.06±174.60; in the right hemisphere: 8799.17±288.10, $p < 0.001$). Post-hoc matched-pairs t-tests confirmed that this asymmetry was present for both males and females (Fig 8A) and indeed, was evident in all four treatment groups (all *p*s < 0.005). I again found more astrocytes in the MePD of males than of females (main effect of sex $p < 0.001$, Fig 8A), and replicated the significant interaction of hemisphere by sex ($p < 0.001$), reflecting a larger sex difference in the right hemisphere (males: 9841.72±299.70, females: 7756.62±352.27) than the left (males: 6755.10±245.08, females: 6003.02±220.94). However, the number of astrocytes was greater in males than in females in both hemispheres (left: $p < 0.05$, right: $p< 0.001$, Fig 8C, D). These sex and hemisphere differences in astrocyte numbers in hormone-manipulated animals essentially replicate our previous report of sex differences in gonadally intact wild type rats (Johnson et al., 2008).

Effects of Testosterone Treatment: I also found a main effect of adult T treatment on astrocyte numbers, with T-treated animals having more MePD astrocytes than blank-treated animals (T-treated: 16038.35±530.67, blank-treated: 14318.12±636.86, $p < 0.05$). There was also a significant interaction between treatment and hemisphere ($p < 0.05$) leading us to examine the effects of hormone manipulation in the two hemispheres separately. These analyses revealed that while hormone treatment did not affect astrocyte numbers in the left MePD overall, there was a significant effect of T on astrocyte numbers in the right MePD, with androgen-treated animals possessing more astrocytes than blank-treated animals ($p < 0.01$, Fig 8B). Examining the sexes separately revealed that T treatment did affect astrocyte numbers in the left MePD, but only in females ($p < 0.05$, Fig 8C). Further post-hoc analysis revealed that the effect of T treatment in the right MePD was significant in females ($p < 0.05$, Fig 8D) and marginally significant in males ($p = 0.056$).

Astrocyte Complexity

Effects of Hemisphere and Sex: All measures of astrocyte complexity except average branch length exhibited a main effect of sex, with males having more complex astrocytes overall than females (all ps < 0.05). In addition, all measures of astrocyte complexity except average number of primary branches were greater in the right hemisphere than the left (main effect of hemisphere all ps < 0.05). There were no significant interactions between hemisphere and sex, or hemisphere and treatment for any measure of astrocyte complexity. That the MePD of males contains more complex astrocytes than females replicates previous results (Johnson et al., 2008).

Effects of Treatment: Collapsing across the sexes, androgen treatment affected all measures of astrocyte complexity (main effects of androgen treatment, all *p*s < 0.005) by increasing the complexity of astrocyte arbors compared to blank-treated animals. However, all measures of astrocyte complexity except average branch length showed significant sex by treatment interactions (all *p*s < 0.05) reflecting the fact that T increased astrocyte complexity more in males than in females (Fig 9). Separate analysis of the two sexes revealed that T-treated males had significantly greater primary branches, branch endings, and branch points in both the right and left hemispheres, than did blank-treated males (all ps < 0.05, Fig 9A-C). In contrast, T-treated females differed from blank-treated females only for average branch length, and only in the right hemisphere (p < 0.05), yet T had no effect on this measure in males (Fig 9D).

AR Colocalization

The medial amygdala is particularly rich in AR+ nuclei (Gréco et al., 1998; McAbee and DonCarlos, 1998) but whether such nuclei belong to neurons or glia is unknown. As expected, I observed numerous AR+ nuclei in the MePD and many were large and round, with distinct nucleoli, presumably belonging to neurons. However, I also found distinct AR immunoreactivity in the nuclei of astrocytes in the MePD (Fig 10A). An orthogonal view bisecting the Z stack along X and Y axes centered on an AR+ nucleus clearly reveals that the AR staining is within an astrocyte nucleus, since AR immunoreactivity colocalizes with DAPI staining from which GFAP+ processes emanate (Fig 10B). Our estimates suggest that approximately 50% of astrocytes in the MePD are AR+ in both hemispheres (number of AR+ astrocytes in left hemisphere: 106.33±61.33 out of a total of 200±115.47 astrocytes counted; number of AR+ cells in

the right hemisphere: 183.66±106.04 out of a total of 356±205.54 astrocytes counted). AR+ astrocytes were also seen in previously documented areas, such as the arcuate nucleus (Lorenz et al., 2005, Fig 11A) and hippocampus (Hösli et al., 2001; Tabori et al., 2005, Fig 11B).

Discussion

The MePD is sexually dimorphic and sensitive to adult gonadal hormones (Cooke et al. 1999; 2003; Morris et al. 2005; 2008a). However, some sex differences in MePD neuroanatomy (e.g., regional volume and neuronal soma size: Cooke et al. 1999; 2007) are maintained by circulating testicular hormones in adulthood while others (e.g., neuron number: Morris et al. 2008a) are not. I recently demonstrated that MePD astrocytes are also sexually dimorphic in both number and complexity and that these sex differences depend on functional AR (Johnson et al. 2008). However, when these sex differences in astrocyte morphology in the MePD are determined by androgens is not known. I now report that astrocytes are sensitive to adult hormone levels in the MePD and change in both number and arbor complexity in response to hormones.

I replicated the sex and hemisphere differences in astrocyte numbers found previously in gonadally intact rats (Johnson et al. 2008). Moreover I found that androgens in adulthood affect the number of MePD astrocytes, although the effect is most pronounced in the right MePD of females. This result concurs with our prior report based on Nissl staining that adult hormone manipulations influence the number of total glia in the MePD only in females and only in the right hemisphere (Morris et al. 2008a). I also found that astrocyte complexity was regulated by adult androgens, although in this case, the effects of androgens are seen mainly in males and are symmetrical,

affecting most measures of astrocyte complexity in both hemispheres, as opposed to the asymmetric and rather subtle effect of androgens on astrocyte complexity in females. Specifically, all but one measure (branch length) of astrocyte complexity was increased by androgens in males in both hemispheres, while androgens increased *only* branch length in females and only in the right hemisphere. This measure of astrocyte complexity, which was the only one affected by T in females, was unaffected by T in males. Thus, in females, astrocytes in the right MePD appear most sensitive to adult androgens, changing in both number and arbor complexity while in males, MePD astrocytes on both sides of the brain depend on androgens in adulthood to maintain their complexity but not number. Finally, I also found that about half of MePD astrocytes in gonadally intact adult males are immunopositive for ARs, suggesting that androgens may act directly upon MePD astrocytes to influence their morphology and number.

Astrocytes and Gonadal Hormone Influence

An expanding body of research indicates that astrocytes are not the passive cells once imagined and has linked these cells to a wide range of phenomena including rapid and coordinate responses in the visual system (Schummers et al. 2008) and social exploratory behavior (Lee et al., 2007). In addition, astrocytes are known to play a vital role in synapse formation (Ullian et al. 2001; Hatton 2002; Barker and Ullian 2008). With large, highly mobile arbors, a single astrocyte may contact approximately 300-600 dendrites and 1,000,000 synapses in the cortex (Halassa et al. 2007; Nishida and Okabe 2007). In both the hippocampus (Garcia-Segura et al. 1988; Day et al. 1990; 1993; Leranth et al. 2008) and hypothalamus (Chowen et al. 1995; Mong 1996;

Amateau and McCarthy 2002; McQueen et al. 1990) astrocytes are sexually dimorphic and responsive to gonadal hormone manipulations. Changes in astrocytes in both regions are associated with changes in the surrounding neurons and/or behavior (Emamian et al. 2010; Mong and McCarthy 1999; Micevych et al. 2010). Our results further illustrate the dynamic nature of these cells and add to a growing list of brain regions where hormones can induce changes in astrocytes.

In the amygdala, investigation of astrocytes has been more limited, but hormones have been found to regulate GFAP expression in the region (Legrand and Alonso 1998; Martinez et al. 2006). Our previous work showed sex differences in both the number and complexity of astrocytes in the MePD portion of the amygdala, which are AR-dependent (Johnson et al. 2008). The current data also demonstrate these sex differences in astrocyte morphology and suggest that circulating gonadal hormones in adulthood have a role in maintaining these sex differences.

As far as I am aware, changes in astrocyte numbers in the adult amygdala that may be precipitated by hormones have only been reported once previously. Featherstone and colleagues (Featherstone et al. 2000) found reduced numbers of GFAP+ cells in the medial amygdala of pup-exposed multiparous postpartum females compared to pup-exposed primiparous females. The authors suggest this difference may be due to greater estrogen exposure in the multiparous dams, a conclusion in line with the greater numbers of astrocytes I see in T-treated females. More recent evidence showing that GFAP-immunoreactivity in the MePD is greatest during the proestrus phase and is increased by estradiol or estradiol plus progesterone in ovariectomized females, is also consonant with our current findings (Martinez et

al.2006). However, optical density measures of GFAP-immunoreactivity cannot distinguish whether greater astrocyte numbers, greater arbor complexity or both, contribute to differences.

Our current data are the first to demonstrate that MePD astrocytes in rats respond to adult androgens. The most robust responses to T treatment in males are in the complexity of the astrocyte arbor. Given the proposed link between number of astrocytic processes and synapse density (Pfrieger and Barres 1997; Elmariah et al. 2005; Mong et al. 2001), our results suggest that neuronal connectivity in the amygdala may be changing in response to circulating hormones in adult males. Interestingly, astrocytic complexity has been associated with both *increases* in synapse number (Pfrieger and Barres 1997; Elmariah et al. 2005) and *decreases* in dendritic spines and axonal-dendritic synapses (Mong et al. 2001) making it unclear whether the changes I see in astrocytes would be associated with increases or decreases in synaptic connectivity in the MePD. However, the increased size of neuronal soma induced by adult androgens in the MePD (Cooke et al., 2003) suggests that such increases in astrocyte number and complexity may be associated with increases in synaptic connectivity.

Gonadal Hormone Receptors in Astrocytes

Astrocytes in several other brain regions have been found to express AR, including the cortex, arcuate nucleus and hippocampus (Finley and Kritzer 1999; Lorenz et al. 2005; Sarkey et al. 2008; Nunez et al. 2003; Tabori et al. 2005). While the MePD is particularly rich in hormone receptors, including AR (Shughrue and Merchenthaler 2001; Roselli 1991), astrocyte expression of AR has not been previously reported in this

region. Several aspects of MePD neuroanatomy are dependent upon circulating androgens and/or functional ARs for complete masculinization, including astrocytes (Cooke et al. 2003; Morris et al. 2005; Johnson et al. 2008). I now find that a large fraction of MePD astrocytes express ARs, opening the possibility that ARs in the astrocytes themselves mediate the effects of androgens on these cells and possibly those on the neurons too.

Direct effects of gonadal hormones on astrocytes have been demonstrated *in vitro* (Melcangi et al. 1996; Stone et al. 1998). Although the hippocampus is also rich in hormone receptors, hippocampal astrocytes respond to gonadal hormones apparently independent of either (classical) AR or ER activity (MacLusky and Hajszan 2004; MacLusky et al. 2006). In the hypothalamus estrogens appear to act first on ER+ neurons that influence the surrounding astrocytes through GABA signaling (Mong et al. 2002). A similar scenario in which androgens act via neuronal AR to influence astrocytes may occur in the MePD.

Gliogenesis and Cell Death in the Amygdala

While androgens in adulthood can affect the number of MePD astrocytes, it is not clear what cellular mechanism androgens regulate to control their number. Hormones affect both the proliferation and survival of astrocytes in several systems (Agapova et al. 2006; Gatson and Singh 2007). Moreover, in the rodent amygdala in particular, evidence suggests that gonadal hormones can influence glial proliferation, at least during early development (Dmitar et al. 1995; Drekić et al. 1995). Recent evidence shows that cells proliferate in the rat medial amygdala during puberty, with more cells added in males than in females, of which many of these newly generated cells are glia.

Interestingly, gonadectomy prior to puberty reversed this sex difference, reducing the number of newly generated cells in males to that of females (Ahmed et al. 2008). Thus, androgens may increase the number of astrocytes in the right MePD of females by increasing their proliferation. However, it is also possible that fewer astrocytes survive without gonadal hormones. Both estrogens (Sales et al. 2010) and androgens (Nguyen and Jayaraman 2010; Ahlbom et al. 2001) are capable of preventing cell death in the brain and elsewhere (reviewed in Forger 2006).

Limitations and Implications

A chief limitation of this report is that the effects seen cannot be specifically attributed to the actions of AR or estrogen receptors (ER) alone. Due to the ability of T (an AR ligand) to be aromatized into estrogen (an ER ligand) or dihydrotestosterone (a high affinity AR ligand), it is possible that the treatment effects are due to ER activity alone or a combination or AR and ER activity. Both are implicated in regulating the adult morphology of the MePD (Cooke et al. 2003). However, given that functional ARs are necessary for masculinized MePD astrocytes (Johnson et al. 2008), AR likely mediate some of the effects of adult androgens on MePD astrocytes. One intriguing possibility is that masculinization of astrocyte number may depend on ARs in development, given the stability of astrocyte number in adult, hormone-manipulated males, while both ARs and ERs may regulate their morphology in adulthood.

Conclusions and Additional Questions

The present findings provide new information about the cellular anatomy of the amygdala. The sexually dimorphic volume of the adult MePD is responsive to adult hormone levels and it is now clear that astrocytes are another component of this

hormone-dependent adult plasticity. Future studies will be aimed toward understanding the mechanisms through which gonadal hormones act on MePD astrocytes to control their number and growth and/or regression of their processes and whether such effects are mediated via estrogens or androgens. In addition, the lateralized influence of gonadal hormones on astrocyte number and morphology seen here and in unmanipulated animals (Johnson et al. 2008) is complex and intriguing. Lateralization in the amygdala is common in humans (Kilpatrick et al. 2006) and rodents (Baker 2004). Interestingly, much of the lateralization in amygdala anatomy is sexually dimorphic and developmentally regulated, resulting in complex interactions between age, sex and hemisphere (Ziegler and Lichtensteiger 1992).

Although many aspects of the amygdala are still poorly understood, it is associated with numerous disorders and diseases and that many of these diseases, such as depression, anxiety and autism, exhibit sex-biases in prevalence. Continued examination of hormonal influence on amygdala anatomy and its neuronal and non-neuronal components may be vital to understand these disorders.

APPENDIX

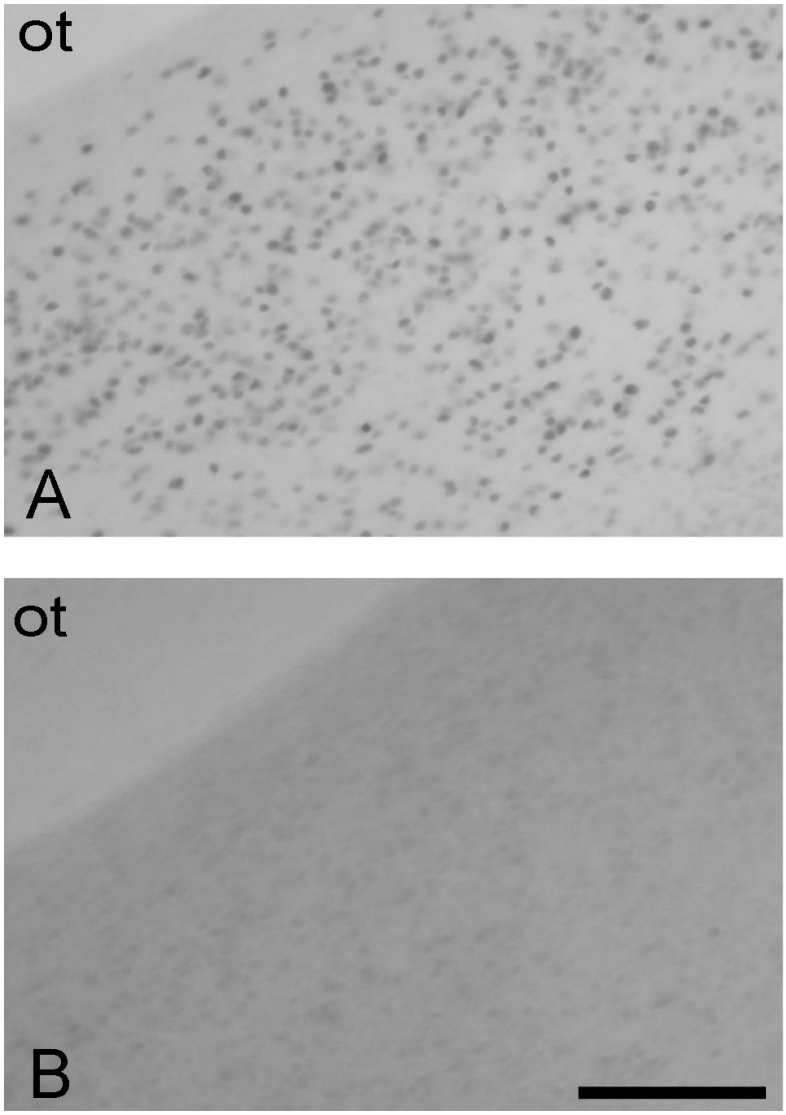

Figure 4 - Representative photomicrographs of coronal sections through the adult MePD of normal (A) and AR-knockout (B) male mice immunostained for androgen receptor (AR; Epitomics #1852-1 rabbit monoclonal anti-AR antiserum). Note the distinct nuclear labeling in the MePD of the normal adult male that is absent in the MePD of an AR knock-out male. Scale bar = 100 microns.

