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Astrocytes in the Rat Medial Amygdala Are Responsive to Adult Androgens

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Abstract

The posterodorsal medial amygdala (MePD) exhibits numerous sex differences including differences in volume and in the number and morphology of neurons and astrocytes. In adulthood, gonadal hormones, including both androgens and estrogens, have been shown to play a role in maintaining the masculine character of many of these sex differences, but whether adult gonadal hormones maintain the increased number and complexity of astrocytes in the male MePD was unknown. To answer this question we examined astrocytes in the MePD of male and female Long Evans rats that were gonadectomized as adults and treated for 30 days with either testosterone or a control treatment. At the end of treatment brains were collected and immunostained for glial fibrillary acidic protein. Stereological analysis revealed that adult androgen levels influenced the number and complexity of astrocytes in the MePD of both sexes, but the specific effects of androgens were different in males and females. However, sex differences in the number and complexity of adult astrocytes persisted even in the absence of gonadal hormones in adulthood, suggesting that androgens also act earlier in life to determine these adult sex differences. Using immunofluorescence and confocal microscopy, we found robust androgen receptor immunostaining in a subpopulation of MePD astrocytes, suggesting that testosterone may act directly on MePD astrocytes to influence their structure and function.

Indexing Terms

androgen; glia; plasticity; astrocytes; amygdala; hormone

The amygdala, a highly conserved brain region, is primarily associated with two major types of behaviors: fear/anxiety behaviors (Nagy et al., 1979; Adamec, 1990; Rogan et al., 1997; LaBar et al., 1998) and social/mating behaviors (Lehman and Winans, 1982; Baum and Everitt, 1992; Kondo and Arai, 1995; Sanders and Shekhar, 1995; Adolphs et al., 2002;

Adolphs and Tranel, 2003; Faber-Zuschratter et al., 2009). The amygdala as a whole is also implicated in several diseases and disorders related to these behaviors, including autism, depression, schizophrenia, and anxiety disorder. Interestingly, all of these diseases and disorders exhibit sex biases in prevalence rates (American Psychiatric Association [DSM-IV-TR] 2000).

The posterodorsal division of the medial amygdala (MePD) of adult rodents shows marked plasticity in response to gonadal hormones (Gomez and Newman, 1991; Morris et al., 2005, 2008a,b). For example, the volume of the MePD and size of neuronal somata in this region are 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). Castration in adulthood also reduces both the size and dendritic branching of neurons in the posterior medial amygdala of adult male hamsters (Gomez and Newman, 1991), but treatment with androgens and/or estrogens prevents these morphological responses to castration (Gomez and Newman, 1991; Cooke et al., 2003), indicating that gonadal hormones play a critical role in regulating adult MePD anatomy and function. Whereas the influence of adult androgens on MePD neurons has been demonstrated, there are fewer reports regarding the influence of circulating androgens on other cellular components such as glia in the MePD.

We previously reported sex differences in the number and complexity of astrocytes in the adult rat MePD and these sex differences showed striking lateralization (Johnson et al., 2008). Moreover, we found that the sex differences in astrocyte morphology depend on androgen receptors (ARs), since astrocytes were entirely female-typical in both number and complexity in genetic (XY) male rats that have dysfunctional ARs due to the testicular feminization mutation (Tfm) (Johnson et al., 2008). The highly plastic nature of astrocytes in other adult systems (Garcia-Segura et al., 1989; Mong, 1996; Emamian et al., 2010), together with their androgen dependence in the MePD, suggests that MePD astrocytes may also exhibit androgen-dependent adult plasticity. To test this hypothesis, we examined the number and morphology of MePD astrocytes in male and female rats after adult castration and testosterone (T) treatment.

We 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 levels. Intriguingly, these responses are highly lateralized, mirroring hemisphere-specific responses of MePD regional volume, and providing further evidence that the two amygdalae respond differentially to circulating hormones. Finally, given that astrocytes have been reported to express AR in other brain regions (Finlay and Kritzer, 1999; Lorenz et al., 2005; Tabori et al., 2005), we asked whether androgens have the potential to directly affect MePD astrocytes. This possibility was supported by the finding that many astrocytes in the MePD are immunoreactive for AR.

Materials and Methods

Animals and surgeries

Ninety-day-old male and female Long Evans rats were obtained from Charles River (Charles River Laboratories, Wilmington, MA). 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. Animals were cared for in accordance with the guidelines set forth by the National Institutes of Health and all procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University (MSU).

