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Androgen Receptors Mediate Masculinization of Astrocytes in the Rat Posterodorsal Medial Amygdala During Puberty

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Abstract

Astrocytes in the posterodorsal portion of the medial amygdala (MePD) are sexually dimorphic in adult rats: males have more astrocytes in the right MePD and more elaborate processes in the left MePD than do females. Functional androgen receptors (ARs) are required for masculinization of MePD astrocytes, as these measures are demasculinized in adult genetic males carrying the testicular feminization mutation (Tfm) of the *AR* gene, which renders AR dysfunctional. We now report that the number of astrocytes is already sexually dimorphic in the right MePD of juvenile 25-day old (P25) rats. Because Tfm males have as many astrocytes as wildtype males at this age, this prepubertal sexual dimorphism is independent of ARs. After P25, astrocyte number increases in the MePD of all groups, but activation of ARs augments this increase in the right MePD, where more astrocytes are added in males than in Tfm males. Consequently, by adulthood, females and Tfm males have equivalent numbers of astrocytes in the right MePD. Sexual dimorphism in astrocyte arbor complexity in the left MePD arises after P25, and is entirely AR-dependent. Thus, masculinization of MePD astrocytes is a result of both AR-independent processes before the juvenile period and AR-dependent processes afterward.

Keywords

androgen; amygdala; astrocytes; glia; hormone; puberty

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, in several species the developmental trajectory of the amygdala is influenced by gonadal hormones that can act both early in life and during puberty (Ahmed et al. 2008; Merke et al. 2003; Rubinow and Juraska 2009; Nishizuka and Arai 1981; 1982).

The mammalian amygdala consists of multiple subregions including the medial posterodorsal amygdala (MePD), which is highly sexually dimorphic. MePD volume in rats

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is greater in males than females, and the male MePD contains more and larger neurons (Cooke et al. 1999; 2003). Astrocytes in the adult rat MePD are also markedly sexually dimorphic, differing in both number and process complexity (Johnson et al. 2008). Furthermore, in male rats insensitive to androgens due to the testicular feminization mutation (Tfm) of the androgen receptor (AR) gene, both the number and complexity of astrocytes are feminized in adulthood, demonstrating that masculinization of MePD astrocytes depends on ARs (Johnson et al. 2008). However, when during development ARs initiate the masculinization of MePD astrocytes is not known.

Some morphological sex differences in the adult MePD of rats depend on circulating gonadal hormones in adulthood. For example, when adult males are castrated, overall MePD volume decreases, as does the size of individual neurons and complexity of the astrocyte arbor (Cooke et al. 1999; Johnson et al. 2012). Sex differences in other cellular features however, such as neuronal number, do not appear to depend on adult androgens, raising questions about the role of gonadal androgens at earlier stages in establishing a fully masculine MePD.

While 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), little is known about astrocytes in the juvenile MePD. Because astrocytes play a critical role in modulating the formation, maintenance and efficacy of synapses (Halassa et al. 2007; Ullian et al. 2001; Nishida and Okabe 2007; Hatton 2002), determining when sex differences in astrocyte morphology arise in the MePD is important for understanding the development of this region. To address this issue, we counted and traced astrocytes in the MePD of juvenile male, female, and Tfm male rats using methods we previously applied to the adult MePD (Johnson et al. 2008).

Our results demonstrate that while astrocyte numbers are sexually dimorphic *prior* to puberty, astrocyte arbor complexity is not. However, both the number and complexity of prepubertal MePD astrocytes are well below that seen in adults (Johnson et al. 2008), indicating that both astrocyte number and process complexity increase dramatically during puberty, with sex differences in astrocyte complexity emerging sometime during the pubertal-to-adult transition. Comparisons with Tfm males indicate that functional ARs are not necessary for masculinizing MePD astrocyte number prepubertally, but that AR stimulation after puberty is critical, both for maintaining a sex difference in astrocyte number and for promoting sexually differentiated growth of astrocyte processes. Finally, we continue to see robust lateralization in MePD morphology with juvenile sex differences in astrocyte number only in the right MePD.