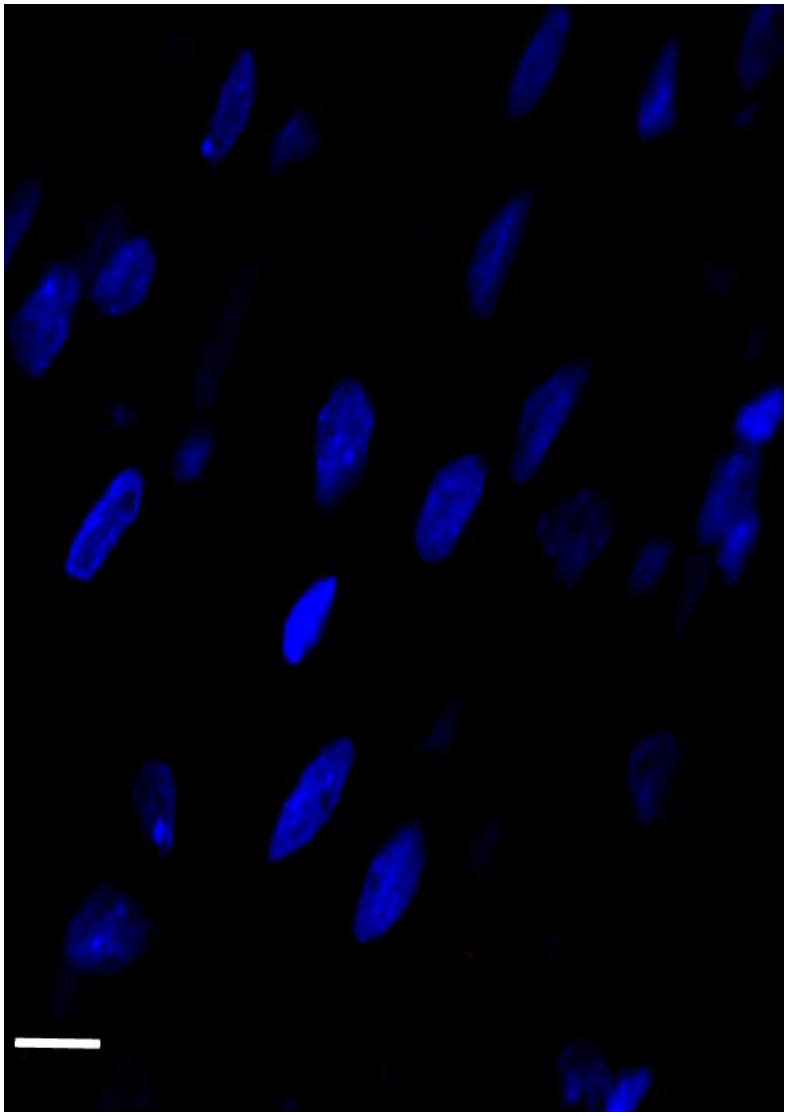

Figure 5 - Confocal image showing that preadsorbing the AR primary antibody with the immunizing peptide (Epitomics P-1852, diluted 1:80 or 1/25 less than the 1:2000 dilution used for the AR primary) eliminated AR labeling in the MePD. Image depicts the result of a sequential scan calibrated to detect DAPI and Cy2 fluorescence. Scale bar = 10 microns.

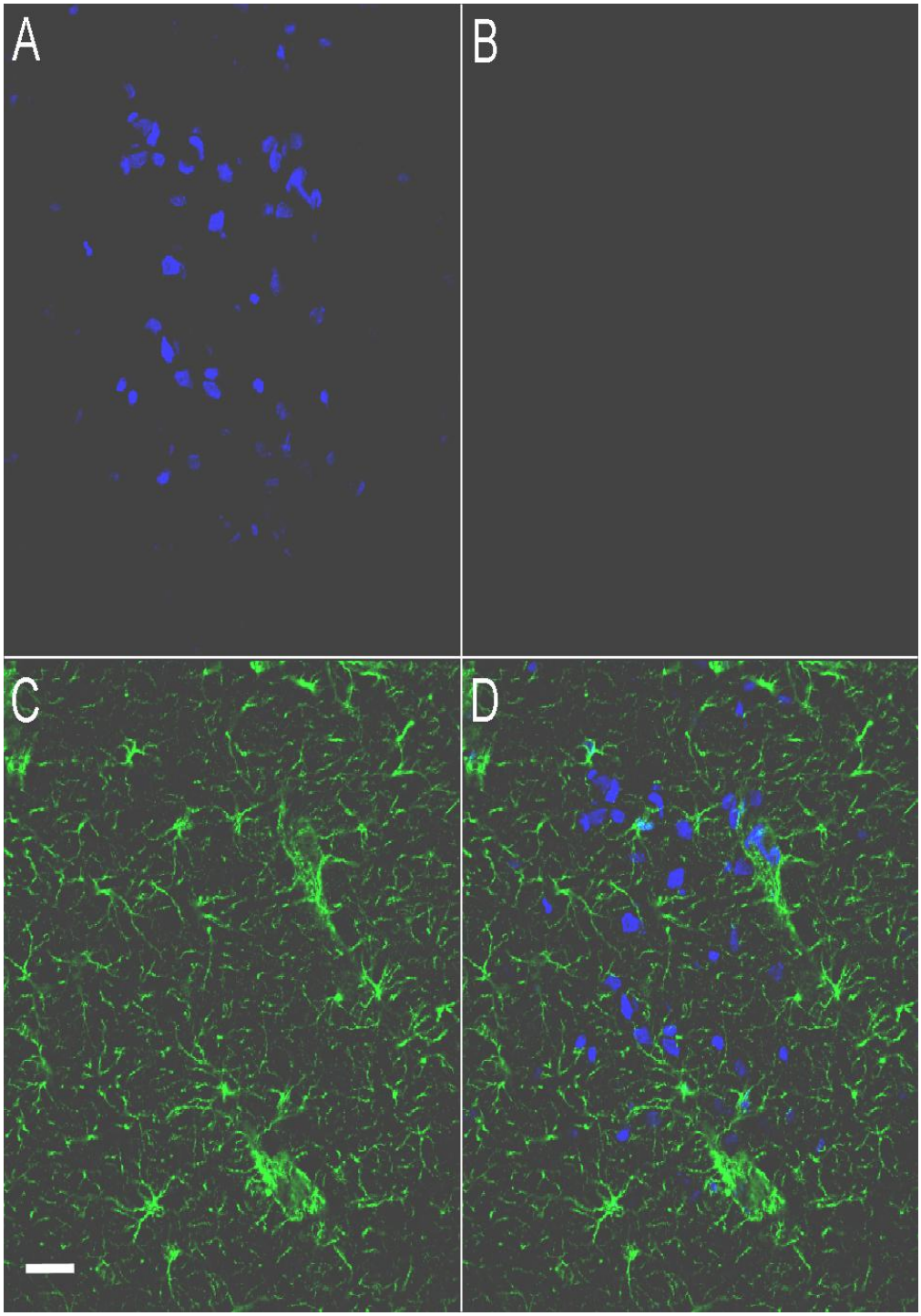

Figure 6 - Confocal image showing that omission of the AR primary antibody eliminated AR labeling in the MePD and other AR-containing brain regions of normal adult male rats. Panels A-C depict the results of individual scans calibrated to detect DAPI (A), Cy2 (B) and Cy5 (C). Both DAPI and GFAP labeling remained intact while Ar labelling was eliminated. Panel D depicts a sequential scan for all three fluorescent labels. Scale bar = 20 microns.

Figure 7

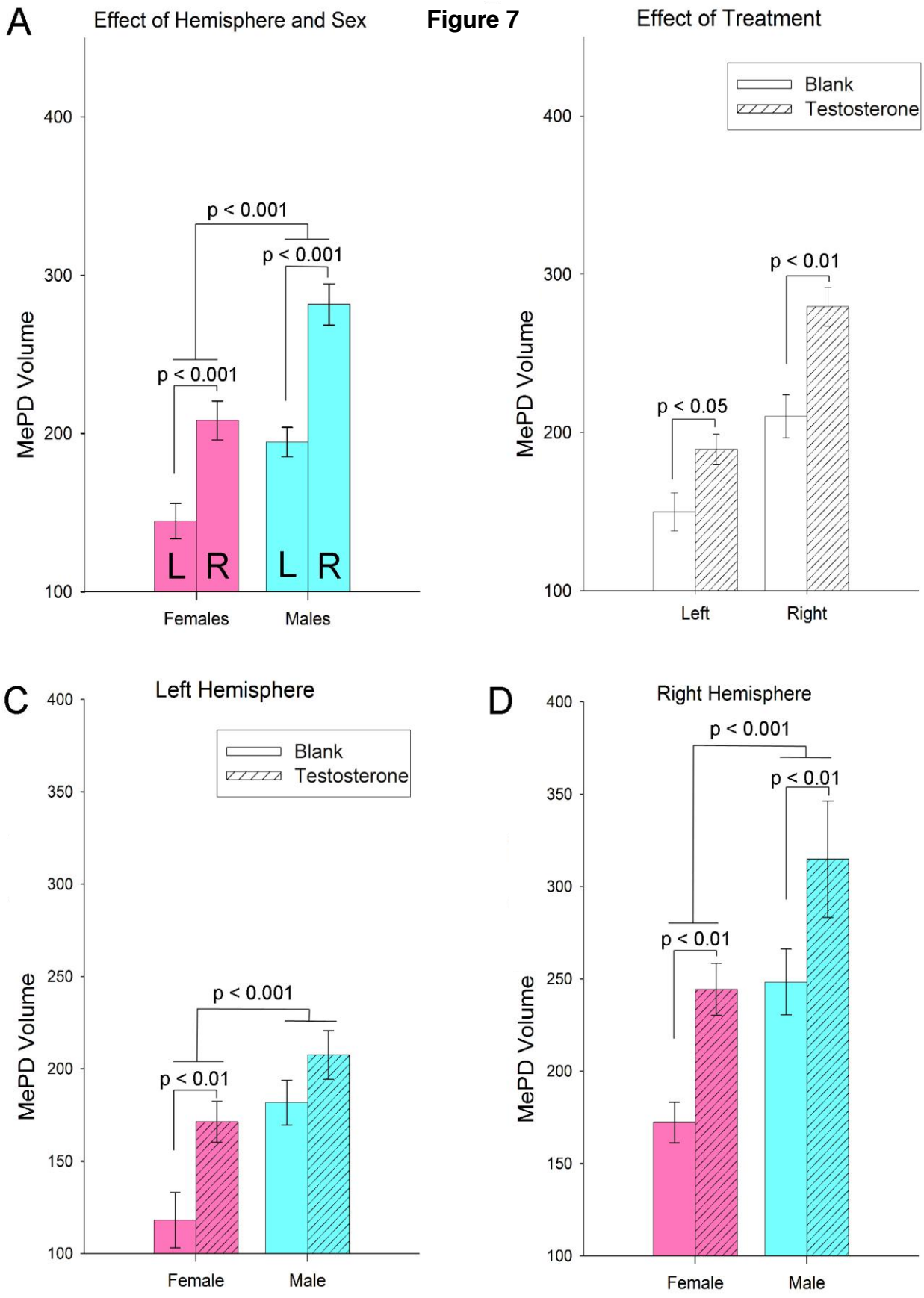
A
Effect of Hemisphere and Sex
MePD Volume
400
300
200
100
p < 0.001
p < 0.001
p < 0.001
L
R
L
R
Females
Males
Effect of Treatment
Blank
Testosterone
p < 0.01
p < 0.05
Left
Right
C
Left Hemisphere
Blank
Testosterone
350
250
150
p < 0.001
p < 0.01
Female
Male
D
Right Hemisphere
p < 0.001
p < 0.01
p < 0.01

Figure 7 - MePD volume is sexually dimorphic and maintained by androgens in adulthood especially in the right hemisphere. (A) Volume of the adult MePD in rats is both sexually differentiated and asymmetric, as males have a larger MePD than females, and the right (R) MePD is larger than the left (L) in both sexes. (B) T treatment increases MePD volume overall in both the left and right hemispheres of rats gonadectomized in adulthood (collapsed across sex). However, analyzing the hemispheres separately reveals that T treatment significantly increases the volume of the left MePD only in females (C) but increases the volume of the right MePD in both sexes (D). Values are means of N = 9 rats/group (± standard error of the mean, SEM)

Figure 8

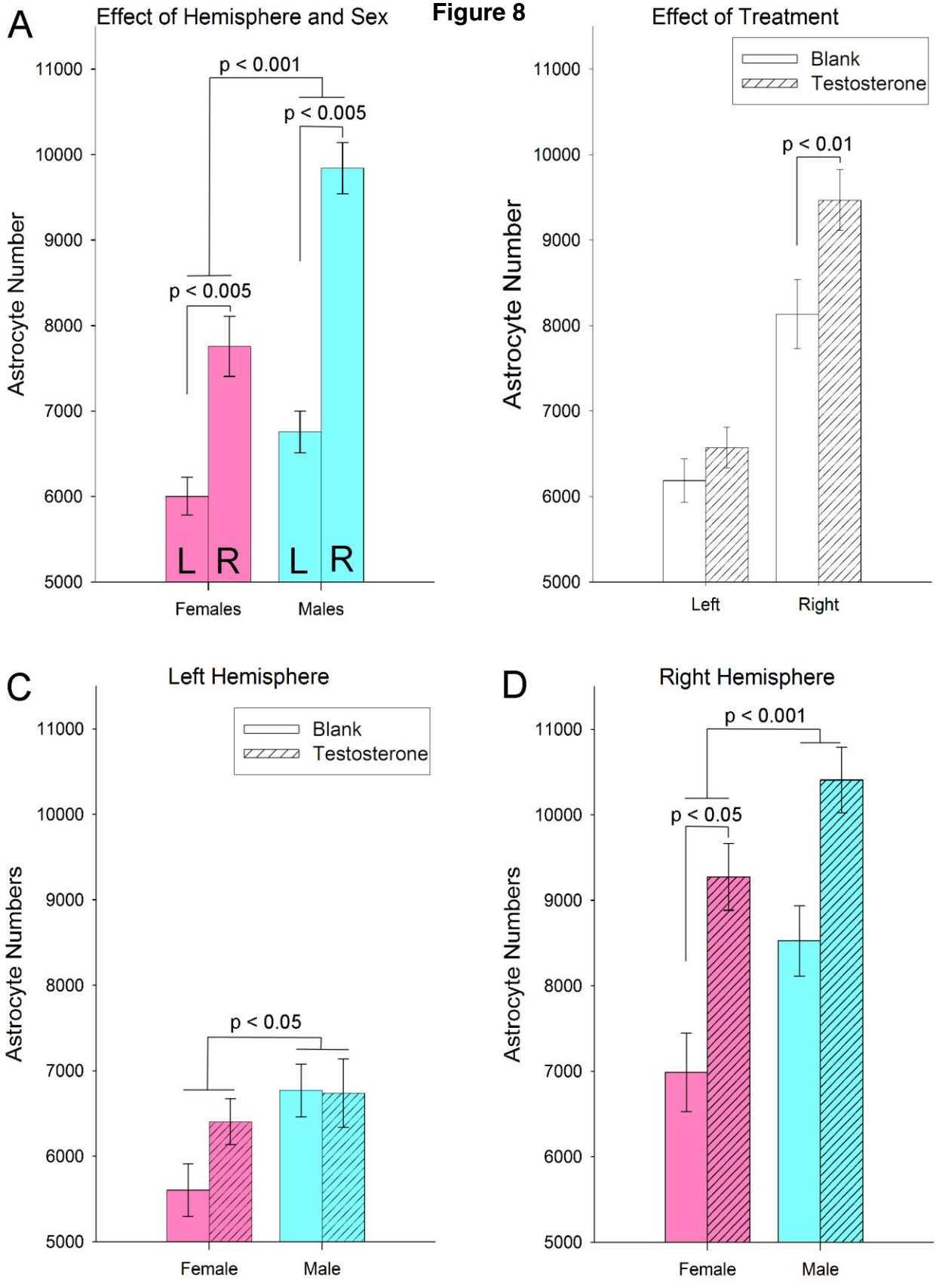
A
Effect of Hemisphere and Sex
Astrocyte Number
11000
10000
9000
8000
7000
6000
5000
p < 0.001
p < 0.005
p < 0.005
L
R
L
R
Females
Males
Effect of Treatment
Blank
Testosterone
p < 0.01
Left
Right
C
Left Hemisphere
Astrocyte Numbers
Blank
Testosterone
p < 0.05
Female
Male
D
Right Hemisphere
p < 0.001
p < 0.05
Female
Male

Figure 8 - Astrocyte numbers are lateralized, sexually dimorphic and maintained by adult androgens in female rats. (A) The number of astrocytes in the adult MePD is both sexually differentiated and asymmetric, with males having more MePD astrocytes overall than females, and with the right (R) MePD containing more astrocytes than the left (L) in both sexes. (B) T treatment increased the number of MePD astrocytes only in the right hemisphere (collapsed across sex). Examining the effect of androgens separately in each hemisphere revealed that in the left hemisphere, males have more MePD astrocytes than females, adult testosterone (T) treatment affects this measure only in females (C). Males also have more astrocytes than females in the right MePD, where T increases astrocyte number in both sexes (D; the difference in males is marginally significant ($p = 0.056$). Values are means of N = 9 rats/group (±SEM). Note that astrocyte numbers in this figure closely mirrors the differences in MePD volume in figure 2.