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 subcutaneously 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 T (Sigma, St. Louis, MO) or nothing (blank). Although T can activate ARs, it can also be converted to estradiol to activate estrogen receptors (ERs). We used T in the present study because while our previous results (Johnson et al., 2008) indicated that masculinization of MePD astrocytes requires functional ARs, they did not eliminate the possibility that, in the presence of functional AR, ER activation may also affect these astrocytes. All capsules were incubated in phosphate-buffered saline (PBS; pH 7.4) for 48 hours at room temperature (RT) prior to implantation. All surgeries were performed using aseptic procedures, and animals were provided post-operative analgesia (0.51 μg buprenorphine/g of body weight).

After 30 days, a duration of hormone treatment sufficient to alter MePD regional volume, average neuronal soma size and total glial numbers (Cooke et al., 1999; Morris et al., 2008a), animals (age 141–142 days) were overdosed with sodium pentobarbital (150 mg/kg, intraperitoneal). 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 collected, trimmed, and weighed. Brains were removed, weighed, and postfixed for 5 hours at 4°C in the same fixative solution before immersion in 30% phosphate-buffered sucrose at 4°C for at least 48 hours. Brains were then sectioned coronally on a freezing microtome at 40 μm through the region of interest with the left cortex scored to mark side. Sections were collected into cryoprotectant (de Olmos et al., 1978) and stored at -20°C . Every third section through the rostrocaudal extent of the MePD was processed for glial fibrillary acidic protein (GFAP) immunocytochemistry (ICC).

Immunostaining for the astrocytic marker GFAP

Sections were transferred from cryoprotectant to Net-well plates (Corning Life Sciences, Corning, NY) and thoroughly rinsed in 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 minutes in 0.5% sodium borohydride before blocking in 10% normal horse serum containing free avidin (Avidin Biotin blocking kit, Vector Laboratories, Burlingame, CA), rinsed, then incubated 1 hour at RT in mouse anti-GFAP monoclonal antiserum (1:50,000, MAB360, Millipore, Billerica, MA; Neuroscience Information Framework antibody ID: 94939) containing free biotin (Avidin/Biotin blocking kit, Vector Laboratories). Tissue was then incubated overnight in the same solution at 4°C. The GFAP monoclonal IgG antibody was made in mice and prepared against purified glial fibrillary acidic protein from porcine spinal cord. In mouse brain western blots, the antiserum recognizes a single band at ≈ 51 kDa (per the manufacturer's technical information), consistent with the known molecular weight of rat GFAP. Following incubation in primary antiserum, tissue was incubated in biotinylated rat adsorbed horse antimouse secondary antiserum (1:500, Vector Laboratories), followed by incubation in peroxidase avidin-biotin complex solution (Elite ABC kit, Vector, at half the recommended concentration). Horseradish peroxidase (HRP) was visualized using diaminobenzidine (DAB, Sigma) in a 0.05M Tris buffer (pH 7.2). After rinsing to quench the peroxidase reaction, tissue was mounted on gel-subbed slides, dried, and counterstained using Harris hematoxylin solution (Sigma) to stain cell nuclei, and 10% lithium carbonate as the bluing agent. Slides were then rinsed, dehydrated, cleared, and coverslipped with Permount. The morphology and distribution of GFAP-immunoreactive cells labeling through the amygdala and hypothalamus were consistent with other descriptions (Mong et al., 1999; Martinez et al., 2006; Johnson et al., 2008).

Stereological Analysis

A Zeiss Axioplan II microscope equipped with 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 100 $\times$ Plan-Neo-Fluar, 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 (Stereo Investigator v. 8.0) 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 $35 \times 35 \mu\text{m}$ with a height of $11 \mu\text{m}$. 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 two GFAP-labeled processes 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 ($n = 9$ rats/group). The number of cells counted to produce these estimates ranged from ≈ 250 –500 per hemisphere depending on the hemisphere and the sex of the subject. We also estimated astrocyte density for the left and right MePD based on the total number of astrocytes divided by the volume of each side.

Astrocyte process complexity—Following previously described methods (Johnson et al., 2008), we used Neurolucida software (v. 7.0, MBF Bioscience) to trace and measure the entire visible arbor of 20 randomly selected astrocytes per subject (10 astrocytes per hemisphere) with five 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, 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. While detection of GFAP within astrocytic processes is known to underestimate actual arbor length of astrocytes (Bushong et al., 2002), this approach nonetheless allows us to detect relative differences in astrocyte morphology between treatment groups and has yielded new information about how astrocytic processes in the adult MePD change in response to adult androgens.