Methods

Animals

Twenty-five-day-old (P25) 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 as described elsewhere (Fernandez et al. 2003). 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, rats 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 (ICC).

Histology

Immunocytochemistry for Visualizing Astrocytes—Using methods previously described (Johnson et al., 2008), sections were transferred from cryoprotectant to Netwell plates (Corning Life Sciences) and thoroughly rinsed in 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, Billerica, MA; Neuroscience Information Framework antibody ID: 94939) with biotin (Avidin biotin blocking kit, Vector Laboratories). Tissue was then incubated overnight at 4°C in the same 1:6000 primary antiserum. 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 approximately 51 kDa (per manufacturer's technical information), consistent with the known molecular weight of rat GFAP.

The next day, tissue was rinsed and incubated in biotinylated rat-adsorbed horse anti-mouse secondary antiserum (1:500, Vector Laboratories) for 1 hr at room temperature, followed by 1 hr incubation in peroxidase avidin-biotin complex solution at half the concentration recommended by the vendor (Elite ABC kit, Vector). Horseradish peroxidase was visualized using an Impact 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 before counter-staining with Harris hematoxylin solution (Sigma) to visualize cell nuclei, using 10% lithium carbonate as the bluing agent. Sections were then dehydrated, cleared and cover-slipped.

Astrocytes were stained using the above protocol to estimate their number in the MePD of juvenile brains. However, to optimize visualization of their arbors, we modified the staining protocol by decreasing the concentration of the primary antiserum to 1:10,000 and the DAB reaction time to 2.5 min to reduce background staining. The remaining alternate set of sections from the same brains was stained using this modified protocol and used for measuring astrocytes processes.

Stereological Analysis—The approach used for stereological analysis of astrocytes in the juvenile MePD was based on our previous study in adult rats (Johnson et al. 2008; Johnson et al. 2012). 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; Johnson et al. 2012) and others (Hines et al. 1992). After tracing the boundaries of the MePD, astrocytes were counted and traced using a 100× 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—As in our previous studies (Johnson et al. 2008), an optical fractionator probe (West 1993) was used to generate an unbiased estimate of astrocyte numbers within the MePD, with adjustments in probe and sampling frame size necessitated by the smaller MePD volume. Probe dimensions were 37×37 μm with a Z-depth of 12 μm and a 1 μm guard zone. Probe spacing was on a 115×115 μm grid. The coefficient of error (CE; Gundersen $m=1$) for each hemisphere was at or below 0.10.

As before (Johnson et al. 2008; Johnson et al. 2012), criteria for identifying an astrocyte included a distinct and recognizable nucleus in a plane of focus within the sampling probe and had at least 2 GFAP labeled fibers extending from it (Fig 1). 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 per animal.

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 randomly selected using a fractionator probe. 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 2). The average number of primary processes, average number of branch points, average number of branch endings, and average branch length per astrocyte were calculated based on these astrocyte traces for each hemisphere of each animal. While GFAP-labeling may not reveal the entire extent of astrocyte processes (Bushong et al., 2002), our measures of arbor complexity are nevertheless informative about when during ontogeny adult sex differences in MePD astrocytes emerge (based on the same methodology, Johnson et al., 2008) and the role of ARs in this process.

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 $p < 0.05$ as statistically significant.

Results

Regional Volume

A mixed design 2-way ANOVA (left and right hemisphere as repeated measures) revealed a main effect of genotype on prepubertal MePD volume ($p < 0.001$, Fig 3A), a main effect of hemisphere ($p < 0.001$) and a genotype by hemisphere interaction ($p < 0.05$). Post-hoc

analysis revealed that wt males have a greater MePD volume than females ($p < 0.05$) and Tfm males have a greater MePD volume than either wt males ($p < 0.05$) or females ($p < 0.001$, Fig 3A).