Figure 9

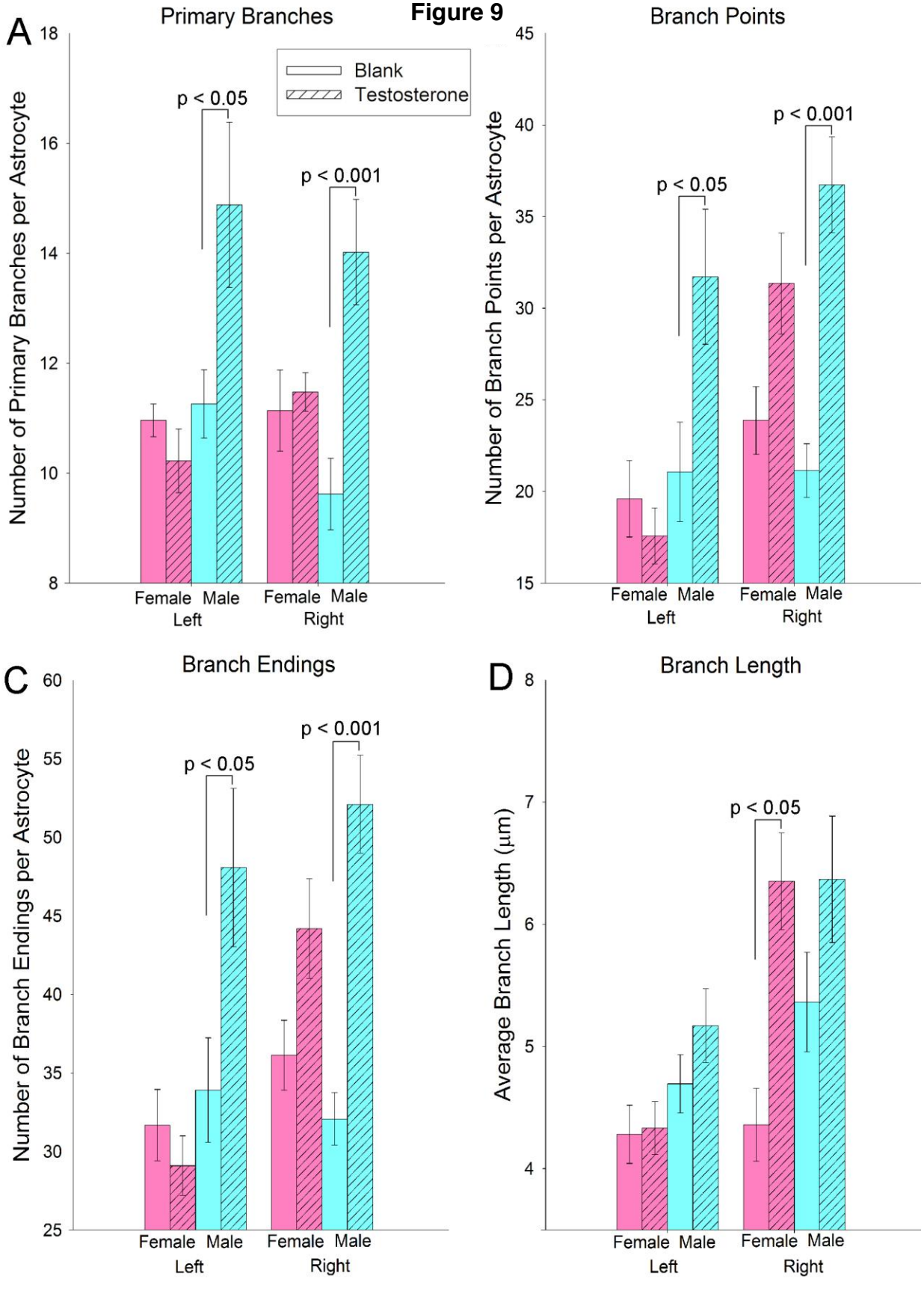
A
Primary Branches
Blank
Testosterone
p < 0.05
p < 0.001
Number of Primary Branches per Astrocyte
18
16
14
12
10
8
Female Male
Left
Female Male
Right
B
Branch Points
p < 0.05
p < 0.001
Number of Branch Points per Astrocyte
45
40
35
30
25
20
15
Female Male
Left
Female Male
Right
C
Branch Endings
p < 0.05
p < 0.001
Number of Branch Endings per Astrocyte
60
55
50
45
40
35
30
25
Female Male
Left
Female Male
Right
D
Branch Length
p < 0.05
Average Branch Length (µm)
8
7
6
5
4
Female Male
Left
Female Male
Right

Figure 9 - Astrocyte complexity is increased by androgens in adulthood, predominantly in males. Number of primary branches (A), branch points (B), branch endings (C) and average branch length (D) of astrocytes were measured in the MePD of adult gonadectomized male and female rats treated with testosterone (T) or blank capsules for 30 days. In males, astrocyte process complexity in both the left and right MePD is increased by T treatment, suggesting that adult androgens may normally maintain the elaborate processes of adult MePD astrocytes. The exception to this pattern is average branch length of MePD astrocytes (D), which was unaffected by androgens in males and was the only measure of MePD astrocyte complexity affected by T treatment in females, and that only in the right hemisphere. Values are means of N = 5 rats/ group, (±SEM).

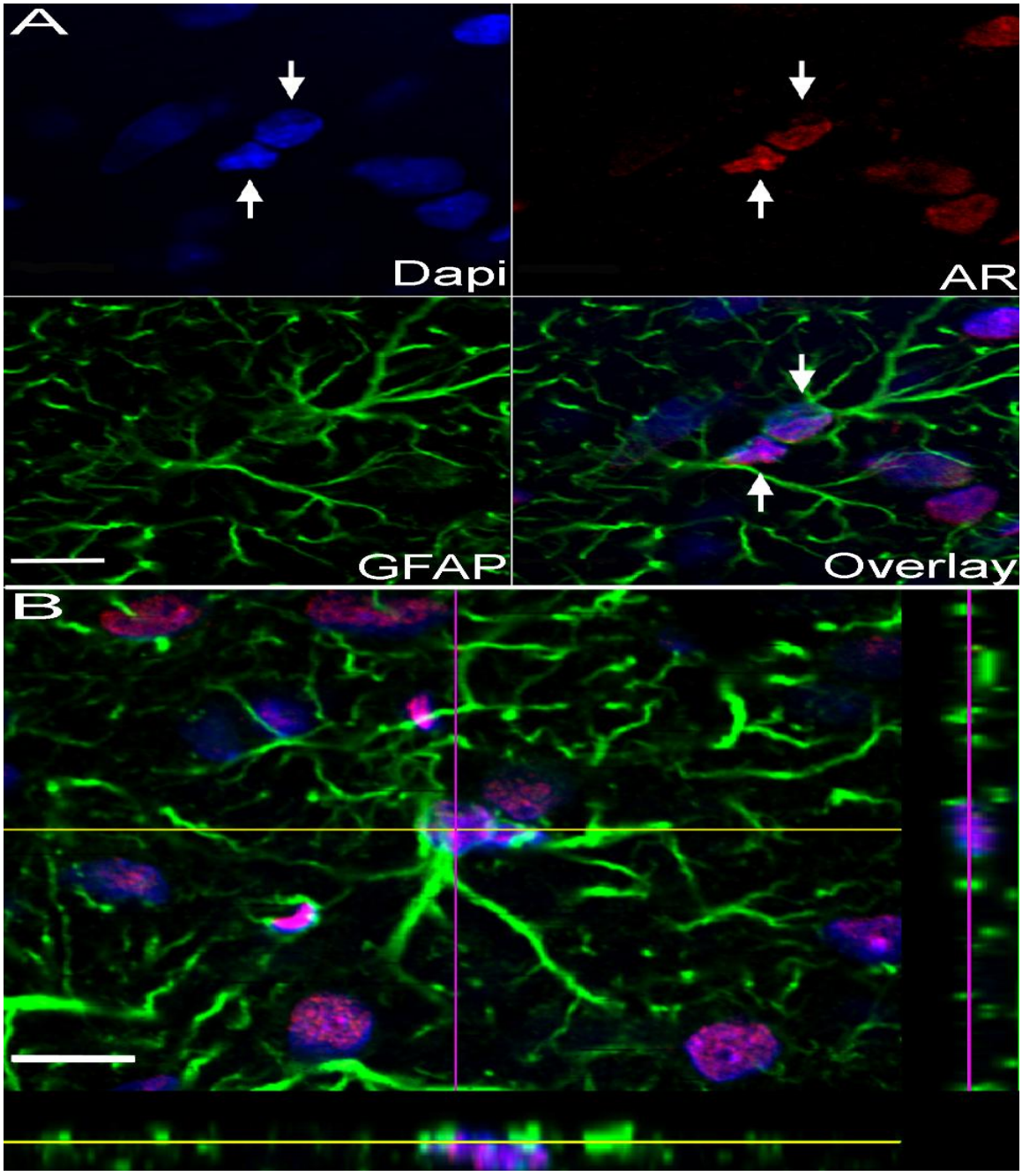

Figure 10 - Confocal images showing androgen receptor (AR) immunoreactivity within nuclei of MePD astrocytes. In panel (A), DAPI, AR and GFAP reactivity is shown for the same field of view in addition to a combined sequential scan for these three labels and indicates colocalization of DAPI and AR staining within the same nuclei (arrows) of GFAP+ astrocytes. The intensity of AR labeling in astrocytes varied and was often less intense than what was seen in presumptive neuronal nuclei. In panel (B), an astrocyte nucleus with AR immunoreactivity is shown with orthogonal views (along the yellow and pink lines) demonstrating that AR immunoreactivity is within the same Z-plane as the astrocytic nucleus. Scale bars = 10 microns.

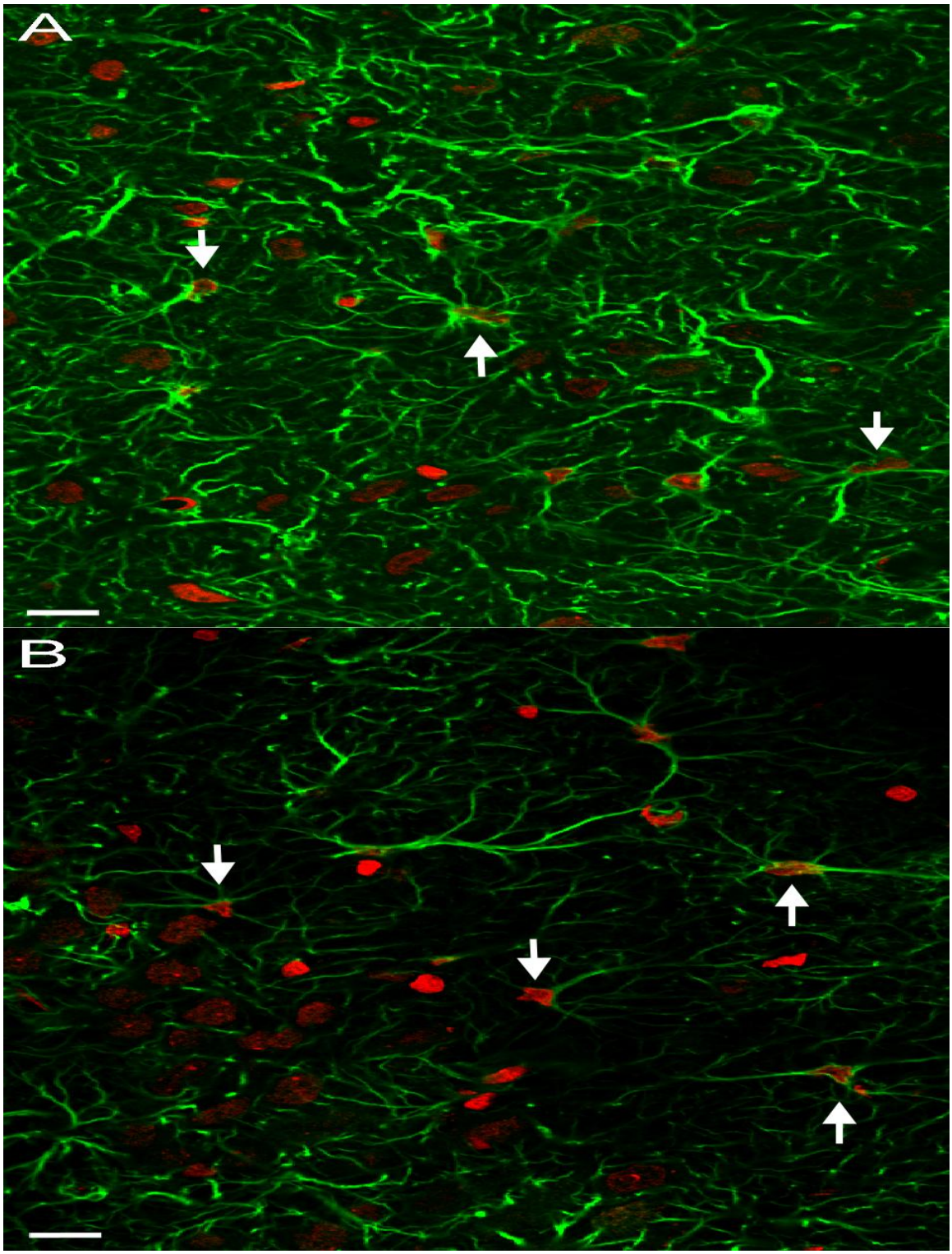

Figure 11 - Confocal image showing nuclear AR immunoreactivity within astrocytes of the arcuate nucleus (A) and CA1 hippocampus (B). Arrows point to AR+ immunoreactivity (red) in nuclei of GFAP expressing cells (green). Scale bars = 10 microns.

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CHAPTER 3: ANDROGEN DEPNDENT SEX DIFFERENCES IN ASTROCYTE NUMBER BUT NOT ASTROCYTE ARBOR COMPLEXITY IN THE JUVENILE MEDIAL POSTERODORSAL AMYGDALA

Abstract

The medial posterodorsal amygdala (MePD) is sexually dimorphic both in juvenile and adult rats and requires androgen receptor (AR) stimulation for complete masculinization. I previously reported that astrocytes in this region are more numerous and more complex in adult males than in females, and that these sex differences are androgen-dependent. I now report that MePD astrocyte number, but not arbor complexity, is sexually dimorphic in juvenile 25-day old rats. Using stereological analysis of tissue immunocytochemically labeled for glial fibrillary acidic protein (GFAP), male juvenile rats displayed more MePD astrocytes than females, but no sex differences were seen for any measure of arbor complexity. Both astrocyte number and astrocyte complexity were reduced in juveniles compared to previous estimates in adults. Furthermore, the sexual dimorphism in astrocyte number at 25 days is independent of ARs since testicular feminized mutant (Tfm) males, which lack functional ARs, nevertheless exhibited masculine numbers of MePD astrocytes. This is in contrast to adults, where Tfm males display a feminine number of astrocytes, indicating that some time after 25 days, AR activation is required for full masculinization of astrocyte number. As seen in adult animals, the sexual dimorphism in astrocyte number was also asymmetric, with the MePD having more astrocytes in the right hemisphere than the left in all three genotypes. These findings demonstrate that several cellular constituents of the MePD are sexually dimorphic and influenced by gonadal hormones, likely acting through both estrogen receptors (ERs) and ARs, throughout the lifespan.

Introduction

The amygdala is a complex brain region involved in fear, anxiety and social behaviors (Klüver and Bucy 1938; LaBar et al. 1998; Aggleton and Passingham 1981). In many species, including humans, the amygdala undergoes marked structural and functional changes prior to, and during, puberty (Giedd et al. 1996; Neufang et al. 2009; Blakemore 2006; Ziegler and Lichtensteiger 1992). Moreover, evidence suggests that in some species the developmental trajectory of amygdala neuroanatomy is at least partially controlled by gonadal hormones such as estrogens and androgens acting both early in life and during puberty (Ahmed et al. 2008; Merke et al. 2003; Rubinow and Juraska 2009; Nishizuka and Arai 1981; 1982). Investigation of how and where gonadal hormones act to alter cellular components within the amygdala will yield important information about this dynamic brain region.

The mammalian amygdala consists of several subregions with extensive interconnectivity. In rodents the medial posterodorsal subregion (MePD) is highly sensitive to gonadal hormone influence throughout the lifespan and contains a particularly dense supply of both ERs and ARs (Simerly et al. 1990; Wood and Newman 1995). Some aspects of MePD neuroanatomy are sexually dimorphic in juvenile animals, including regional volume and synaptic organization (Mizukami et al. 1983; Cooke and Woolley 2005). Several additional sex differences are found in adult animals including total number of glia, astrocyte number and astrocyte arbor complexity (Morris et al. 2008; Johnson et al. 2008). These results suggest that hormonal influence may further alter MePD neuroanatomy during puberty, and indeed androgen-dependent

gliogenesis in the rodent medial amygdala during puberty has been demonstrated (Ahmed et al. 2008).

The timing of sex differences in astrocytes (a subtype of glia) in the amygdala is particularly intriguing due to the important role these cells play in neural network modulation and synapse formation (Halassa et al. 2007; Ullian et al. 2001; Nishida and Okabe 2007; Hatton 2002). In some brain regions, including the hypothalamus and amygdala, astrocytes contain gonadal hormone receptors and respond to hormonal manipulations with changes in morphology (Lorenz et al. 2005; Pfrieger and Barres 1997; Elmariah et al. 2005; Mong et al. 2001; Sarkey et al. 2008), Johnson et al. in prep. b).

Interestingly, in the hypothalamus astrocyte sex differences are present very early in development and persist into adulthood (Mong 1996) suggesting hormones influence these cells during early development. In the MePD, astrocyte number and arbor complexity are both sexually dimorphic in adults but little is known about when this sex difference arises (Johnson et al. 2008). Given the amygdala's role in several diseases and disorders that exhibit strong sex differences in population prevalence, such as schizophrenia and autism, both of which are linked to critical periods of hormonal influence, it is likely that understanding hormone-induced neuroanatomical changes in the amygdala, including changes in astrocytes, will be an important step in the development of treatments.

Methods

Animals

Twenty-eight-day-old wildtype (wt) male, female and testicular feminized mutant

(Tfm) male rats were obtained from our breeding colony at Michigan State University and genotyped using PCR. The colony has been infused with Long Evans sires from Charles River for over 20 generations. Animals were housed five to nine per cage with siblings in standard rat cages with food and water available ad libitum. Lights were on at 0700 and off at 1900 hours. Animals were cared for in accordance with the guidelines set forth by the National Institute of Health and all procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University.

On the day of sacrifice, animals were given an overdose of sodium pentobarbital (150mg/kg, ip). Once animals were deeply anesthetized (showing no reflex response to either tail or foot pinch) they were intracardially perfused with 0.9% saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4, ~100 mL/animal). Brains were removed and weighed, postfixed for five hours at 4°C in the same fixative solution, then stored in 20% phosphate-buffered sucrose at 4°C for at least 48 hrs. The left cortex of each brain was scored to mark that hemisphere and then sectioned coronally on a freezing microtome at 40µm through the region of interest. Sections were collected into cryoprotectant (de Olmos et al. 1978) and stored at -20°C until stained. Every second section through the rostrocaudal extent of the MePD was processed for immunocytochemistry.