Double immunofluorescence for AR and GFAP

To test for colocalization of ARs in astrocytes 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. Sections were stained first for AR followed by staining for GFAP using established 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 hours of incubation at 4°C in rabbit anti-AR monoclonal antiserum (0.3 μ g/ml; Epitomics, Burlingame, CA; #1852-1, lot #YD072702, monoclonal IgG raised in rabbit directed against amino acids 901–919 on the C-terminus of the human AR) containing free biotin. Following incubation in AR primary antiserum, tissue was incubated in biotinylated goat antirabbit secondary antiserum (1:2,000; Vector Laboratories) followed by Cy2-conjugated streptavidin (1:48,000; Jackson ImmunoResearch, West Grove, PA). Next, to detect astrocytes tissue was incubated overnight at 4°C in GFAP antiserum (1:2,000) as described above and again blocked for nonspecific binding of streptavidin and biotin (Streptavidin/Biotin blocking kit; Vector Laboratories). After incubation in biotinylated goat antimouse secondary antibody (1:500, Vector Laboratories) for 2 hours, tissue was incubated in Cy5-conjugated streptavidin (1:32,000), then thoroughly rinsed and mounted in dim light on gel-subbed slides and allowed to dry in the dark. Cell nuclei were counterstained with DAPI (1 μ g DAPI / 1 ml distilled H₂O) for 5 minutes before dehydrating, clearing in Citrisolv, and coverslipping with DPX (Sigma-Aldrich, St. Louis, MO), which we find preserves fluorescent signals for more than a year when slides are

stored at -20°C (Monks et al., 2004). The distribution of AR-immunoreactive nuclei was consistent with previous descriptions from AR mRNA expression (Simerly et al., 1990) and autoradiography of androgen binding (Brown et al., 1995).

AR immunoreactivity 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, which eliminated immunostaining (data not shown), and scans of singly labeled tissue indicated no excitation or emission overlap. Second, preadsorbing the AR primary antibody with the immunizing peptide (Epitomics P-1852, 2.5 $\mu\text{g/ml}$) eliminated AR labeling in the MePD and other AR-containing brain regions of normal adult male rats.

Third, we compared AR immunostaining in wildtype male mice and mice carrying the Tfm of the AR gene, which because of a frame shift mutation disrupts AR protein production (Charest et al., 1991). We maintain a colony of Tfm mice, originally derived from Jackson Laboratories, on a C57 background. Using the same AR staining protocol, we see staining in the MePD of wildtype mice that is comparable to what we see in rats, but no immunolabeling in the brains of the Tfm male mice (Fig. 1).

AR/GFAP colocalization using confocal microscopy

DAPI-labeled astrocyte nuclei within the MePD were examined for AR immunoreactivity. Analysis was conducted with 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 (10 $\times$ objective) using the same well-established criteria (Hines et al., 1992; Paxinos and Watson, 2005; Morris et al., 2008a).

Twenty to thirty sites per animal were assayed within the MePD using a 60 $\times$ Plan-Apo, 1.42 N.A. oil-immersion objective. Sites were chosen semirandomly throughout the MePD. At each site sequential scans were run every 2 μm up to a depth of at least 18 μm for each of the three fluorophores. Image stacks were then saved 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 when immunoreactivity was found in the nucleus. The software's z-stack orthogonal viewer was also used to confirm colocalization of markers for a subsample of nuclei. From this analysis, we 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 (San Jose, CA) for production of figures. Images were unaltered in Photoshop other than contrast and brightness adjustments to Figure 1, color matching in Figure 4, and the addition of appropriate labels. When necessary, images were resized using "nearest neighbor" resampling to achieve the requisite DPI for the desired image size.

Hormone assay

Plasma T concentrations were measured in duplicate from 50- μ l samples using the Coat-a-Count Total Testosterone Kit (Diagnostic Products, 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 intraassay coefficient of variation was 9.0%.

Statistical analysis

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), treating left and right hemispheres as a repeated measure and sex (male, female) and treatment (T, blank) as between-group measures. When appropriate, two-way ANOVAs were also conducted within each hemisphere and least significant difference (LSD) post-hoc analysis to assess differences between two groups. For all analyses, results are expressed as mean $\pm$ standard error of the mean (SEM), with n = number of animals/group, and statistical significance set at $P = 0.05$.