Given the significant interaction of genotype and hemisphere, we then conducted separate one-way analyses of variance for each hemisphere and found a main effect of genotype in the left hemisphere ($p < 0.05$) with Tfm males having a greater MePD volume than females ($p < 0.01$, Fig 3B), but wt males were not significantly different from either group. 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 p s < 0.01 , Fig 3B). Thus at P25, 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 juvenile Tfm male rats. Comparing the volume of right MePD versus left revealed that the right MePD is larger than the left in both wt and Tfm males (p s < 0.005) with no significant laterality of MePD volume in females (see Figure 3B), thus indicating that lateralization in prepubertal MePD volume is a male trait established independent of AR.

Astrocyte Numbers

We find a significant main effect of genotype on the number of astrocytes with males having more astrocytes than females, and Tfm males being no different from wt males (p s < 0.05 , Fig 3C). Similar to previous reports in adults (Johnson et al. 2008), there was also a main effect of hemisphere ($p < 0.001$) with more astrocytes in the right MePD than the left in all groups (p s < 0.05 , Fig 3D). The complex pattern of laterality previously seen in adult animals prompted separate analysis of the two hemispheres. In the right MePD, there was a significant effect of genotype on astrocyte numbers ($p < 0.05$, Fig 3D). Post-hoc analyses indicated that wt males have significantly more astrocytes than females (p s < 0.05 , Fig 3D), while Tfm males have as many astrocytes as wt males on this side at this age. While there was no main effect of genotype on the left MePD, post-hoc analyses revealed a sex difference on this side, with males having more astrocytes than females ($p < 0.05$, Fig 3D). The number of astrocytes in the left MePD of Tfm males is intermediate between wt males and females, and not significantly different from either. Thus, MePD astrocytes at P25 are sexually dimorphic in number on both sides, with wt males having significantly more astrocytes than females. The number of astrocytes in the right MePD of Tfm males is equivalent to wt males, indicating that at least on this side, the sex difference in astrocyte number in juveniles does not require functional AR.

Astrocyte Complexity

Astrocyte complexity was assessed based on measures previously used for adult MePD astrocytes (Johnson et al. 2008, 2012). However, unlike our findings in adult MePD astrocytes, we find no main effects of genotype or interactions of genotype with hemisphere on any measure of astrocyte complexity in 25 day-old rats (Fig 4). Thus, neither the sex difference nor the laterality of astrocyte arbors evident in adult rats (Johnson et al. 2008) were present prepubertally. Moreover, we find that the complexity and length of astrocytic processes in the MePD of juvenile males is well below those for adult males (Johnson et al. 2008), 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 their adult morphology in a sex- and hemisphere-specific manner.

Discussion

Astrocytes are sexually dimorphic in both complexity and number in the MePD of adult rats (Johnson et al. 2008), prompting our inquiry into when these sex differences emerge.

Because circulating androgens in adulthood do not appear to fully explain these dimorphisms in adult MePD astrocytes (Johnson et al. 2012), we examined MePD astrocytes in developing male, female and Tfm male rats at 25 days of age. We find that the sex difference in astrocyte number is present by P25, but unexpectedly, and in contrast to our findings in adults, does not depend on functional ARs at this prepubertal stage. In contrast, astrocyte arbor complexity in the MePD at P25 is neither sexually dimorphic, nor affected by a dysfunctional AR. These findings indicate that gonadal hormones act via ARs during the pubertal-to-adult transition to 1) maintain a sex difference in astrocyte number that is established prepubertally in the right MePD independent of AR, and 2) promote the sexually differentiated growth of astrocyte processes in the left MePD.