Histology

Immunocytochemistry for Counting Astrocyte Numbers

Archived sections were transferred from cryoprotectant to Netwell plates (Corning Life Sciences) and thoroughly rinsed in 0.1 M phosphate buffered saline (PBS; 140 mM NaCl, 10.7 mM KCl, 1 mM KH2PO4, 10 mM Na2HPO4, pH 7.4) containing 0.3% Triton

X-100 and 0.1% Knox gelatin (PBS-GT). PBS-GT was used throughout as the vehicle for ICC reagents and for rinsing. Sections were then incubated for 1 hr in 10% normal horse serum (Vector Laboratories) with avidin (Avidin Biotin blocking kit, Vector Laboratories) followed by 1 hr incubation at room temperature in mouse anti-GFAP monoclonal antiserum (1:6000, Chemicon, MAB360) with biotin (Avidin biotin blocking kit, Vector Laboratories). Tissue was then incubated overnight at 4°C in the same 1:6000 primary antiserum without biotin. The antiserum was prepared against purified glial fibrillary acidic protein from porcine spinal cord, and in western blots recognizes a single band at approximately 51 kDa (manufacturer's technical information). Following incubation in primary antiserum, tissue was incubated in biotinylated rat-absorbed horse anti-mouse secondary antiserum (1:500, Vector Laboratories) for 1 hr at room temperature. This was followed by 1 hr incubation in peroxidase avidin-biotin complex solution at half recommended strength (Elite ABC kit, Vector). HRP was visualized using an Immpact diaminobenzidine kit (DAB, Vector Laboratories) with a 4.5 min reaction time. Tissue was rinsed to quench the peroxidase reaction, mounted on gel-subbed slides, and allowed to dry for at least 24 hrs. Sections were counter-stained using Harris hematoxylin solution (Sigma) to visualize cell nuclei, using 10% lithium carbonate as the bluing agent, before dehydrating in a graded series of ethanols, clearing in Citrisolv and cover-slipping using Permount.

I found that the processes of juvenile astrocytes were not well stained using the above protocol, and thus, modified the staining protocol to optimize visualization of their arbors. Specifically, dilution of the primary antiserum was increased to 1:10,000 and the DAB reaction time was reduced to 2.5 min. The remaining alternate set of tissue

sections was stained using this modified protocol and used for measuring arbor complexity of juvenile astrocytes. The original set was used to assess astrocyte number.

Stereological Analysis

A Zeiss Axioplan II microscope with the visual field captured by an Optronics MicroFire digital video camera was used to quantify the number and complexity of astrocytes within the region of interest. Using StereoInvestigator software (v. 8.0, MBF Bioscience; Williston, VT), the perimeter of the MePD was traced in serial sections at low magnification. MePD boundaries were identified using the standard rat atlas (Paxinos and Watson 2005) in conjunction with previously established standards within our laboratory (Morris et al. 2008; Johnson et al. 2008, in prep. b) and others (Hines et al.1992). After tracing the boundaries of the MePD, astrocytes were counted and traced using a 100X Plan-NeoFluar, 1.3 N.A oil-immersion objective. Slides were coded to ensure that all measures were performed “blind” to group membership. Images of astrocytes were captured using the image capture option in StereoInvestigator.

Astrocyte Number

An optical fractionator probe (West 1993) was used to generate an unbiased estimate of astrocyte numbers within the MePD. Probe dimensions were 37x37 µm with a Z-depth of 12 µm and a 1 µm guard zone. Probe spacing was on a 115x115 µm grid. The coefficient of error (CE; Gunderson m=1) for each hemisphere was at or below 0.10.

Criteria for identifying an astrocyte included a distinct and recognizable nucleus in a plane of focus within the sampling probe and from which at least 2 GFAP labeled

fibers extended (Fig 12). Such nuclei were often, although not always, smaller than surrounding (presumably neuronal) nuclei, often elliptical in shape, and lacking a clear nucleolus. These methods produced estimates of overall MePD volume and number of astrocytes per hemisphere for each subject. The number of cells counted per hemisphere ranged from approximately 150 - 300 depending on the sex and hemisphere of the subject.

Astrocyte Process Complexity

Neurolucida software (v. 7.0, MBF Bioscience; Williston, VT) was used to trace and measure the entire visible arbor of 24 randomly selected astrocytes per subject. The MePD was traced via the criteria described above and 12 sites per hemisphere were selected. At each of these sites, the astrocyte nearest the randomly placed marker was traced in its entirety using a Wacom Cintiq 12WX tracing display (Wacom Co.; Saitama, Japan, Fig 13). The average number of primary processes, average number of branch points, average number of branch endings, and average branch length per astrocyte were calculated for each hemisphere of each animal.

Statistical Analysis

Separate two-way mixed-design analyses of variance (ANOVA) were conducted for each dependent variable (MePD volume, astrocyte number, number of primary processes, number of branch points, number of branch endings, and branch length). The left and right hemispheres served as a repeated measure and genotype (male, Tfm, female) as a between group measure. This was followed by one-way ANOVAs for each individual hemisphere and LSD post hoc analysis to determine differences between genotypes on each side. For all analyses, results are expressed as mean ±

SEM (standard error of the mean), with N = number of animals, and a *p* value of 0.05 was considered statistically significant.

Results

Regional Volume

Mixed design 2-way ANOVA (left and right hemisphere as repeated measures) revealed a main effect of genotype on MePD volume ($p < 0.001$) in addition to a main effect of hemisphere ($p < 0.001$) and a genotype by hemisphere interaction ($p < 0.05$). Post-hoc analysis revealed that Tfms have a greater MePD volume than wt males ($p < 0.05$) and females ($p < 0.001$) and males to have a greater MePD volume than females ($p < 0.05$). Separate one-way analysis of variance in each hemisphere revealed a main effect of genotypes in the left hemisphere ($p < 0.05$) with Tfms having a greater MePD volume than females ($p < 0.01$, Fig 14A) but with wt males being no different than either females or Tfm males. There was also a main effect of genotype in the right hemisphere ($p < 0.001$) with all groups being significantly different from each other (all ps ≤ 0.01, Fig14A). Thus Tfms have greater MePD volumes than females in both hemispheres and a greater volume than males in the right hemisphere. These represent the first measures of MePD volumes in Tfm male rats. Paired-samples t-tests revealed that the right hemisphere had greater regional volume than the left in both wt and Tfm males (ps < 0.005) with no significant laterality of MePD volume in females.

Astrocyte Numbers

Similar to previous reports in adults (Johnson et al. 2008) there was a main effect of hemisphere ($p < 0.001$) with greater numbers of astrocytes in the right MePD

compared to the left in all groups (*p*s < 0.05). There was also a main effect of genotype on the number of astrocytes as wt and Tfm males have more astrocytes than do females (*p*s < 0.05). Although there was no genotype by hemisphere interaction, the complex pattern of laterality previously seen in adult animals prompted separate analysis of the two hemispheres. While there was no main effect of genotype in the left MePD there was a significant effect in the right MePD ($p < 0.05$, Fig14B). Nevertheless, post-hoc analysis of the left MePD did reveal that males had greater numbers of astrocyte than females ($p < 0.05$, Fig 14B) and Tfms were intermediate. In contrast, post-hoc analysis of the right MePD revealed males and Tfms had greater numbers of astrocytes than females (*p*s < 0.05, Fig 14B). Thus, in the right hemisphere astrocyte numbers are sexually dimorphic, and Tfm males are masculinized. Since Tfm male rats lack functional AR but have circulating androgens (Roselli and Salibury 1987), these results suggest that the sex difference is not dependent on AR at this age.

Astrocyte Complexity

Astrocyte complexity was assessed using previously established measures from adults (Johnson et al. 2008, in prep. b) and analyzed using mixed design 2-way ANOVAs (left and right hemisphere as repeated measures). No main effects or interactions of hemisphere and genotype were found for any measure of astrocyte complexity (Fig 15). Thus none of the sex differences or laterality of astrocyte arbors seen in adult animals were present in these juveniles. Moreover, estimates of astrocytic arbor complexity and length in the juvenile MePD are below those obtained for adult MePD astrocytes, suggesting that puberty may represent a time when astrocytes not only extend processes to achieve their full adult extent, but also critically depend on

androgens acting via ARs to develop processes in a sex- and hemisphere-specific manner.

Discussion

Astrocytes During Neonatal Development

Early prenatal development is a critical period during which hormonal influence serves to masculinize much of the central nervous system in male mammals (Mizukami et al. 1983; Segovia and Guillamón 1993; Gorski et al. 1978; Merke et al. 2003; Gilmore et al. 2007; Chura et al. 2010; Cooke et al. 1998; reviewed in Breedlove 2010; Hamson et al. 2010). Although reliable patterns have emerged, the pathways mediating masculinization are complex and still poorly understood. Reports from the past three decades reveal that gonadal hormones influence astrocytes and that this influence may occur early in development (Weiner 1983; Tranque et al. 1987; Day et al. 1993; Mong 1996). In the arcuate nucleus the masculinization of astrocyte arbors has been attributed to estrogens and linked to changes in surrounding neurons (Naftolin 1978; Leedom et al. 1994; Mong et al. 1999; 2001; 2002). Like the arcuate nucleus, the MePD exhibits sexually dimorphic astrocytes in adulthood (Martinez et al. 2006; Johnson et al. 2008). However, the timing of these sex differences and the role of AR and ER in their early development has not been previously investigated. Furthermore, and in contrast to the arcuate nucleus, the MePD requires functional ARs for complete anatomical masculinization (Morris et al. 2005; Cooke et al. 2003). This information, combined with the growing appreciation of astrocytes in brain plasticity led us to investigate possible sex differences in juvenile astrocytes, using Tfm rats to probe for any role of ARs in MePD sex differences.

The current findings provide further evidence that MePD astrocytes are sexually dimorphic in number and reveal that this sex difference is present prior to puberty. The sex differences in astrocyte number in the left MePD are concordant with previously reported sex differences in volume occupied by glia in the left MePD (females < males, Cooke et al. 2007). However, sex differences in glia in the juvenile right MePD have not previously been reported.

In the right hemisphere, Tfm males are masculinized in terms of astrocyte number, suggesting that this sex differences is not dependent upon functional AR prepubertally. This result raises the question of how the sex differences originate. Do estrogens during early development lead to increased astrocyte numbers in the MePD? Interestingly, there are sex differences in astrocyte proliferation in the juvenile rat MeA with females exhibiting greater numbers of BrdU+ cells, which co-label with GFAP in the MeA, suggesting increased astrogenesis in *females* at these times (Krebs-Kraft et al. 2010). These findings at PND 4 and 14 in the MeA contrast with the greater number of astrocytes I see in *males* in the MePD. Stereological counts of astrocyte numbers, which also account for possible hemisphere effects are needed at these time points to see how proliferation at PNDs 4 and 14 relate to the sex differences I see at PND 25.

Intriguingly, the sex difference in astrocyte proliferation in the medial amygdala at PNDs 4 and 14 is dependent upon endogenous cannabinoid receptor activation, since administration of a cannabinoid receptor agonist to females eliminated the sex differences (Krebs-Kraft et al. 2010). This result suggests that sex differences in the cannabinoid system may be responsible for the sex differences in MeA astrocyte numbers; sex differences in metabolism of endogenous cannabinoids have also been

implicated (Krebs-Kraft et al. 2010). While these results provide clues to a possible receptor system modulating astrocyte proliferation, they also raise additional questions about how and when the sex differences in cannabinoid synthesis and/or receptor activation originate.

Although few comparisons are available for the sex differences I see in astrocyte numbers, there are previous reports of sex differences in juvenile astrocyte complexity. For example, while astrocyte arbor complexity is sexually dimorphic in the hypothalamus of neonates, and this sex differences is retained into adulthood, this pattern is not present in the MePD. I found no sex differences in any measure of astrocyte arbor complexity in the juvenile MePD, suggesting that the sex difference in arbor complexity seen in adult animals arises during puberty. Are changes in astrocytes in the amygdala during puberty part of a synaptic reorganization of this region? Further research examining synapse formation in the MePD during puberty might answer this question.

Implications and Additional Questions

While the current experiment does not include adult animals, previous reports using similar methodologies from the same animal colony found androgen dependent, and AR dependent, sex differences in both MePD astrocyte number and arbor complexity in two separate studies from our lab (Johnson et al. 2008; Johnson et al. in prep. b). I therefore feel comparison of the current results in juvenile animals to previous reports in adults is valid, and suggests that while the sex difference in MePD astrocyte number is present at PND 25, the absolute numbers of astrocytes increases between that age and adulthood.

The current evidence demonstrates that prior to puberty, Tfm males are masculinized, as both wt males and Tfms having more astrocytes than females. In adult animals, Tfm males are feminine in astrocyte number, suggesting an AR-dependent increase in MePD astrocyte numbers in wt males during puberty. Ahmed and colleagues (Ahmed et al. 2008) found androgen-dependent proliferation in non-specific glia in the rat medial amygdala during puberty. In contrast, I found no sexual dimorphism in astrocyte arbor complexity prior to puberty. However, in adulthood the sexual dimorphism in astrocyte arbor complexity is pronounced and Tfm males are feminized. Are androgens acting on AR during puberty to promote arbor elaboration in the MePD? Hormonal regulation of both GFAP-immunoreactive optical density and astrocyte arbor complexity has been previously demonstrated in the MePD of adult animals, so hormonal regulation during puberty seems plausible (Martinez et al. 2006, Johnson et al. in prep. b).

Despite this new information, numerous questions remain. For example, are estrogens acting directly on astrocytes to promote cell proliferation and arbor elaboration during early development? Evidence from the hypothalamus suggests that estrogens act indirectly through ER+ neurons to influence astrocytes morphology (Mong et al. 2002). A similar pathway is possible in the MePD but cannot be confirmed or eliminated based on the currently available data. While I found evidence of AR+ immunoreactivity in MePD astrocytes of adult animals, the current report suggests that estrogens establish early sex differences in the number of these cells. Do MePD astrocytes contain ERs as well? Astrocytes in both humans and rodents are known to express ERs in some brain regions, and the MePD is known to contain ER

immunoreactivity (Donahue 2000; Langub and Watson 1992; Milner et al. 2001; Simerly et al. 1990). It is possible that ER+ astrocytes in the MePD respond to hormonal signals early in development to prevent cell death or promote cell proliferation in developing males. What role does this early estrogen-induced sexual dimorphism play in MePD function and animal behavior?

Additionally, how early does this sexual dimorphism arise? Increasing evidence suggests that early prenatal hormones may shape the amygdala in important ways in disorders such as autism (Sweeten et al. 2002; Baron-Cohen et al. 2005). Single-nucleotide polymorphisms (SNPs) in the human ER are correlated with autistic-like traits, and the same report found SNPs in synapse formation genes linked to autistic-like traits as well (Chakrabarti et al. 2009). In rodents, sex differences in excitatory post-synaptic potentials have been reported in the medial amygdala (Cooke and Woolley 2005) suggesting hormones may somehow influence the function of synapses in the region. Do hormone responsive astrocytes affect synapse formation in the amygdala?

What is clear from the present findings is that gonadal hormones play a role in the masculinization of astrocytes during critical periods in development. While currently the functional consequences of this influence remain elusive, continued investigation may reveal whether changes in astrocytes lead to alterations in the neuronal network in this important brain region. Changes in astrocyte morphology are associated with several diseases and disorders including depression (Gosselin et al. 2007; Hercher et al. 2009) and Alzheimer's disease (Scott et al. 1992). Further investigation in both human and rodent models is needed to understand how astrocytes may alter amygdala function

and how gonadal hormone may alter astrocytes during critical periods in development.

APPENDIX

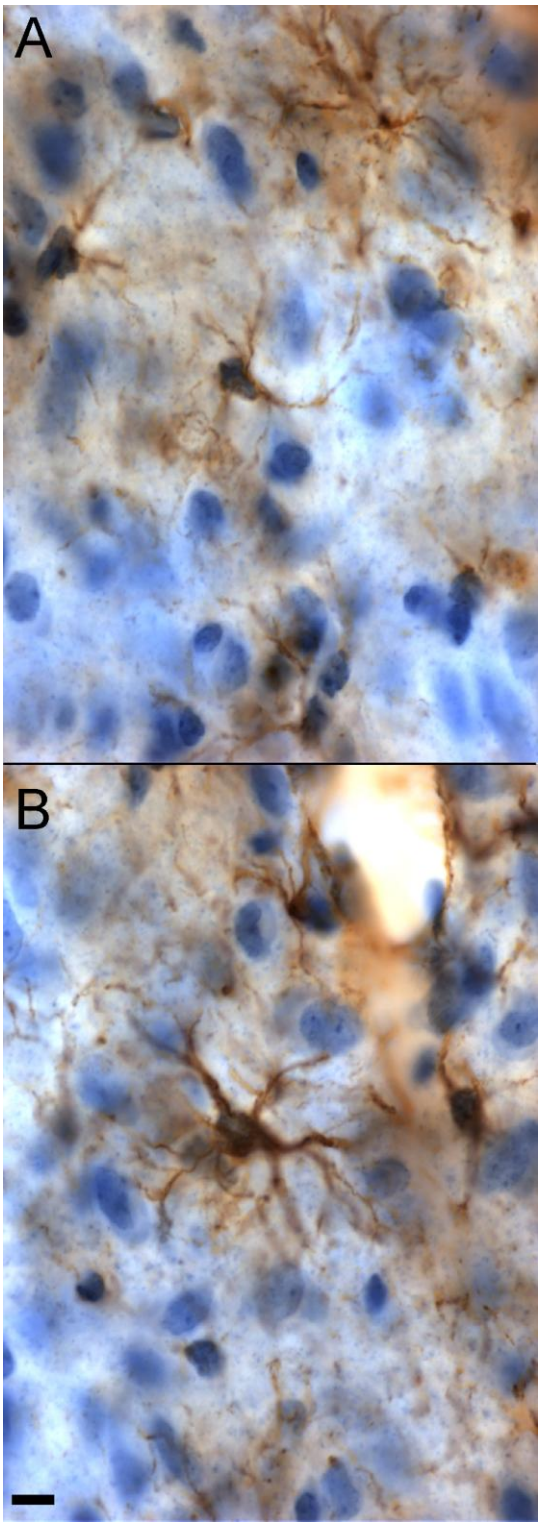

Figure 12 - Photomicrographs depicting astrocytes of varying complexity in the juvenile MePD. Panel A depicts a simple astrocyte, which has few and short processes. Panel B depicts a more complex astrocyte that has multiple, elaborated processes. Scale bar = 10 microns.