Results

Serum testosterone levels

As expected, serum T levels were significantly different between T-treated (11.18 ± 0.14 ng/ml) and blank-treated animals (0.00 ± 0.00 ng/ml; $P < 0.001$). The T capsules resulted in slightly higher physiological levels than reported for gonadally intact adult male rats (Roselli et al., 1987). Furthermore, possibly because of the sex difference in body weight between males and females, T-treated males had significantly lower levels of serum T (8.19 ± 0.68 ng/ml) than T-treated females (14.17 ± 0.79 ng/ml). These results, in conjunction with a significant difference in seminal vesicle weight between T-treated (1.62 ± 0.14 g) and blank-treated males (0.18 ± 0.01 g; $P < 0.001$), confirmed that the hormone manipulations were successful.

Regional volume

Effects of hemisphere and sex—MePD volume overall was larger in males than in females (476.14 ± 20.45 in males vs. 353.03 ± 21.34 mm³ $\times 10^{-3}$ in females, $P < 0.001$) and was greater in the right hemisphere than the left for both sexes ($P_s < 0.001$; Fig. 2A). We also found main effects of sex within each hemisphere, with males having a greater MePD volume than females for both ($P_s < 0.005$, Fig. 2C,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—We also confirmed that T-treated rats (grouped across sex) have a larger MePD volume than blank-treated rats (T-treated: 468.93 ± 19.49 , blank-treated: 360.24 ± 24.33 in mm³ $\times 10^{-3}$, $P < 0.001$) and found a significant treatment by hemisphere interaction ($P < 0.05$) indicating a greater effect in the right MePD than the left, leading us to analyze the effect of treatment on MePD volume separately for each hemisphere. Although T treatment significantly affected MePD volume in both hemispheres

($P < 0.01$; Fig. 2B), we found that the effects of T on MePD volume in the left hemisphere depended on sex, as only females displayed a significant difference between T-treated and blank-treated animals ($P < 0.01$; Fig. 2C). T did not significantly affect the volume of the left MePD in males. However, in the right MePD, T treatment maintained larger MePD volume in both sexes ($P < 0.01$ for both; Fig. 2D). The greater MePD volume in T-treated animals, and the greater responsiveness of MePD volume on the right than the left 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 number

Effects of hemisphere and sex—GFAP staining in the amygdala and hypothalamus produced a pattern of GFAP immunoreactivity matching that reported previously (Mong et al., 1999; Martinez et al., 2006; Johnson et al., 2008). We replicated our previous finding that there are more MePD astrocytes overall in the right hemisphere than the left ($8,799.17 \pm 288.10$ in the right vs. $6,379.06 \pm 174.60$ in the left; $P < 0.001$). Post-hoc matched-pairs *t*-tests confirmed that this asymmetry was present in both males and females (Fig. 3A) and, indeed, was evident in all four treatment groups (all $P < 0.005$). We again found that males have more astrocytes in the MePD than females (main effect of sex $P < 0.001$; Fig. 3A), and replicated the significant interaction of hemisphere by sex ($P < 0.001$), reflecting a larger sex difference in astrocyte number in the right hemisphere than the left. However, the number of astrocytes was greater in males than in females in both hemispheres (left: $P < 0.05$, right: $P < 0.001$; Fig. 3). As seen previously (Johnson et al., 2008), MePD astrocyte density was neither sexually dimorphic nor lateralized (Table 1). These sex and hemisphere differences in astrocyte numbers in hormone-manipulated animals essentially replicate our previous report of sex differences in gonadally intact rats (Johnson et al., 2008).

Effects of testosterone treatment—We also found a main effect of adult T treatment on astrocyte numbers, with T-treated animals having more MePD astrocytes overall than blank-treated animals ($16,038.35 \pm 530.67$ vs. $14,318.12 \pm 636.86$, respectively; $P < 0.05$). There was also a significant interaction between treatment and hemisphere ($P < 0.05$) leading us to examine the effects of hormone in the two hemispheres separately. These analyses revealed that while hormone treatment did not affect astrocyte numbers in the left MePD overall, it did on the right, with androgen-treated animals possessing more astrocytes on the right than blank-treated animals ($P < 0.01$; Fig. 3B). Further post-hoc analysis revealed that the effect of T treatment on the number of astrocytes in the right MePD was significant in females ($P < 0.05$) and marginally significant in males ($P = 0.056$, Fig. 3C,D).

Interestingly, whereas T treatment did not significantly influence astrocyte density in males, blank-treated females had greater astrocyte density than T-treated females in the right MePD only ($P < 0.05$; Table 1). This finding suggests that the reduction in right MePD volume in blank-treated females (Fig. 2C) may be greater than the reduction in astrocyte numbers there (Fig. 3D), causing increased astrocyte density in this hemisphere.

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 ($P < 0.05$ for all). That the MePD of males contains more complex astrocytes than females replicates previous results (Johnson et al., 2008). 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 $P < 0.05$). There were no significant interactions between hemisphere and sex, or hemisphere and treatment for any measure of astrocyte complexity.

Effects of testosterone treatment—Collapsing across the sexes, androgen treatment affected all measures of astrocyte complexity (main effects of androgen treatment, all $P < 0.005$) with T-treated rats having more complex astrocyte arbors than blank-treated rats (Fig. 4). However, all measures of astrocyte complexity except average branch length showed significant sex by treatment interactions (all $P < 0.05$) reflecting the fact that T affected astrocyte complexity more in males than in females (Fig. 3). 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 $P < 0.05$; Figs. 4A–C, 5). In contrast, T-treated females differed from blank-treated females only for average branch length, and only in the right hemisphere ($P < 0.05$), where T had no effect on this measure in males (Fig. 4D).

AR colocalization

The medial amygdala is particularly rich in AR+ nuclei (Greco et al., 1998; McAbee and DonCarlos, 1998; Romeo et al., 2000) but whether such nuclei belong to neurons or glia was unknown. As expected, we observed numerous AR+ nuclei in the MePD and elsewhere and many were large and round, with distinct nucleoli, presumably belonging to neurons. Moreover, in agreement with earlier reports, AR+ astrocytes were observed in areas where they had been previously documented, such as the arcuate nucleus (Lorenz et al., 2005; Fig. 6A) and hippocampus (Hösli et al., 2001; Tabori et al., 2005; Fig. 6B). In addition, we found distinct AR immunoreactivity in the nuclei of astrocytes in the MePD (Fig. 7A), not previously reported. 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. 7B). Our estimates suggest that $\approx 50\%$ of astrocytes in the MePD of gonadally intact adult male rats 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).

Discussion

The MePD is sexually dimorphic and sensitive to gonadal hormones (Gomez and Newman 1991; Cooke et al., 1999, 2003; Morris et al., 2005, 2008a). Some sex differences in MePD neuroanatomy, such as regional volume and neuronal soma size depend on circulating

testicular hormones in adulthood (Cooke et al., 1999, 2007). Whereas other sex differences, such as neuron number, appear independent of hormone manipulations in adulthood (Morris et al., 2008a). These data suggest that adult hormones underlie some sex differences in the MePD, whereas hormones earlier in development may determine others.

We recently demonstrated that adult MePD astrocytes are sexually dimorphic in both number and complexity and that these sex differences depend on functional AR (Johnson et al., 2008). However, whether these sex differences in astrocyte morphology in the MePD are determined by androgens during early development, puberty, and/or adulthood was an open question. We now report that the number of MePD astrocytes and their complexity are sensitive to androgens in adult males and females. We also find that many MePD astrocytes express AR, suggesting that androgens may act directly upon adult MePD astrocytes, in a cell autonomous fashion, to influence their morphology.

Influence of gonadal hormones on astrocyte number and complexity

Our current data are the first to demonstrate that MePD astrocytes in adult rats respond to circulating androgens. The most robust responses to T treatment in males were in the complexity of the astrocyte arbor, in both the left and right MePD. In females, by contrast, T treatment had little effect on astrocytic processes. For the *number* of MePD astrocytes, the pattern is reversed, as T treatment had no significant effect in males, but did affect astrocyte numbers in females, at least in the right hemisphere. Overall, adult androgens seem to exert different influences on MePD astrocytes in male and female rats. The hormones appear to affect astrocyte complexity, but not numbers, in males, while exerting a subtle effect on astrocyte numbers in females, with little effect on astrocyte complexity. These sex differences in astrocyte responsiveness could be due to a number of factors including sex differences in steroid hormone receptor expression (McAbee and DonCarlos, 1998; Xiao and Jordan, 2002), differences in hormonal history (von Ziegler and Lichtensteiger, 1992) or possibly differences in steroid hormone coregulator expression and/or activity (Auger et al., 2002).

The functional significance of testosterone-dependent changes in astrocyte number and complexity in the adult MePD are not presently clear. Given the link between number of astrocytic processes and synapse density (Pfrieger and Barres, 1997; Mong et al., 2001; Elmariah et al., 2005), our results suggest that the neural network in the amygdala may be changing in response to circulating hormones in adults. Computer simulations in artificial neuron–neuron and neuron–glia networks suggest that the presence and number of astrocytes improves network performance, particularly as the network increases in complexity (Porto-Pazos et al., 2011). However, astrocytic complexity in vivo has been associated with both *increases* in synapse number (Pfrieger and Barres, 1997; Elmariah et al., 2005) and *decreases* in dendritic spines and axodendritic synapses (Mong et al., 2001), making it unclear whether the changes we see in astrocytes would be associated with increases or decreases in synaptic connectivity in the MePD. Given that the size of somas and dendritic arbors are correlated in androgen-responsive motoneurons (Breedlove and Arnold 1981; Kurz et al., 1986), and that adult androgens increase neuronal soma size in the MePD (Cooke et al., 2003), the androgen-induced increase in MePD astrocyte number and

complexity may be associated with increased synaptic connectivity, possibly enhancing network function. This speculation will need to be tested.