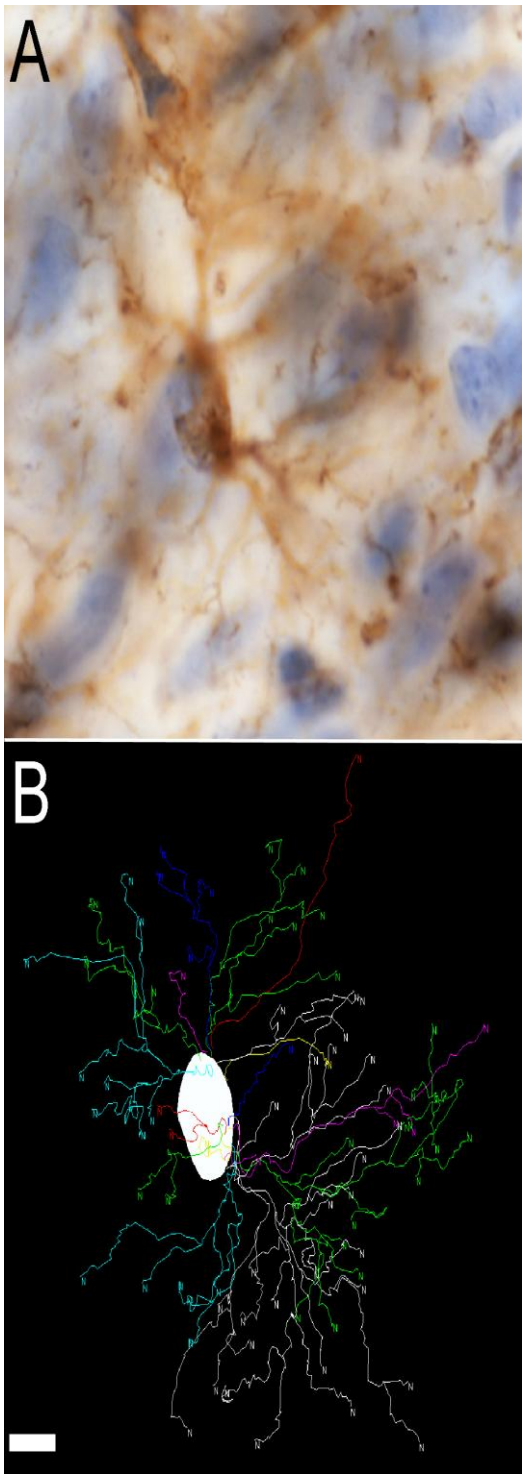

Figure 13 - Photomicrograph depicting a complex astrocyte in the juvenile MePD (A) and the resulting tracing (B). Because of the narrow plane of focus, only a portion of the full extent of arbor complexity is visible at a time. Complex astrocytes frequently demonstrated multiple branches that emerged as the plane of focus was moved through the tissue. Scale bar = 10 microns.

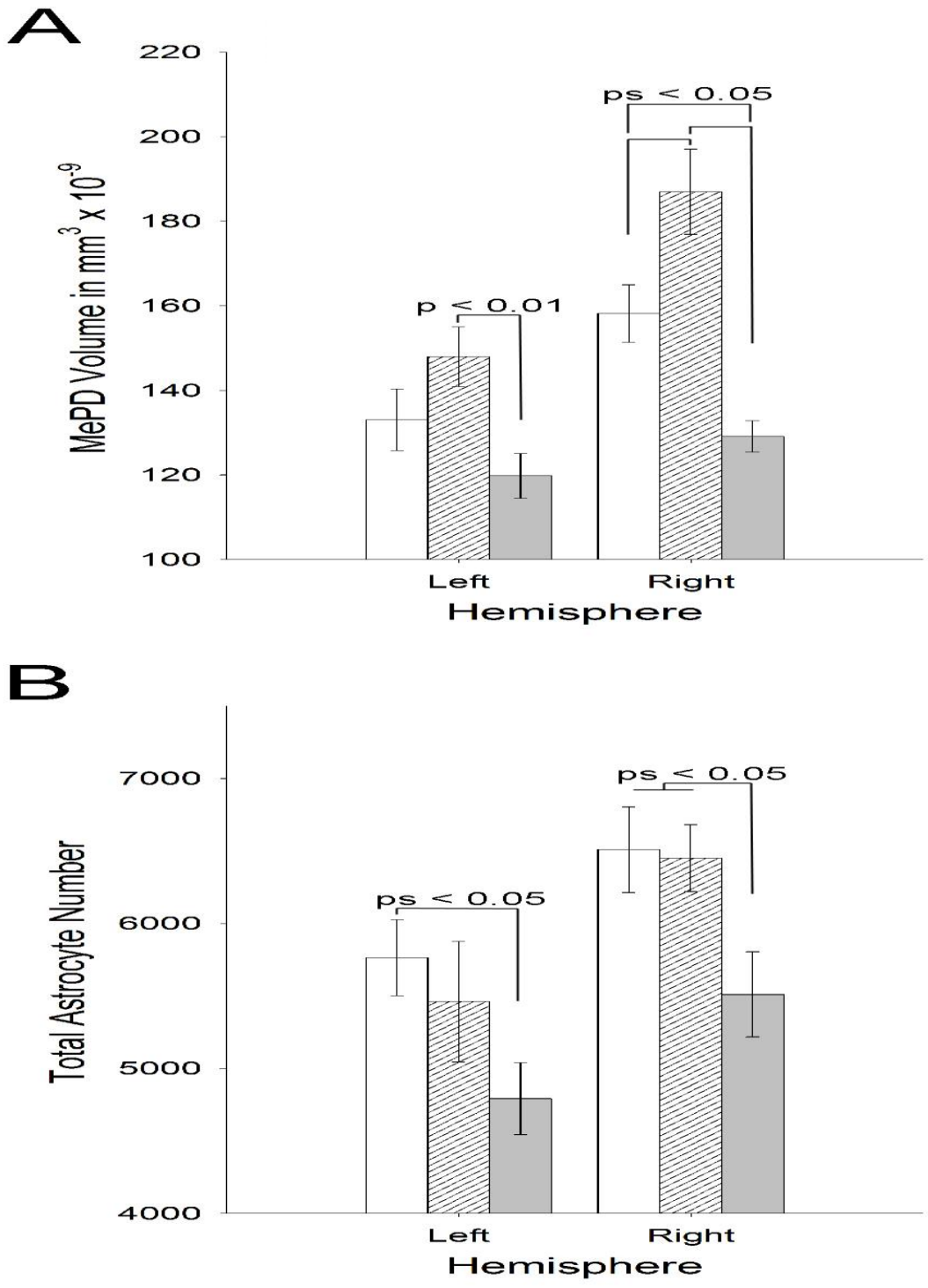

Figure 14 - Regional volume and astrocyte number are sexually dimorphic in the posterodorsal medial amygdala (MePD) of juvenile wildtype male, female and testicular feminized mutant (Tfm) male rats. MePD volume is larger on the right than the left in juvenile wildtype and Tfm males but not in females (A). Values are means of N = 9 rats/group (+ standard error of the mean, SEM).

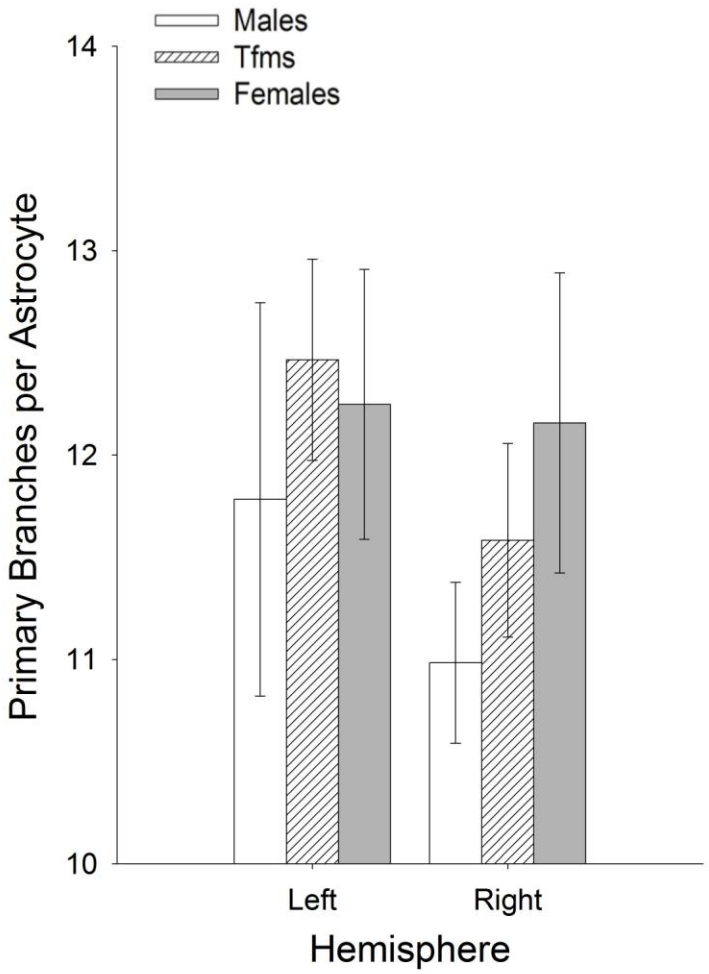

Figure 15 - Estimated number of primary branches in astrocytes of the MePD in juvenile wildtype male, Tfm male and female rats. No hemisphere or sex differences were seen in any group. All other measures of astrocyte complexity exhibited a similar pattern. Values are means of N = 5 rats/ group (+ SEM).

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CHAPTER 4: DIFFERENTIAL HORMONE ACTION IN THE LEFT AND RIGHT AMYGDALAE

Abstract

The amygdala is a highly connected and plastic region involved in numerous behaviors and implicated in disorders including depression, schizophrenia and autism. Research has repeatedly demonstrated that the anatomy and function of the amygdala is sexually dimorphic in humans and laboratory species. Sexual dimorphisms in the amygdala have been important in our understanding of how gonadal hormones influence the brain. In this review, I summarize literature demonstrating that the amygdala of the two hemispheres may also be distinct in their response to gonadal hormones and argue that this distinction may be informative to future research concerning the amygdala and gonadal hormone influence.

Introduction

Gonadal hormones alter many aspects of brain neuroanatomy and function in complex ways; from modulation of GABAA receptor subunit expression (McIntyre, Porter, & Henderson, 2002) and optic-nerve head astrocyte activity (Agapova et al., 2006) to producing sex differences in corpus callosum structure (Chura et al., 2010) and stimulus processing networks (Kilpatrick et al., 2006). While it is understood that gonadal hormones are responsible for many sex differences throughout the brain (Gorski et al., 1978; Hofman and Swaab, 1989; Allen et al., 1991; Allen and Gorski, 1991; Cooke and Woolley, 2005; Alonso-Nanclares et al., 2008; Morris et al., 2008a) several complications prevent a complete understanding of gonadal hormone influence. One often ignored issue is laterality of gonadal hormone effects. Hemisphere-specific hormone effects exist in multiple brain regions (Chura et al., 2010; Neufang et al., 2009;

Xiao and Jordan, 2002), but the amygdalae in particular appear highly lateralized.

In a variety of species, the amygdalae are responsive to gonadal hormones during development and in adulthood (humans: Giedd et al., 1997; cats: Rees and Michael, 1984; mice: Morris et al., 2008b; hamsters: Zehr et al., 2006; rats: Cooke et al., 2003). As is the case with many sexually dimorphic regions, it has often been assumed that gonadal hormones act symmetrically upon the amygdalae in the two hemispheres. However, both human and rodent models indicate that sex hormones may influence the anatomy and function of the amygdala in a hemisphere-specific manner. In this review I present an overview of examples where the amygdala exhibits sex differences in conjunction with hemisphere differences and discuss findings in both humans and rodents.

Hemispheric Sex Differences in the Human Amygdala

The primate amygdala contains both estrogen receptor (ER) and androgen receptor (AR; Donahue, 2000; Abdelgadir et al., 1999) and several reports document sex differences in the size of the amygdala relative to other brain regions (Giedd et al., 1996; Goldstein et al., 2001; Gur et al., 2002; Peper et al., 2009). Furthermore, sex differences in amygdala volume using fMRI are related to serum testosterone levels (Neufang et al., 2009), and men with Klinefelter syndrome (XXY sex chromosome complement) show reduced amygdala volumes with the strongest differences in the left hemisphere (Shen et al., 2004; Patwardhan et al., 2002).

Unfortunately, results from imaging techniques and tissue analysis often differ. Brabec and colleagues (Brabec et al., 2010) assessed male and female mice amygdalae using two tracing parameters in post-mortem tissue and found no sex

differences in amygdala volume and argue for refinement of imaging measurements. It's possible that sex differences in human amygdala volume may be masked by natural hormone fluctuations. There is evidence for menstrual-cycle influence on amygdala function (Dreher et al., 2007; Goldstein et al., 2010) and portions of the rodent amygdala are highly plastic and hormone-responsive (Cooke, 2007). Additional studies with hormonal-controls may be necessary before conclusions can be reached. Despite this difficulty in clear delineation of sex differences in human amygdala anatomy, imaging work continues to demonstrate sex differences in amygdala function. Moreover, many of these reports reveal lateralization of amygdala function, which is sex-specific.

Humans - Faces and Emotional Memory

As evidence of its role in social behavior, the amygdala is active in most reports of brain response to facial affect and emotional stimuli, and several well-documented lateralized sex differences come from this area of research. For example, while exposure to fearful faces activated the left amygdala in both sexes, exposure to happy faces produced a laterality effect with the right amygdala being more activated than the left, but only in males. Interestingly, females showed balanced amygdala activation during exposure to happy faces (Killgore and Yurgelun-Todd, 2001). Subsequent reports found similar sex differences and laterality when examining fearful faces and fearful eyes (Williams et al., 2005; Hardee et al., 2007) and demonstrated that women with Turner syndrome, who lack an X chromosome, show greater right amygdala and less left amygdala activation in response to fearful faces compared to control subjects, suggesting a possible role for sex chromosomes in this laterality (Skuse et al., 2005).

Results in adolescents show similar instances of laterality. Males had greater right hemisphere activation when viewing faces, and this laterality effect was strongest when viewing angry faces. Adolescent females equally activated both amygdalae (Schneider et al., 2011). Additionally, there is an age-related increase in left cortical activation relative to left amygdala activity when viewing fearful faces in adolescent girls, but not in boys (Killgore and Yurgelun-Todd, 2004; Yurgelun-Todd and Killgore, 2006) suggesting as females age the cortex becomes increasingly involved in facial processing but that this does not occur in males. However, two additional reports illustrate the complexity of laterality in facial processing.

Schneider and colleagues (Schneider et al., 1997) found that mood induction, during which subjects were asked to mimic the mood shown in a stimulus face, resulted in greater activation in the left amygdala in men and women. This may represent a shift to left-processing for men when asked to imitate a mood or it could be due to a small sample where results from both sexes were combined. Secondly, there is evidence that sex of the stimulus face may influence hemisphere effects although the female-left and male-right activation pattern persisted (Armony and Sergerie, 2007).

A sex difference in the pattern of lateralized amygdala response extends beyond response to faces. Positron emission tomography reports examining the sexes separately were the first to notice a lateralized response of the amygdala to emotionally arousing pictures. Both Cahill (Cahill et al., 1996) and Hamann (Hamann et al., 1999) found right-preferential amygdala activation in males while separate MRI reports found left amygdala activation in response to similar stimuli in females (Canli et al., 2000; Canli, 1999). These reports were followed by demonstrations that amygdala activation

in the left hemisphere for women and right hemisphere for men was related to memory for emotional films (Cahill et al., 2001; 2004). Similar patterns of sex-specific lateralization were seen in response to negative images (Canli et al., 2002; Mackiewicz et al., 2006). Additionally, after exposure to a disturbing image or story, an immediate early spike of fMRI activity was strongest in the right amygdala for men and left amygdala for women (Gasbarri et al., 2007).

Interestingly, although emotional memory is lateralized in this manner, Canli and colleagues (Canli et al., 2000) found that self-reports of emotional arousal correlated with left amygdala activity in both men and women. This left shift for men may be similar to the left shift seen when males are asked to mimic the mood of stimulus faces (Schneider et al. 1997) and suggests that when awareness of self is required, males shift to using the left amygdala. Finally, a male-right and female-left bias in amygdala activation also influences long term memory for central story information in men and peripheral story detail in women (Cahill, 2003).

Two more interesting and related findings include a study of psychosis that found left amygdala enlargement in women with mood-incongruent delusions but not in similar males (Gibbs et al., 2008). In concordance with this, the administration of procaine, an anesthetic agent that reliably produces psycho-sensory and hormonal responses, including delusions, resulted in greater left amygdala activity in women compared to men (Adinoff and Devous, 2003). Thus, there is repeated evidence of a female-left, male-right bias in the perception of various types of emotional/social material even when such perceptions may be psychotic or pharmacologically induced. There is also evidence to suggest that the left amygdala may be tied to self-awareness, since it is

activated in both sexes during mood induction and self-reports of arousal. Additional evidence from humans illustrates sex differences in the amygdala's connections with other brain regions, which may be related to this effect.

Humans - Functional Connectivity

Kilpatrick and colleagues (Kilpatrick et al., 2006) examined which brain regions exhibit correlated activity with the amygdala during rest; a measure understood to imply connectivity between the regions. The right amygdala in males showed greater resting state functional connectivity to other brain regions than in females, whereas the left amygdala in females showed greater functional connectivity than in males. Intriguingly, the male-right amygdala bias was strongly associated with regions linked to the detection of external stimuli, such as the sensorimotor cortex and striatum. In contrast, the female-left bias of the female amygdala involved regions linked to internal monitoring such as the hypothalamus (Kilpatrick et al., 2006). As the authors note, these results suggest not only a male-right and female-left division of amygdala utility, but also that the role of the two hemispheres may be divided into monitoring external versus internal information.

Importantly, these findings were later replicated in a homosexual sample (Savic and Lindström, 2008). Specifically, homosexual men are more like heterosexual women in exhibiting more widespread connections from the left amygdala whereas the opposite is true for homosexual women, who are more like heterosexual men. Interestingly, gonadal hormone levels during development are linked to sexual orientation in adulthood (Finn et al., 2002; Manning et al., 2007) perhaps the same hormones may alter patterns of amygdala connectivity in a hemisphere-specific manner

(Kilpatrick et al., 2006). A link between the amygdala, sexual behavior and hormones is present in several species (Green et al., 1957; Lehman and Winans, 1980; Cooke et al., 2003; Sartorius et al., 2008), but the human amygdala sexual response is complex and possibly hemisphere specific.