An expanding body of research indicates that astrocytes are not the passive cells as once imagined and have been linked to the release of gonadotropin releasing hormone (Witkin et al., 1997) and formation of synapses (Ullian et al., 2001; Hatton, 2002; Barker and Ullian, 2008). Moreover, in both the hippocampus (Garcia-Segura et al., 1988; Day et al., 1990, 1993; Leranth et al., 2008) and hypothalamus (McQueen et al., 1990; Chowen et al., 1995; Mong, 1996; Amateau and McCarthy, 2002), astrocytes are sexually dimorphic and responsive to gonadal hormone manipulations. Rapid hormone-induced extension and retraction of astrocytic processes in the hypothalamus and hippocampus are associated with changes in synaptic communication in a manner that promotes or inhibits release of neuropeptides in the hypothalamus, and affects the number of dendritic spines in the hippocampus (Azcoitia et al., 2010). Our results further illustrate the dynamic nature of astrocytes and add to a growing list of brain regions where hormones can induce changes in these cells which likely affects the function of neurons and their connections.

As far as we are aware, changes in astrocyte numbers in the adult amygdala caused by changes in adult hormone levels has only been reported once previously. Featherstone et al. (2000) found reduced numbers of GFAP+ cells in the medial amygdala of pup-exposed multiparous postpartum females compared with pup-exposed primiparous females. The authors suggest that this difference may be due to greater estrogen exposure in the multiparous dams. More recently, optical density measures showed that GFAP immunoreactivity in the MePD is greatest during the proestrus phase and is increased by estradiol or estradiol plus progesterone in ovariectomized females (Martinez et al., 2006). While consistent with our current observations, it is not clear whether such increases in GFAP expression reflect increases in astrocyte number and/or their complexity. However, our data indicating that the aromatizable androgen T primarily increases astrocyte numbers in females suggest that such estrogen-induced increases in GFAP may well reflect changes in number.

Gliogenesis and survival in the amygdala

While it is clear that androgens in adulthood can affect the number of MePD astrocytes in female rats, it is not clear what cellular mechanism androgens regulate to control their number. Androgens affect both the proliferation and survival of astrocytes in vitro. For example, synthetic androgens increase proliferation of astrocytes derived from human optic nerve head (Agapova et al., 2006), and dihydrotestosterone (DHT) protects primary cortical astrocytes from cell death due to metabolic and oxidative insults (Gatson and Singh, 2007). In the rodent amygdala in particular, evidence suggests that gonadal hormones can influence glial proliferation during early development and puberty (Dmitar et al., 1995; Dreki et al., 1995; Ahmed et al., 2008). During puberty, cells proliferate in the rat medial amygdala, with more new cells added in males than in females; many of these newly generated cells are astrocytes. Gonadectomy prior to puberty eliminated 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 males by increasing

their proliferation. However, it is also possible that astrocyte survival is affected and that fewer of the newly produced astrocytes survive without gonadal hormones (Ahmed et al., 2008). Both estrogens (Sales et al., 2010) and androgens (Ahlbom et al., 2001; Nguyen and Jayaraman, 2010) are capable of preventing cell death in the brain and in vitro (reviewed in Forger, 2006; Gatson and Singh 2007). Thus, it is currently unclear if gonadal hormones promote MePD astrocyte proliferation only or a combination of proliferation and cell survival.

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; Nunez et al., 2003; Lorenz et al., 2005; Tabori et al., 2005; Sarkey et al., 2008). While the MePD is particularly rich in hormone receptors, including AR (Roselli, 1991; Wood and Newman 1995; Shughrue and Merchenthaler, 2001), expression of AR by astrocytes in the MePD has not been previously demonstrated. Several aspects of MePD neuroanatomy are dependent on circulating androgens and/or functional ARs for complete masculinization, including masculinization of astrocytes (Cooke et al., 2003; Morris et al., 2005; Johnson et al., 2008). We now find that a large fraction of MePD astrocytes in adult male rats express ARs, opening the possibility that ARs in the astrocytes themselves mediate the effects of androgens on these cells and possibly on neurons as well. On the other hand, it is also interesting that the astrocyte population in the MePD appears to be heterogeneous with respect to AR expression with only about 50% of them expressing AR. Thus, astrocytes are not only heterogeneous across brain regions in both structure and function, but appear to be heterogeneous locally within the MePD. Whether MePD astrocytes in adult female rats show the same level of AR expression has not yet been determined. Differences in AR expression may help to explain the differences in MePD astrocyte response to androgens in adult males and females.