Humans - Sexual Response

Given the laterality in social processing described above and the amygdala's long association with sexual behavior (Klüver and Bucy, 1938), one might expect lateralized sex differences in the amygdala's response to sexual stimuli. The available evidence is sparse but intriguing. One group using fMRI found that males demonstrated greater amygdala activation in response to visual sexual stimuli compared to females with the sex difference being greatest in the left hemisphere (Hamann et al., 2004; Hamann, 2005). However, sexual stimulation of the penis resulted in decreased regional cerebral blood flow in the right amygdala and orgasm results in deactivation of the left amygdala in males (Georgiadis and Holstege, 2005; Holstege et al., 2003). These findings might suggest that in males appetitive sexual responses are related to left amygdala activation and consummatory behavior may require some balance of right and left amygdala deactivation. However, subsequent examination failed to find a relationship between male orgasm and the amygdala (Georgiadis et al., 2007) and other reports of appetitive response have been conflicting. For example, homosexual men demonstrated less activity in the left amygdala compared to heterosexual men when both groups where shown male homosexual erotic films (Hu et al., 2008). However, Paul and colleagues (Paul et al., 2008) found the opposite result with right amygdala

activation in heterosexual men shown male homosexual erotic films, which they attribute to an aversive response.

Overall, the amygdala's involvement in human sexual response remains difficult to assess and inconsistent (Karama et al., 2002; Kim et al., 2006; Miyagawa et al., 2007; Rupp and Wallen, 2007). Amygdala activity during sexual stimuli may underlie a general emotional arousal (Walter et al., 2008) and sociological effects can influence the region during sexual stimuli presentation (Beauregard et al., 2001). Until further reports address these issues, reaching conclusions about hemisphere-specific effects remains difficult.

Conclusions from Humans

Although facial processing, emotional memory and functional connectivity exhibit reliable lateralized sex differences in humans, there are several concerns. For example, hemisphere effects in the human imaging literature do not appear to be particularly robust. Recent meta-analyses of emotional processing are discrepant, with hemispheric differences seen in some (Baas, 2004; Fusar-Poli et al., 2009) and not in others (Wager et al., 2003; Sergerie et al., 2008). One explanation put forth is that laterality in the human imaging literature may not indicate separate hemisphere functions but may be due to different activation decay in the left (slow decay) verse right (faster decay) amygdalae. Sergerie and colleagues (Sergerie et al., 2008) argue that the commonly used block-design trial format could produce artificial laterality if activation decays differently in the two hemispheres.

Some studies demonstrating hormonal influence on the amygdala in humans have not supported hemisphere effects. For example, adolescent females with

congenital adrenal hyperplasia (CAH), a genetic disorder that results in elevated androgen exposure prenatally, displayed amygdala responses to faces indistinguishable from similar aged control males. Furthermore, no laterality was seen in control males or CAH females (Ernst et al., 2007). Similarly, a positive correlation was found between subjects' testosterone levels and amygdala activation to fearful and angry faces, suggesting circulating hormones may influence amygdala functionality (Derntl et al., 2009). However, no laterality was seen. While both these studies provide evidence for hormonal influence on amygdala functionality, the lack of laterality during processing of faces diverges from other reports (Schneider et al., 2011; Killgore and Yurgelun-Todd, 2001) and casts doubt on a hypothesis of lateralized hormone action in the amygdala.

Thus, the human literature currently provides an unclear picture of lateralized sex differences in the amygdala. However, including only studies which examined males and females, and which found both sex and hemisphere differences, does provide fairly consistent evidence of a male-right, female-left bias (Table 2). Additional studies using male and female subjects engaging in wide variety of amygdala-related tasks are needed. Additionally, evidence of lateralized sex differences in human anatomy are needed to bolster support for hemisphere specific gonadal hormone action, but as the rodent literature demonstrates, these may require increased resolution in imaging techniques or a focus on specific subregions in human tissue.

Hemispheric Sex Differences in the Rodent Amygdala

Rodents - Amygdala Function

Contrasting the human literature, lateralized sex differences in rodent amygdala anatomy are prevalent, and a few interesting reports related to amygdala function also

reveal lateralized sex differences. Gerendai and colleagues (Gerendai et al., 1995) used unilateral surgical deafferentation of the temporal lobes, including portions the corticomedial amygdala, in combination with left or right hemicastration to investigate neural control over gonadal functions. In females, right or left temporal lobe deafferentation in conjunction with right or left ovariectomy reduced serum luteinizing hormone levels. In males, temporal lobe deafferentation was effective at reducing testosterone production only if the left testis was removed, but hemisphere deafferentation effectiveness varied with age. Specifically, in prepubertal males unilateral orchidectomy (left testis) reduced serum testosterone (measured in vitro) only with right temporal lobe deafferentation while in adult males unilateral orchidectomy (left testis) reduced testosterone only with left temporal lobe deafferentation (Gerendai et al., 1995). These results suggest that while both temporal lobes are involved in gonadal hormone regulation in females, in prepubertal males the right hemisphere may be important and this role switches to the left hemisphere in adulthood.

Furthermore, although reduction of luteinizing hormone levels was hemisphere independent, compensatory ovarian hypertrophy after unilateral ovariectomy was eliminated only with right temporal lobe lesions (Gerendai et al., 1995). Similar evidence shows that lesions of the right medial amygdala disrupt ovulation more effectively than lesions of the left amygdala in adult females (Sanchez and Dominguez, 1995). Together the findings demonstrate some right hemisphere dominance in control of the ovaries. Given the important role of the hypothalamus in ovulation and the strong connections between the amygdala and hypothalamus, gonadal hormones may alter

connectivity between the right amygdala and hypothalamus to produce this hemispheric dominance.

Another aspect of amygdala function, the stress response, may also exhibit lateralized sex differences. After hemisphere specific 6-hydroxydopamine lesions to the basolateral amygdala, right-lesioned males had increased time in the open arms and reduced time in the closed arms of an elevated plus maze compared to sham-lesioned animals. Interestingly, right-lesioned females spent less time in the open arms and more time in the closed arms compared to shams, suggesting that right amygdala lesions are anxiolytic in males and anxiogenic in females (Sullivan et al., 2009).

Additionally, plasma ACTH levels in male rats with lesions to the right amygdala were significantly lower than left-lesioned males. However, in females left and right lesions reduced ACTH levels equally. Finally, after 30 minutes of restraint stress, left-lesioned males trended towards a higher stress response compared to right lesioned males while right-lesioned females had higher stress responses than left-lesioned animals, resulting in a significant sex by hemisphere effect (Sullivan et al., 2009).

These reports demonstrate that lesions of the amygdala are able to influence both gonadal hormone and corticotropin production in complex hemisphere and sex-specific patterns, and that the stress response is also sensitive to opposing hemisphere-specific amygdala lesions in male and female rats. Several other domains of amygdala function such as fear have been thoroughly researched and there is evidence of laterality in the rodent amygdala fear response (Baker, 2004; Scicli et al., 2004; Coleman-Mesches and McGaugh, 1995; Adamec et al., 2005; Berlau and McGaugh 2006). Unfortunately, while several of these reports show a right-bias in their male

subjects, they lack female subjects, precluding detection of sex differences. Therefore, it is unclear whether there is lateralized gonadal hormone influence in the amygdala's fear response.

Rodents - Amygdala Anatomy

Modern imaging techniques have been used to examine amygdala volume in non-humans and, as in humans, results are conflicting. Magnetic resonance microscopy examining amygdala volume in male and female mice revealed a sex difference in amygdala volume (females < males) with the greatest sex difference in the right hemisphere after corrections for total brain volume (Koshibu, 2004). However, a larger study found no sex or hemisphere differences in the amygdala in the mouse brain (Spring et al., 2007). As in humans, it is possible that current imaging techniques lack the resolution to reliably identify sex differences in amygdala anatomy, especially if sex differences are prominent only in certain amygdala subregions.

The medial amygdala (MeA) may be such a region. The MeA contains a dense concentration of both ER and AR and exhibits several lateralized sex differences (Canteras et al., 1995; Simerly et al., 1990; Cooke et al., 2003). Moreover, several of these lateralized sex differences are present before adulthood. Early in development, the rat MeA displays sex differences in aromatase activity, an enzyme that converts testosterone, an AR agonist, into estradiol, an ER agonist. At gestational day 22 males have greater aromatase activity in the left MeA than the right, while females have greater aromatase activity in the right MeA than the left. By postnatal day (PND) 6, the hemisphere difference in males has reversed (right > left), but by PND 15 the two sides seem equivalent. As in males, the female hemisphere difference reverses from right >

left to left > right by PND 6. However, this laterality in aromatase activity remains present at PND 15 in females (von Ziegler and Lichtensteiger, 1992). While these results suggest estrogen action in the medial amygdala is intricately arranged and may occur at different times for each hemisphere, the anatomical and behavioral implications of these findings are unclear.

Early gonadal hormones influence many aspects of MeA neuroanatomy. For example, the right posterodorsal medial amygdala (MePD) is larger in 25-day-old male rats than in females, but only in the right hemisphere (Johnson et al., in prep. a). Furthermore, neither sex differences nor laterality in MePD volume appear dependent upon functional ARs in juveniles (Johnson et al., in prep. a). While the right hemisphere displays greater sex differences in MePD volume at this age, this is not true for other measures of MePD anatomy.

Dendrite length and complexity are greater in the left amygdala of juvenile males compared to females, and male rats have more excitatory synapses in the left MePD than females (Cooke et al., 2007; Cooke and Woolley, 2005). There is also a sex difference in miniature excitatory post-synaptic current frequency (females < males) and synapse number found only in the left hemisphere but no difference in the number of neurons in this hemisphere (Cooke et al., 2007; Cooke and Woolley, 2005). In contrast, in the right MePD, juvenile males have more neurons than juvenile females but there are no sex differences in dendritic morphology (Cooke and Woolley, 2005; Cooke et al., 2007). Finally, there are hemisphere and sex differences in juvenile MePD astrocytes as well. Juvenile male rats have more astrocytes in the left and right hemisphere than do juvenile females. However, both sexes have more astrocytes in the right

hemisphere than the left (Johnson et al., in prep. a). Interestingly, functional ARs are not necessary for the lateralization of astrocyte numbers in the juvenile MePD (Johnson et al., in prep. a).

These complex sex by hemisphere interactions are also prominent in the adult medial amygdala. MePD volume is sexually dimorphic in rats, with males having larger MePD regional volume than females (Hines et al., 1992), which is also lateralized as the right MePD is larger than the left (Morris et al., 2005; Johnson et al., 2008). The right MePD also has a greater rostral-caudal extent than the left in male rats and evidence from Tfm male rats shows that while functional ARs are necessary for masculinized MePD volume, they are not necessary for the lateralization of MePD volume (Morris et al., 2005). In mice there is also a sex difference and lateralization, but only in females is the left MePD larger than the right (Morris et al., 2008b).

Although in male rats the right MePD is larger in volume than the left, the left MePD contains more neurons than the right in both sexes (Morris et al., 2008a). There are also sex differences in MePD neuronal soma size but reports of laterality are conflicting. Cooke and colleagues (Cooke et al., 2003) found larger somata in the left hemisphere compared to the right in male rats but this laterality was not seen in subsequent experiments (Morris et al., 2008a; b). Unspecified glia numbers are also lateralized in adults, with more in the right MePD than the left of both male and female rats, contrasting the greater number of neurons in the left MePD (Morris et al., 2008a).

In the adult rat, MePD astrocytes display particularly intriguing patterns of lateralization. As in juveniles, both male and female adults have more astrocytes in the right MePD than the left, and within the right hemisphere males have more astrocytes

than do females. In contrast, there is no sex difference in astrocyte number in the left hemisphere. However, the left MePD exhibits more complex astrocytes compared to the right. Furthermore, the hemisphere difference in astrocyte complexity occurs in the context of a sex difference in the left hemisphere, where males have more complex astrocytes than do females (Johnson et al., 2008).

Thus, the laterality in astrocyte number (left < right) mirrors MePD regional volume (left < right), while the laterality in complexity of astrocyte arbors (right <left) may mirror neuron number (right < left); Morris et al., 2008a; Johnson et al., 2008). Importantly, the sex differences in astrocyte number and arbor complexity are both AR dependent, but the hemisphere differences appear to be AR independent (Johnson et al., 2008). These findings in adults contrast with juveniles where ARs are not necessary for masculinized astrocyte numbers, and astrocyte arbors are neither lateralized nor sexually dimorphic (Johnson et al. in prep. a). These differences between juveniles and adults suggest lateralized sex differences in astrogenesis and arbor proliferation during puberty, which are AR dependent. Specifically, functional ARs appear to mediate astrogenesis in the right MePD of males and females during puberty, but to a greater extent in males, therefore maintaining the sex differences seen in this hemisphere in juveniles. In the left MePD, ARs mediate an increase in astrocyte arbor complexity during puberty, generating new sex differences in adulthood.

Perhaps the sex differences and laterality in MePD astrocytes indicate different inter- and intra-amygdala connections in the left and right hemispheres brought on by gonadal hormone action. Since astrocytes are closely linked to synapse formation (Nishida and Okabe, 2007; Halassa et al., 2007) such an explanation fits with the

laterality in synapses and excitatory post-synaptic currents seen in juvenile animals (Cooke and Woolley, 2005). However, multiple reports suggest that dendritic spines are neither sexually dimorphic nor lateralized in juvenile or adult animals (Arpini et al., 2010; Cooke et al., 2007), and that gonadal hormones may specifically promote sex differences in MePD synapses on dendritic shafts (Nishizuka and Arai, 1981).

Conclusions from Rodents

Available reports indicate that the amygdala's involvement in testosterone and luteinizing hormone release, as well as stress response, may be lateralized (Gerendai et al., 1995; Sullivan et al., 2009). Furthermore, several aspects of amygdala neuroanatomy exhibit lateralized sex differences, primarily in the MePD (Table 3). Many of these effects are lateralized before puberty, demonstrating lateralized hormonal influence during development in the two amygdalae (Johnson et al., in prep. a; Cooke and Woolley, 2005; Cooke et al., 2007).

While many of the sexual dimorphisms in MePD measures are at least partially dependent upon ARs, whenever Tfm males are examined, they exhibit similar lateralization compared to males (Johnson et al., 2008, in prep. a; Morris et al., 2005), which suggests that ERs mediate the masculinization of laterality in this nucleus. In the case of astrocytes, it appears that ARs mediate an increase in astrocyte number in the right MePD and astrocyte complexity in the left MePD sometime during puberty. However, the hemisphere differences are present in both juvenile and adult Tfm males; they are simply smaller in absolute terms in juveniles than in adults (Johnson et al., 2008, in prep. a). Therefore it is unlikely that AR action alone is responsible for

differences between the left and right MePD. Similar conclusions may hold for MePD regional volume (Morris et al., 2005).

Perhaps the most intriguing clue comes from the previously mentioned lateralization of aromatase activity in the MeA during development (von Ziegler and Lichtensteiger, 1992). If aromatase activity indicates early estrogen action, then it may be the lateralization of aromatase that establishes the earliest hemisphere differences in the region. Furthermore, hormonal influence during this initial lateralization may orchestrate the progression of additional laterality as gonadal hormones act on the amygdalae during puberty and adulthood. Identifying the mechanisms through which this priming may occur will be challenging but potentially informative.

It should be noted that the lateralized sex differences in the rodent amygdala literature are generally restricted to the medial subregion. Although laterality is present in other subregions (Ji, 2009; Berlau and McGaugh, 2006; LaLumiere, 2005) the lack of sex differences in these regions may be due to frequent use of only male animals. It is possible that if both sexes were examined, lateralized sex differences would emerge for the basolateral or central amygdala. While this outcome would greatly strengthen the case for hemisphere-specific gonadal hormone action in the amygdala, given the interconnectedness of the amygdala, the effects in the MeA alone may lead to lateralized function in the region as a whole.

Combining the Human and Rodent Literature

Both the human and rodent literature suggests that gonadal hormones influence the amygdala in a hemisphere-specific manner. The human literature shows lateralized sex differences in amygdala function, with the most reliable and robust findings in the

amygdala's response to fearful faces and emotionally disturbing imagery. The consistent male-right and female-left pattern in these reports suggests that men and women use different hemispheres for processing the same information, and results from homosexual and clinical populations appear to corroborate this assumption (Savic and Lindström, 2008; Skuse et al., 2005).

Evidence from rodents also indicates lateralized gonadal hormone influence in the amygdala. Hormone manipulations and Tfm males repeatedly illustrate that gonadal hormones are responsible for sex differences in several components of MeA neuroanatomy and that these measures are frequently lateralized (Cooke et al., 2003; Morris et al., 2005; Johnson et al., 2008). Therefore, it appears that in both humans and rodents the same gonadal hormones that masculinize the brain during development influence the neuroanatomy of the left and right amygdala differentially. Still, the most difficult questions remain: how are hormones achieving this hemisphere-specific influence and to what purpose?

Possible Mechanisms

One explanation of lateralized sex differences in the amygdala is that sex chromosomes are primarily responsible for amygdala laterality with minimal influence of traditional gonadal hormone action. Indeed, there is evidence for regulation of cerebral laterality by sex chromosomes (van Rijn et al., 2006; Annett, 2008; Agate, 2003). For example, women with Turner syndrome (X chromosome monosomy) exhibit increased leftward brain asymmetry mostly in posterior brain regions while men with Klinefelter's syndrome (XXY) trended toward reduced brain asymmetry in frontal regions (Rezaie et al., 2009).

Another report specifically implicated human genes coding for Protocadherin11 on the X and Y chromosomes in establishing cerebral asymmetry (Crow, 2010). However, the Protocadherin11 hypothesis is based on verbal and spatial ability variances in sex chromosome aneuploidies, and related studies suggest these effects may be restricted to the neocortex (Murphy et al., 1994). Perhaps the genes responsible for cortical laterality (Protocadherin11X,Y) are not responsible for lateralization of sub-cortical structures. In support of this, evidence from homosexual populations suggests sex chromosome complement alone does not determine male or female typical patterns of amygdala laterality (Kilpatrick et al., 2006; Savic and Lindström, 2008) and differences in behavior between XXY and XY mice are due mostly to androgen deficiency rather than genetic complement (Liu et al., 2010).

A second possibility is that gonadal hormone receptors control the development of sex differences and laterality in the amygdala. In humans, AR polymorphisms and handedness are related; reduced AR binding efficiency is associated with increased left-handedness (Medland et al., 2005). However, evidence from Tfm male rats and mice shows that while functional ARs are necessary for many of the sex differences seen in portions of the amygdala, a loss of AR function does not eliminate laterality of these measures (Morris et al., 2005; Johnson et al., 2008, in prep. a).

It is possible that estrogens and ER are mediating lateralized sex differences in the amygdala. The hemisphere-specific sex differences in aromatase activity in the MeA during development suggest estrogen influence (von Ziegler and Lichtensteiger, 1992). Estrogens have cell and region-specific effects on neuroanatomy (Raz et al., 2008; Micevych et al., 2010) reviewed in (McCarthy, 2010), but exactly how estrogens

might, for example, increase astrocyte numbers in the MePD only in the right hemisphere is still unclear.

An explanation of lateralized gonadal hormone receptor activity could involve differential expression of gonadal hormone co-regulators in the left and right MePD. These co-regulators are found in both neurons and astrocytes (Grenier et al. 2006), and are capable of altering the actions of steroid receptors in complex ways (reviewed in Tetel 2009). Members of the steroid receptor co-activator family (SRCs) are found in the amygdala of rodents, including the MePD, and modify gene expression and influence behavior (Meijer et al. 2000; Tetel et al. 2004; Molenda et al. 2002; Auger et al. 2000). However, I am not aware of any reports demonstrating laterality in gonadal hormone co-regulator expression or activity in the amygdala. It is possible that such differences are present and awaiting identification.

Tentative Explanations and Conclusions

At this time, explaining the significance of lateralized sex differences in the amygdala involves mostly speculation. Lateralization is proposed to enhance cognitive capacity and brain efficiency (reviewed in Vallortigara and Rogers, 2005), and sex differences in the brain and behavior often (although certainly not always, see De Vries and Boyle 1998) reflect adaptations necessary for each sex's reproductive needs. Perhaps, for reproductive purposes, gonadal hormones act differently in the left and right amygdala.

Humans and rodent males primarily use stimulus information from the external environment to locate and assess potential mates. In contrast, females often assess internal information to determine if mating is a viable option. Indeed, human females

alter mating strategies and choices depending upon current ovulatory state (Penton-Voak, 2000; Penton-Voak et al., 1999; Haselton et al., 2007; Gangestad et al., 2002). In rodents, the influence of internal status on female mating behavior is clearly demonstrated by the ability to make an unreceptive female rat receptive simply by injecting steroid hormones. In contrast male rats with greatly reduced levels of testosterone still retain reproductive function.

The lateralized sex differences in the amygdala may be the result of a fine tuning allowing a specific amygdala hemisphere to emphasize important information relevant to the animal's sex. The difference in connectivity demonstrated by Kilpatrick and colleagues (Kilpatrick et al., 2006), in which the male amygdala is strongly linked to regions that monitor external stimuli and the female amygdala is linked to internal monitoring regions, supports this idea.

Importantly, while these lateralized sex differences may have initially evolved to allow efficient processing of sexually specific social/mating information, it is possible other amygdala behaviors have become lateralized and sexually dimorphic as well. In humans the functional connectivity data along with mood induction and self-report results suggest that the left amygdala may be biased towards homeostatic activity and that males utilize this hemisphere only when self awareness is necessary. Is this the result of a relative silencing of the left amygdala sometime during development in males? Furthermore, are the numerous sexual dimorphisms in cellular anatomy unfolding during puberty in the rodent's left medial amygdala also indicative of a rewiring of this hemisphere in males?

Anxiety disorders, depression, schizophrenia and autism are all linked to the amygdala (Charney et al., 1993; Canli et al., 2005; Phillips et al., 1999; Amaral et al., 2008) and show sex biases, with greater prevalence in males (schizophrenia, autism) or in females (anxiety, depression; American Psychological Association, 2000). Furthermore, depression, autism and schizophrenia have shown interesting laterality effects in neuroanatomy (Versace et al., 2010; Nacewicz et al., 2006; Hulshof et al. 2001f). If sex hormones are reshaping the amygdalae in a hemisphere-dependent manner, understanding laterality and sex differences in amygdala neuroanatomy may be important for developing more effective treatments for amygdala-related disorders. Paraphrasing Larry Cahill (Cahill, 2006), the available evidence indicates that studies of amygdala sexual dimorphisms risk conclusions that are incomplete at best, and wrong at worst, if they fail to address potential influence of both sex and hemisphere. It is our hope that this collection of findings will compel researchers to record and report hemisphere information in the future when investigating gonadal hormone influence in the amygdala.

APPENDIX

Table 2 - Lateralized sexual dimorphisms in the human amygdala from experiments examining both men and women

Subjects and Reference.	Left Amygdala	Right Amygdala
Male adolescents[1]		Angry faces
Female adolescents	Angry faces	Angry faces
Male adults[2,3]		Memory for negative stimuli
Female adults	Memory of negative stimuli	
Male adults[4,5]		Functional connectivity
Female adults	Functional connectivity	
Male adults[6]		EEG spike/negative images
Female adults	EEG spike/negative images	
Clinical males[7]	Mood incongruent delusions	Mood incongruent delusions
Clinical females	Mood incongruent delusions*	
Male adults[8]	Response to procaine	Response to procaine
Female adults	Response to procaine	

Differences in color indicate significant sex differences. In cases where the sex difference favors women (males < females) text is colored pink. In cases where the sex difference favors men (females < males) text is colored blue. 1 Schneider et al., 2011, 2 Cahill et al., 2001, 3 Canli et al., 2002, 4 Kilpatrick et al., 2006, 5 Savic and Lindström, 2008, 6 Gasbarri et al., 2007, 7 Gibbs et al., 2008, 8 Adinoff and Devous, 2003 *Sex differences was in the volume of the left amygdala when females and males with mood incongruent delusions were compared.

Table 3 - Lateralized sexual dimorphisms in the rodent medial posterodorsal amygdala (MePD).

Subjects and Reference.	Left MePD	Right MePD
Juvenile Males		Regional Volume
Juvenile Females	Regional Volume*	Regional Volume
Juvenile Males		Neuron number
Juvenile Females	Neuron number	Neuron number
Juvenile Males	Dendrites**	Dendrites
Juvenile Females	Dendrites	Dendrites
Juvenile Males	Miniature EPSCs Hz	Miniature EPSCs Hz
Juvenile Females	Miniature EPSCs Hz	Miniature EPSCs Hz
Juvenile Males		Astrocyte Number
Juvenile Females		Astrocyte Number
Adult Males		Regional Volume
Adult Females		Regional Volume
Adult Males		Rostrocaudal extent
Adult Females	Rostrocaudal extent	Rostrocaudal extent
Adult Males	Neuron number	
Adult Females	Neuron number	
Adult Males		Astrocyte number
Adult Females		Astrocyte number
Adult Males	Astrocyte complexity	
Adult Females	Astrocyte complexity	

For each measure, differences in size indicate significant hemisphere differences within sex. Differences in color indicate significant sex differences. In all cases the sex difference favors male rodents. 1 Johnson et al. in prep. a, 2 Cooke et al. 2007, 3 Cooke et al. 2005, 4 Morris et al. 2005, 5 Johnson et al. 2008, 6 Morris et al. 2008. *Johnson et al. in prep. a found laterality in both sexes but not sex difference in the left MePD. Cooke et al. 2007 found no laterality in females but did find a sex difference in the left MePD. **Dendrite measures were dendritic length, number of dendritic tips and intersections in Scholl analysis. ***Morris et al. 2005 found no laterality in females. Johnson et al. 2008 found laterality in females.

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GENERAL DISCUSSION

Early efforts by Migel Garcia-Segura's team demonstrating changes in the astrocyte marker glial fibrillary acidic protein (GFAP) in response to estrogen led the way in modern investigations of gonadal hormone influence on glial cells (Tranque et al., 1987, also see Zimmerman 1982). Subsequent reports focused on hypothalamic astrocytes, which are hormone responsive and sexually dimorphic (Mong et al. 1996; Amateau et al., 2000; Langub and Watson 1992). The collected findings detailed the role of astrocytes in ovulation and emphasized that astrocytes are a vital component of brain circuitry (reviewed in Garcia-Segura et al., 2008). However, continued exploration has identified gonadal hormone responsive astrocytes in other brain regions, such as the hippocampus (Day et al., 1990) and amygdala (Johnson et al., 2008; in prep. b)

Heavily connected with the hypothalamus, cortex, hippocampus and brain stem, the amygdala is a hub of brain communication and is involved in a wide range of functions including fear, social and reproductive behaviors. Like the hypothalamus, the amygdala contains high concentrations of gonadal hormone receptors, and in laboratory species and humans the amygdala is sexually dimorphic (Simerly et al., 1990; Nishizuka and Arai, 1981; Kilpatrick et al., 2006). Several of these sexual dimorphisms are present in juveniles while others emerge after puberty (Cooke et al., 2007; Zehr et al., 2006). However, little information has been available regarding astrocytes in this important and hormone sensitive brain region.

As their roles in synapse formation, brain injury and disease have become clear, the need to understand how gonadal hormones influence astrocytes has grown (Ullian et al., 2001; Blurton-Jones and Tuszynski, 2001; Garcia-Ovejero et al., 2005). In this

review I discuss several findings in the medial posterodorsal amygdala (MePD), a region responsive to both androgen and estrogen influence (Dugger et al., 2007; Cooke et al., 1999). I find that astrocytes are gonadal hormone sensitive in portions of the amygdala and that, in contrast to the hypothalamus, androgen receptors (ARs) are principally important in establishing astrocytic sex differences in this region. I discuss the implications of these finding for amygdala function in both humans and rodents.

Astrocytes are Sexually Dimorphic in the Juvenile and Adult MePD

The sexual dimorphism in hypothalamic astrocyte complexity is present at postnatal day (PND) 1 (Mong et al. 1996), suggesting that gonadal hormone action during early development is responsible. The juvenile MePD exhibits sex differences in synapse density, neuron number and regional volume (Cooke et al., 2007; Cooke and Woolley, 2005) at PND 28, which also appear to be the result of early gonadal hormone action, leading to the possibility that MePD astrocytes may also be sexually dimorphic in juveniles.

In 25 day old rats, I used glial fibrillary acidic protein (GFAP) immunocytochemistry with a Harris hematoxylin counterstain to stereologically count MePD astrocyte numbers and to manually trace the processes of randomly selected astrocytes, providing quantitative measures of astrocyte arbor complexity (Fig 13). I found that in contrast to the hypothalamus, there are no sex differences in astrocyte arbor complexity in juvenile animals (Fig 15). None of the measures I obtained from manual tracing of astrocyte arbors exhibited any significant sex differences. However, I did find sex differences in the number of astrocytes in the MePD, with males having greater astrocytes than females in both the left and right hemispheres (Fig 14B). Thus,

it appears that gonadal hormones during early development either increase gliogenesis or are protective against cell death in the male MePD, but do not result in increased astrocyte arborization in males or females (Johnson et al., in prep. a).

Although astrocytes are tied to dendritic spine formation (Ullian et al., 2001), the relationship between astrocyte complexity and dendritic spine density is highly region specific (Pfrieger and Barres 1997; Elmariah et al., 2005; Mong et al., 2001). The greater synapse density and miniature excitatory post-synaptic currents in the prepubertal male MePD (Cooke and Woolley, 2005) indicate differences in neural connectivity in the region. Our results suggest that these sex differences in the surrounding neuronal network are either tied to the sex differences in astrocyte number or may be independent of astrocytes.

I investigated astrocytes in the adult MePD as well. As in juvenile animals, adult rats exhibit a sex difference in astrocyte numbers with male rats having more than females but only in the right hemisphere (Fig 2B). Furthermore, the hemisphere differences seen in both sexes for juvenile astrocyte numbers (left < right) are still present in adult animals. However, unlike juveniles, adult MePD astrocyte arbors are also sexually dimorphic, with male astrocytes having more primary branches, branch points, branch endings, and greater branch length on average compared to females (Johnson et al., 2008).

Interestingly, this sex difference also exists in the context of a hemisphere difference. Specifically, sex differences in measures of astrocyte arbor complexity were seen in the left MePD but not the right (Fig 3). Thus both astrocyte number and arbor

complexity are sexually dimorphic in the MePD of adult animals, but these sex differences are present in opposite hemispheres.

Laterality is common in the MePD and the greater numbers of astrocytes in the right MePD mirrors that hemisphere's greater regional volume and total glia numbers (Morris et al., 2008). The left hemisphere's greater arbor complexity in adulthood is accompanied by greater neuron numbers compared to the right (Morris et al., 2008b). However, this opposing laterality of astrocyte number and arbor complexity has not been reported previously and is particularly intriguing given the numerous examples of lateralized function seen in the human amygdala (Cahill et al. 2001; Canli et al. 2000; Schneider et al. 2011)

Androgen Receptors Mediate Gliogenesis and Astrocyte Arbor Proliferation

Testicular feminized mutant (Tfm) males were examined along with wild-type animals, allowing us to identify the contribution of ARs to the sex differences in the MePD. Tfm male rats are genetic males (XY) that lack functional ARs. Tfm males have internalized testes and produce testosterone but due to androgens inability to bind to AR, they exhibit a feminized phenotype with nipples and reduced ano-genital distance (Dugger et al., 2007). Furthermore, prior reports from our lab have demonstrated that in multiple brain regions, including the MePD, Tfm male neuroanatomy is incompletely masculinized (Dugger et al., 2007; Durazzo et al., 2007; Morris et al., 2005).

I found that in juvenile animals Tfm males were masculine for both MePD regional volume and astrocyte number (Fig 13B) indicating that the sexual dimorphisms seen in these measures prior to puberty are not dependent upon AR (Johnson et al., in prep. a). However, in adult animals Tfms are feminized for astrocyte number

suggesting AR sensitive astrogenesis in the MePD during puberty (Johnson et al., 2008, Fig 2B), which is concordant with prior evidence of androgen dependent gliogenesis in the region during puberty (Ahmed et al., 2008).

Interestingly, Tfm males are also feminized for astrocyte arbor complexity once the sex differences in these measures emerge in adulthood (Johnson et al., 2008, Fig 3). Furthermore, there appears to be little increase in Tfm male astrocyte complexity during this period, while wt males exhibit increases in primary branch numbers, branch points, branch endings, and branch length (Fig 17). Together with previous evidence, these findings suggest that puberty is a critical period for the MePD as gonadal hormones act to change the astrocytes in the region in a sexually dimorphic manner, and that ARs play a vital role in masculinization of MePD astrocytes during that period.

MePD Astrocytes are Plastic in Adults and Contain Androgen Receptors

The MePD exhibits a mixture of plastic and static cellular anatomy in adult animals (Morris et al., 2008). While the sexual dimorphisms seen in adult and juvenile animals suggest gonadal hormone action during early development and during puberty, they do not determine if astrocytes respond to circulating hormones in adulthood. In vivo and in vitro evidence shows that astrocytes respond to gonadal hormones in several brain regions, including the hippocampus, hypothalamus and cortex, where astrocytes contain ER and/or AR, suggesting possible cell-autonomous responses (Lu, 2003; Kuo et al., 2010; Gatson and Singh, 2007).

To investigate the influence of circulating hormones on astrocytes in the MePD I used male and female animals that were gonadectomized and implanted with capsules containing either testosterone or nothing (blank) for 30 days. The animals were

analyzed using methodology similar to our previous investigations of MePD astrocytes, and a separate cohort of males was examined for AR immunoreactivity. Using dual-label immunocytochemistry in conjunction with confocal microscopy I found evidence of AR immunoreactivity within nuclei of many MePD astrocytes (Fig 10A, B). This adds the amygdala to a list of regions where AR positive astrocytes have been identified, and opens the possibility that androgens act directly on these cells to induce changes in MePD neuroanatomy.

In hormone manipulated animals I found that removal of circulating hormones resulted in reduced astrocyte numbers compared to animals exposed to testosterone. This effect was primarily seen in females and only in the right hemisphere (Fig 8). Furthermore, I found that circulating hormones maintain arbor complexity in the MePD of males with T-treated males exhibiting more complex astrocytes than blank-treated males in both hemispheres. There was a similar effect in females but only in the right hemisphere and significant only for one measure of astrocyte complexity (Fig 9). Thus, it appears that both astrocyte numbers and arbor complexity are influenced by circulating hormone levels, although the effect is more powerful for arbor complexity.

Comparing juveniles and adults suggests that the development of masculinized astrocyte arbor complexity is androgen dependent (Johnson et al., 2008). However, because testosterone can be converted to estrogen, data from our hormone manipulated animals does not indicate whether ERs or ARs are responsible for maintaining this sex difference in adulthood. Peak estrogen levels during proestrus are linked to higher levels of GFAP-immunoreactivity in the MePD (Martinez et al., 2006) suggesting estrogen control of astrocytes in the region, at least in females. Estrogen

could be maintaining astrocyte complexity in males through aromatization of testosterone. This outcome would indicate an interesting shift in which androgens are responsible for establishing the sex difference, while estrogens maintain it in adulthood.

Conclusions

Interpreting the collected findings is difficult due to the region specific influence of astrocytes and the complex laterality in MePD neuroanatomy. Nevertheless, several findings point to a period of neuronal pruning in the MePD of males during puberty. Our results suggest that this may be mediated by an increase in astrocyte arbor complexity.

During puberty there is a reduction in the number of primary dendrites and terminal spine density in the MePD (Zehr et al. 2006). This reduction is accompanied by reductions in spinophilin protein levels but an increase in synaptophysin levels suggesting that while dendritic spines have been reduced, the amount of synapses has not (Zehr et al., 2006). Furthermore, Nishizuka and Arai (1981) reported a development-related and hormone responsive sex difference in synapses in the medial amygdala using electron microscopy. While they found no sex differences in juveniles, they did find sex differences in the number of dendritic shaft synapses in adults but not in adult dendritic spine synapses. They also found that while treatment of neonatal females with testosterone resulted in masculine numbers of dendritic shaft synapses in adulthood, such treatment did not influence dendritic spine synapses. Finally, they also found that neonatal orchidectomy reduced both dendritic shaft and spine synapses in adulthood. Subsequent reports confirmed juvenile orchidectomy's effectiveness in reducing dendritic spines and altering neural communication in adult animals (Cooke and Woolley, 2009). Together these studies strongly suggest a pubertal reorganization

of MePD neuronal connectivity. Specifically, there is likely a reduction in dendritic spines and an increase in dendritic shaft synapses in males during puberty which is gonadal hormone dependent.