In vitro, both direct effects of gonadal hormones on astrocytes and indirect effects requiring the presence of neurons have been demonstrated (Torres-Aleman et al., 1992; Melcangi et al., 1996; Stone et al., 1998). In vivo work also demonstrates indirect effects of hormones on astrocytes. For example, in the developing hypothalamus, estradiol acts on ER+ neurons, which then influence the surrounding astrocytes through GABA signaling (Mong et al., 2002), and localized intraparenchymal infusions of estradiol decrease GFAP expression in afferent target regions (reviewed in Azcoitia et al., 2010). A similar scenario in which androgens act via neuronal ARs to influence local astrocytes may occur in the MePD. Nonetheless, the substantial number of AR-expressing astrocytes in the MePD opens the possibility that androgens also act via local astrocytes to influence the structure and function of the MePD.

Because testosterone, an AR agonist, can be converted to estradiol, an ER agonist, the astrocytic response to T we observed may be due to activation of AR, ER, or both. We did not use a specific AR or ER agonist for several reasons. First, although long thought of as solely an AR agonist, DHT can be metabolized to 3-beta-diol, which activates ER- β (Handa et al., 2008), eliminating DHT as a pure AR agonist option. Second, in some brain regions,

including the amygdala, estrogen is required to upregulate expression of AR (Handa et al., 1987; McAbee and DonCarlos, 1999), so that DHT alone may not reveal effects seen when androgens and estrogens act together. Finally, because we have previously demonstrated that a lack of functional ARs in Tfm male rats results in feminized MePD astrocyte numbers and arbor complexity (Johnson et al., 2008), we know that ARs are required for T to modify astrocytes. However, this does rule out the possibility that ER activation also affects MePD astrocytes when functional ARs are present. Therefore, we used T treatment in case such supplemental influences of ER stimulation normally affect these measures.

Both ARs and ERs contribute to masculinization of MePD regional volume, average neuronal soma size, dendrite complexity, and sexual behaviors for which the MePD is implicated (Gomez and Newman 1991; Cooke et al., 2003; Morris et al., 2008b). In addition, ERs are responsible for inducing changes in astrocyte arbor complexity in the hypothalamus (Mong et al., 1999). Thus, it is possible that ERs may also mediate some of the astrocytic changes seen in the current set of hormone-manipulated animals. ERs are expressed in astrocytes in many regions (Langub and Watson, 1992; Garcia-Segura et al., 2008; Mitterling et al., 2010), raising the possibility that astrocytes in the MePD may also express ER. Given that ARs and ERs colocalize in medial amygdala neurons (Wood and Newman, 1995), it is possible that ARs and ERs also colocalize in MePD astrocytes, allowing direct integration of hormonal inputs in these cells. Further experiments are needed to evaluate these possibilities.

Hormones and lateralization of the amygdala

The lateralized influence of gonadal hormones on MePD astrocyte number and morphology seen here and the lateralized sex differences in MePD astrocytes seen previously (Johnson et al., 2008) are in concert with studies showing that lateralization of the amygdala and other regions (e.g., Xiao and Jordan, 2002) is sexually dimorphic and developmentally regulated, resulting in complex interactions between age, sex, and hemisphere. Lateralization in the amygdala is common in humans. Both functional connectivity and amygdalae activation to faces are sexually dimorphic and lateralized (Kilpatrick et al., 2006; Schnieder et al., 2011). Laterality is also common in the rodent amygdala, where developmental aromatase expression and amygdalae responses to fear conditioning are lateralized (von Ziegler and Lichtensteiger 1992; Baker, 2004).