Our results demonstrate an increase in the complexity of astrocytes in the left MePD during puberty (Fig 17), which is mediated by androgens. Given these findings and the involvement of astrocyte processes in synapse formation (Elmariah et al., 2005), I propose that androgens during puberty induce MePD astrocyte arbor proliferation, leading to reductions in spine density and possibly new dendritic shaft synapse formation in the MePD. This is concordant with the sex differences seen in adult animals for dendritic shaft synapse number, astrocyte arbor complexity (Nishizuka and Arai, 1981; Johnson et al., 2008) and provides a possible cellular mechanism for the dendritic pruning seen during puberty (Zehr et al., 2006).

It is likely that this process is primed by gonadal hormones earlier in development since neonatal administration of testosterone to females resulted in masculinized numbers of dendritic shaft synapses in adults but not in juveniles, (Nishizuka and Arai, 1981). The sex differences seen in juvenile Tfms suggest possible early ER influence in the region and the intricately timed expression of aromatase levels early in development also indicate estrogen involvement (von Ziegler and Lichtensteiger, 1992). An increase in adult astrocyte arbor complexity in females post puberty resulting from neonatal testosterone treatment would confirm this early hormone priming and strengthen the proposed association between adult synapse arrangement and astrocyte arbor complexity.

Despite indirect evidence for early hormone priming by estrogen, the sex differences in MePD astrocyte complexity that arise during puberty are AR dependent and remain plastic in adulthood (Johnson et al., 2008; in prep. b). Thus, if astrocytes are indeed responsible for the changes in synapse complement this process would be directed by AR action. Our finding of ARs in MePD astrocytes opens the possibility that androgens act directly on astrocytes to change their arbor complexity, which may then cause changes in synapses in the region. A similar pathway has already been described in the preoptic area with estrogens acting on ER positive astrocytes to influence surrounding neurons (Amateau and McCarthy 2002; McCarthy et al., 2003).

An additional complication for our proposal is that the androgen dependent increase in astrocyte complexity seen between prepuberty and adulthood occurs only in the left hemisphere. Therefore, our current hypothesis is restricted to the left MePD. However, this assumes that the combined hemisphere data demonstrating dendritic pruning (Zehr et al., 2006) and synapse sexual dimorphisms (Nishizuka and Arai, 1981) would not be found exclusively in the right hemisphere once analyzed independently and presents the obvious question of what is happening in the right MePD?

Perhaps the increase in astrocyte numbers seen in the right hemisphere (Fig 16) also serves to prune dendritic spines and/or change synapse complement. Alternatively, the increased astrocyte numbers seen in the right MePD may not significantly influence the neural communication network. Unfortunately, reports examining the functional consequences of gliogenesis are scarce. Reduced glial cell numbers are seen in depression, and electroconvulsive therapy, a treatment option in severe depression, activates glia and gliogenesis in the amygdala (Bowley et al. 2002;

Jansson et al. 2009; Rajkowska and Miguel-Hidalgo 2007). It therefore seems likely that there are functional consequences to the increased numbers of astrocytes in the MePD, especially in males, after puberty.

Fortunately, results from human functional imagine studies may provide insight into this laterality. In the human facial perception and emotional memory literature there is support for a male-right and female-left amygdala activation model (Killgore and Yurgelun-Todd, 2001; Hardee et al., 2007; Cahill, 2006, Table 2). This laterality is also seen in resting state functional connectivity, presumed to reflect lateralized sex differences in amygdala connectivity to other brain regions (Kilpatrick et al., 2006). Interestingly, the human male right amygdala exhibits high resting state functional connectivity with areas involved in sensory perception while the female-left amygdala exhibits such connections with homeostatic centers. Results from homosexual and clinical populations strengthen the hypothesized gonadal hormone influence on lateralized amygdala function (Savic and Lindström, 2008; Skuse et al., 2005).

If amygdala laterality plays a similar role in humans and rodents, then perhaps the consequence of an astrocyte mediated synaptic reorganization of the left amygdala during puberty is to “silence” this hemisphere in males. In contrast, the increase in astrocyte numbers in the right MePD may be involved in biasing amygdala activity to this hemisphere in males, producing the male-right bias seen in adults.

Not surprisingly, given its central role in brain communication, the amygdala has been implicated in several diseases and disorders, including autism, which exhibits a strong male bias in the population and may be related to androgen influence (American Psychological Association, 2000; Baron-Cohen, 2002). Demonstrations that autistic

individuals exhibit deficits in empathizing while displaying a propensity for systematizing (Lombardo et al., 2010; Auyeung et al., 2009a; b) seem in line with greater amygdala functional connectivity with homeostatic regions in females and sensory regions in males (Kilpatrick et al., 2006). If gonadal hormones during early development are priming the amygdala for synaptic reorganization during puberty, leading to a male-right/sensory-monitoring amygdala network, then what are the consequences for early disruption or acceleration of this process? Continued investigation of how gonadal hormones influence amygdala cellular anatomy may provide answers. Given the increasing importance of astrocytes, understanding how they influence the synaptic organization of the amygdala should be a high priority.

Future Directions and Closing Remarks

The sexual dimorphisms I have reported in MePD astrocytes underscore the fact that the cellular components of the amygdala are influenced by gonadal hormones acting upon both ERs and ARs. What I have proposed is that androgens act on MePD astrocytes during puberty to promote astrogenesis and/or arbor proliferation and this leads to changes in MePD synapse complement, altering MePD connectivity and amygdala functionality. Although these reports provide new evidence of complex cellular sexual dimorphisms and hormone responsiveness in an important brain region, additional research is necessary to test these hypotheses and detail the mechanisms through which gonadal hormones activate MePD astrocytes. Therefore a wide range of future research directions is available.

Questions regarding the ontogeny of astrocyte sex differences are particularly intriguing and may have important implications for disease research. A series of

experiments centered on ideas put forth by Nishizuka and Arai (1981) would be informative. Because a single PND 5 injection of T failed to masculinize synapses in juvenile females but did masculinize synapse in adult females, it appears that neonatal gonadal hormones provide a priming effect, leading to changes that occur later, during puberty.

Is there a priming effect of neonatal gonadal hormones for astrocyte sex differences in adulthood? If neonatal T treatment masculinizes astrocytes before puberty or fails to result in masculinized astrocytes in adult females, this would suggest that the changes I see in astrocytes during puberty are orthogonal to the previously reported changes in MePD synapses. However, if T treatment leads to masculine astrocytes in adult females then several more experiments should follow.

Orchidectomy of male rats on PND 1, which are then given recurring testosterone treatment starting on day 28, could be examined. If these animals exhibited masculinized astrocytes, one might deduce that gonadal hormone influence between PNDs 1 and 28 is not necessary for the pubertal increase in astrocyte numbers and arbor complexity. Including a group of males who were orchidectomized just prior to puberty but who received no testosterone supplementation would produce a dichotomy of “no T at puberty” and “T only at puberty” animals useful for isolating when gonadal hormones must be available to produce masculinized astrocytes in adult animals.

Assuming sustained evidence of a link between astrocyte sex differences and MePD neuropil, investigating the mechanisms and consequences of changes in MePD

astrocytes would be valuable. My results using Tfm males demonstrate that ARs contribute to both astrocyte complexity and numbers in the MePD.

Although astrocytes in the region contain AR, it is not clear if the sex differences seen are due to androgens acting directly on astrocytes. Use of the cre-lox system in which ARs are selectively disabled in GFAP- expressing cells would explore this question. Finding that removal of AR from astrocytes in males resulted in feminized astrocytes in the MePD would immediately lead to questions about the consequences for surrounding neurons, but would also open the door for further exploration of the molecular and genetic mechanisms behind AR influence in astrocytes.

Another intriguing investigation might examine how ARs mediate cellular proliferation in some astrocytes while promoting arbor elaboration in other populations. Work in the hypothalamus has identified prostaglandins and endogenous cannabinoids as intermediaries in pathways leading to astrocytic sex differences (Amateau and McCarthy, 2002; Krebs-Kraft et al., 2010). This evidence, in combination with the diverse actions of gonadal hormone co-regulators (Tetel, 2009; Auger et al. 2000; Meijer et al. 2000) suggests a wide array of possible molecular signaling pathways. Astrocytes express some of these co-regulators (Grenier et al. 2006) and their activation has been linked to sexually dimorphic gene expression (Molenda et al. 2002). Furthermore, the co-regulator SRC-1 has already been identified as responsive to seasonal fluctuations that also affect mating behavior and cellular anatomy in the MePD of male hamsters (Tetel et al., 2004; Gomez and Newman 1991).

Finally, besides cellular and molecular questions, my dissertation raises questions about the function of the amygdala as a whole. At the broadest level,

questions should now be asked about astrocytes in other regions of the amygdala. Are astrocytes in other subregions sexually dimorphic as well? If so, when do these sex differences arise? Do astrocytes throughout the amygdala increase in arbor complexity during puberty? Moreover, what are the consequences of astrocyte-induced changes in medial amygdala connectivity? Are MePD astrocytes strengthening connections to the hypothalamus and/or olfactory bulbs in males or are the changes in connectivity more local, resulting in altered communication between the medial amygdala and other amygdala subunits? How are these changes integrated into the functioning of the amygdala as a whole?

My dissertation shows that astrocytes are an important and dynamic part of the medial amygdala and likely play an important role in shaping this region as it responds to gonadal hormones throughout life. The novel sexual dimorphisms and complex laterality I found emphasize the need for continued exploration of brain neuroanatomy, including examination of cell types beyond neurons and careful documentation of both sex and hemisphere. The amygdala in particular appears to present challenging levels of sex and hemisphere differences in numerous aspects of cellular anatomy, all of which make the region more intriguing. Despite decades of research and a constantly expanding role in important brain function, the amygdala remains poorly understood and mysterious. Continued exploration of the region will likely provide information useful in our understanding of disorders such as autism and depression and may lead to insight regarding the function of the brain as whole.

APPENDIX

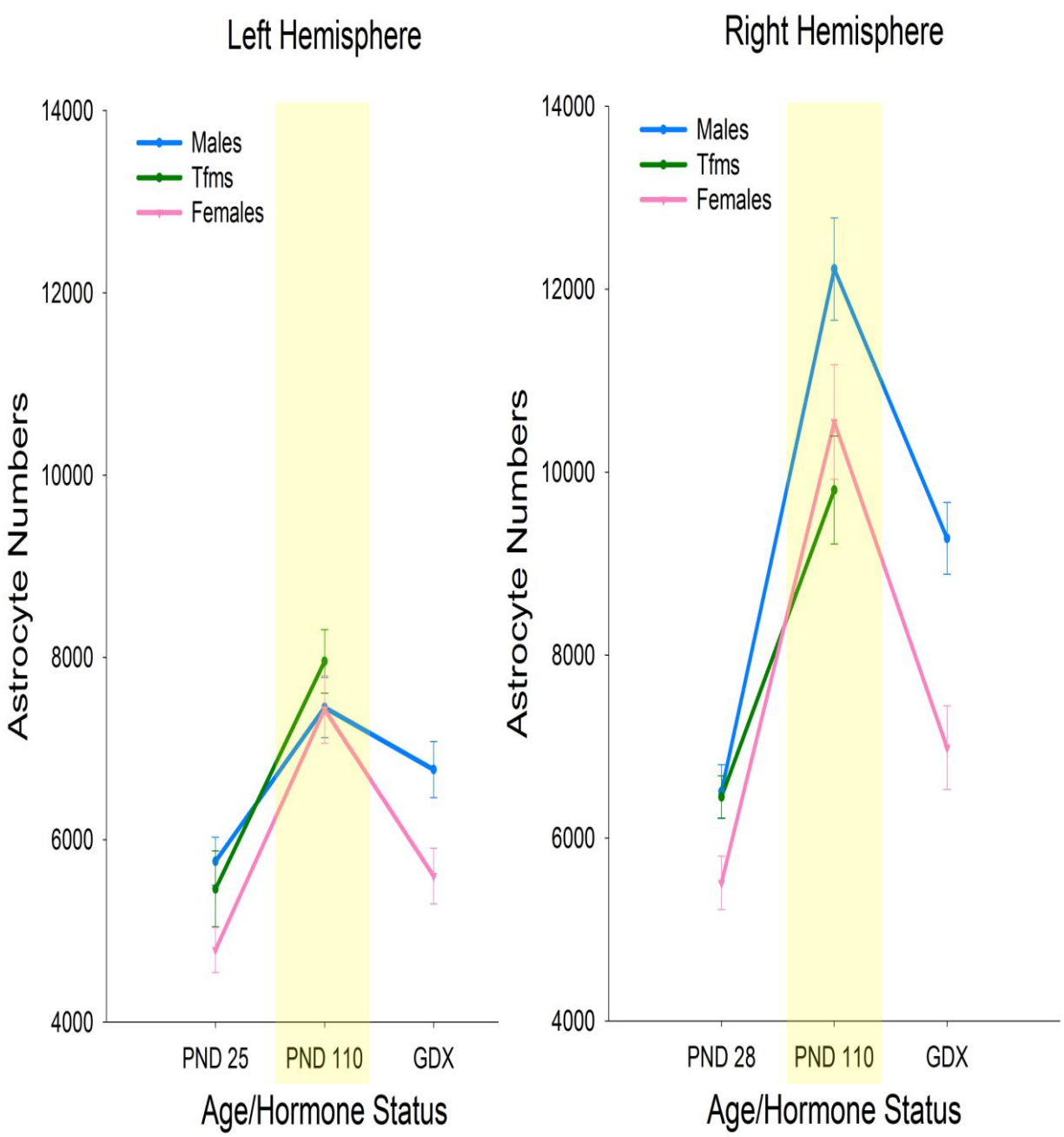

Figure 16 - MePD astrocytes increase in number during puberty in both the left and right hemispheres. In the left hemisphere Tfm males do not differ from wt males before or after puberty and astrogenesis does not depend on functional androgen receptors. Astrogenesis in the right hemisphere is greater compared to the left and is at least partially dependent upon functional androgen receptors. Tfm males are masculine before puberty but feminine in adulthood. The right hemisphere displays a more dramatic decline in astrocyte numbers when adult circulating hormone levels are reduced and this effect is especially prominent in females. Values are means of N = 8-10 rats/ group (+ SEM).

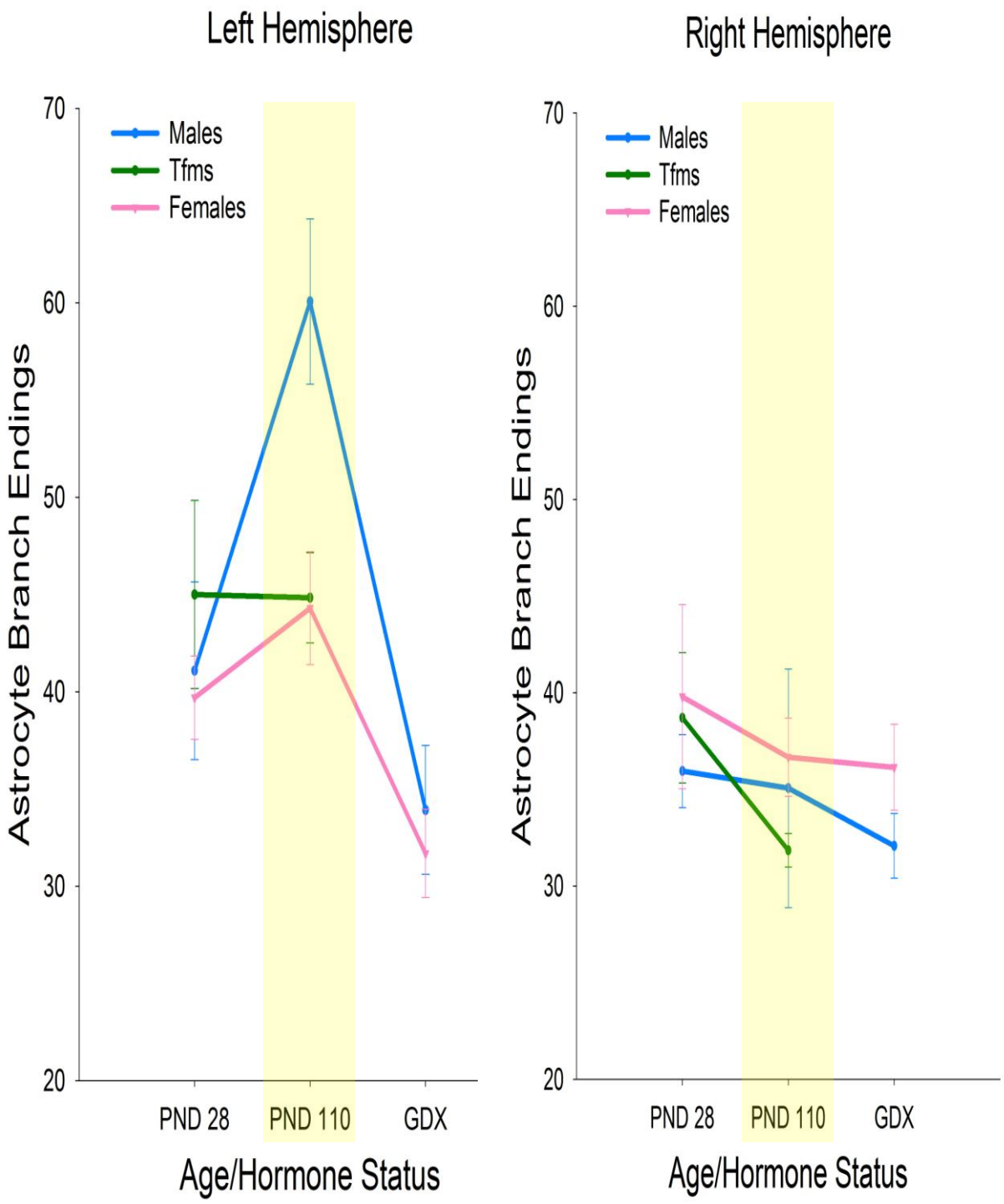

Figure 17 - Astrocytes exhibit an androgen-dependent increase in the number of branch endings during puberty in the left MePD. Other measures of astrocyte complexity such as number of primary branches, branch points, and average branch length exhibit similar patterns. Eliminating adult circulating hormone through gonadectomy reduces astrocyte complexity in the left hemisphere. Values are means of N = 5 rats/ group (+ SEM).

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