The lateralized sex difference in astrocyte numbers found previously (Johnson et al., 2008) persisted in the current experiments. Moreover, we found that the effect of androgens on the number of MePD astrocytes is most pronounced in the right MePD, consistent with our prior estimates of total glia number based on Nissl staining (Morris et al., 2008a). On the other hand, astrocyte complexity was affected by androgens to a greater extent in males than in females, and the effects were largely symmetrical in males, increasing most measures of astrocyte complexity in both hemispheres, while asymmetric in females. While this might indicate a uniform strengthening of neural connectivity in both hemispheres in males, an additional intriguing possibility is that hormone-induced changes in astrocyte arbor complexity act to silence one hemisphere and strengthen the other, given that increases in astrocyte complexity are associated with both increases and decreases in synaptic

connectivity, depending on the brain region (Pfrieger and Barres, 1997; Mong et al., 2001; Elmariah et al., 2005). In human males, the right amygdala is dominant in several functional measures including emotional memory, response to fearful faces, and resting state functional connectivity (Cahill et al., 2001; Kilpatrick et al., 2006; Schneider et al., 2010). Perhaps the lateralized response of amygdalar astrocytes to gonadal hormones contributes to or reflects the lateralized function of this region.

Conclusions and Additional Considerations

The present findings provide new information about the cellular organization of the amygdala. Regional volume and neuronal soma size in the MePD are responsive to hormones in adult rats, and it is now clear that the number and complexity of astrocytes are also hormone-sensitive components of this highly plastic brain region. Future studies will be aimed toward understanding the mechanisms by which gonadal hormones control the number of MePD astrocytes and the growth and/or regression of their processes. A final intriguing possibility is that the specific effects of hormones on astrocytes in the MePD depend on the developmental stage, as is true for other sex differences in the amygdala (Nishizuka and Arai, 1981). While the current results make it clear that MePD astrocytes respond to androgens in adulthood, differences in adult androgens do not explain the sex differences in astrocyte morphology in the MePD, given that sex differences in astrocyte number and complexity persist despite comparable T treatment in adult male and female rats. These data suggest that androgens also play a role earlier in development, influencing adult characteristics of MePD astrocytes, including both their number and complexity. The question of whether and how perinatal and/or pubertal androgens contribute to establishing adult sex differences in MePD astrocytes awaits future studies.

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Figure 1.

Representative photomicrographs of coronal sections through the adult MePD of a genetic male mouse with the testicular feminized mutation (Tfm) of the androgen receptor (AR) gene (**A**) and a normal wildtype male mouse (**B**) immunostained for androgen receptor (AR; Epitomics #1852-1 rabbit monoclonal anti-AR antiserum). Note the distinct and robust nuclear labeling (arrows) in the MePD of the normal adult male that is absent in the MePD of the Tfm male. Scale bar = 100 μ m.

Figure 2.

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 ($\pm$ SEM).

Figure 3.

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:** Testosterone (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 do females. **(C).** Males also have more astrocytes than females in the right MePD, where T treatment appears to result in more astrocytes in both sexes **(D)** (the difference in males is marginally significant, $P = 0.056$). Values are means of $n = 9$ rats/group ($\pm$ SEM). Note that astrocyte numbers in this figure closely mirror the differences in MePD volume in Figure 1, indicating that the density of astrocytes per unit volume is maintained when androgens affect volume in the right MePD.

Figure 4.

Astrocyte complexity is increased by androgens in adult male rats. 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 greater with 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 only in the right hemisphere. Values are means of $n = 5$ rats/group, ($\pm$ SEM).

Figure 5.

Photomicrograph depicting a relatively simple astrocyte from a male rat gonadectomized for 30 days (**A**) and two relatively large and complex astrocytes from a male rat gonadectomized and treated with testosterone for 30 days (**B**). Testosteronetreated males generally had more complex astrocytes compared with blank-treated males, but simple and complex astrocytes could be found in the MePD of both treatment groups. Scale bar = 10 μm .

Figure 6. Confocal image showing nuclear AR immunoreactivity (magenta) within astrocytes of the arcuate nucleus (**A**) and CA1 hippocampus (**B**). Arrows point to AR+ immunoreactivity in nuclei of GFAP expressing cells (green). Scale bars = 10 μ m.

Figure 7.

Confocal images showing androgen receptor (AR) immunoreactivity (red) within nuclei of MePD astrocytes. In (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 (B), an MePD 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 μ m.

Table 1
Astrocyte Density in the MePD of Testosterone and Blank Treated Animals

	Astrocytes per $\mu\text{m}^3 \times 10^5 \pm \text{SE of the mean}$	
	Left hemisphere density	Right hemisphere density
Males+T	3.33 $\pm$ 0.24	3.31 $\pm$ 0.09
Males+B	3.81 $\pm$ 0.24	3.83 $\pm$ 0.21
Females+T	3.83 $\pm$ 0.22	3.55 $\pm$ 0.20 *
Females+B	8.73 $\pm$ 4.53	4.06 $\pm$ 0.09 *

* Significant difference between females treated with blank capsules (B) and with testosterone (T).