Sexual differentiation of MePD volume and astrocytes prepubertally

The current findings represent the first description of MePD neuroanatomy in the juvenile TFM-male and raise interesting questions about the mechanisms by which adult sex differences in MePD volume emerge. Previous reports indicate that the adult sex difference in MePD volume favoring males is established prior to puberty (Nishizuka and Arai 198; Mizukami et al. 1983; Cooke, Stokas and Woolley 2007). Moreover, adult TFM males exhibit a partially-masculinized phenotype for some characteristics including MePD volume, presumably reflecting a shared role of AR and ER in masculinizing the amygdala (Morris et al. 2005; Cooke, Breedlove and Jordan 2003). We now find that MePD volume is larger than the wild-type male size in prepubertal TFM males, especially in the right hemisphere. While we are not aware of any reports detailing the level of circulating gonadal hormones in *juvenile* TFMs, in adults, circulating androgens are in the high-male range while aromatase activity in the medial amygdala is normal (Rosselli et al. 1987). Thus, perhaps the enlarged MePD in juvenile TFM males reflects the conversion of excess circulating androgens to estrogens, which then act on ER to push growth of the MePD beyond what is typical of wt males. This possibility is supported by the finding that estradiol can maintain MePD volume in castrated adult males, especially in the right hemisphere (Cooke, Breedlove and Jordan 2003).

The current findings also demonstrate that sexual dimorphic arbor complexity of adult MePD astrocytes is not present prepubertally, likely reflecting the significant growth of processes that has not yet happened in wt males (Fig 5). Because detection of GFAP is known to underestimate actual arbor length (Bushong et al. 2002), there may be sexual dimorphisms in juvenile MePD astrocytes not captured by the current analysis. However, the current data accurately describe relative differences in astrocyte arbors using techniques and subject numbers which previously identified robust sex differences in adult MePD astrocytes (Johnson et al. 2008; 2012).

The sexually monomorphic pattern of astrocyte arbors in the MePD contrasts with observations in the arcuate nucleus and pre-optic regions of the hypothalamus, where astrocyte arbors are already sexually dimorphic in neonatal rats due to estrogen action (Mong et al. 1996; McCarthy et al. 2002). This regional variation is not surprising given that even within the hypothalamus astrocyte differentiation is region specific (Mong, Glasser and McCarthy, 1999) and maturation rates are variable across the brain (Toga et al. 2006; Neufang et al. 2009).

We suspect that MePD astrocytes may not receive or be responsive to the early estrogen signals that masculinize astrocyte arbors in the arcuate nucleus and pre-optic area. Differences in aromatase activity, which would regulate the amount of androgen and estrogen activity at key developmental time points may play a role in the rate of maturation of astrocytes in different brain regions (Roselli et al. 1985; von Ziegler and Lichtensteiger 1992).

In contrast to the development of MePD astrocyte arbors, the sex difference in astrocyte number seen in the right MePD of adults *is* present in juvenile animals, with males having more astrocytes than females (Fig 3A, B). Interestingly, the number of astrocytes at P25 in Tfm males is masculinized, demonstrating that this prepubertal sex difference in astrocyte number does not depend on functional ARs. These data raise the question of whether testicular androgens in males act via estrogen receptors prepubertally act to boost astrocyte numbers. Alternatively, the prepubertal sex difference in MePD astrocyte number could be achieved through a mechanism that is independent of circulating gonadal hormones (see Arnold 2012).

Unfortunately, there is scant information about the mechanisms by which sex differences in astrocyte number develop. However, one study reported a sex difference in the proliferation of astrocytes in the neonatal medial amygdala. Specifically, female rats had more bromodeoxyuridine (BrDU)-labeled astrocytes than males at P4 after exposure to BrDU from P1 – P4 (Krebs-Kraft et al. 2010). The implication that proliferation of medial amygdala astrocytes may be greater in females than in males before P4 (Krebs-Kraft et al. 2010) contrasts with our current findings of a greater number of MePD astrocytes in males at P25. Three possible explanations could reconcile these results. First, the sex difference at P25 may be specific to the MePD and not be representative of the medial amygdala overall. Thus, more astrocytes may accumulate in males than in females in the MePD due to a male bias in proliferation, whereas in the surrounding regions, the sex difference may be reversed (Krebs-Kraft et al. 2010). Second, a female bias in astrocyte proliferation up to P4 may indeed occur in the MePD, but after P4, the rate of proliferation in the MePD of males may overtake that of females, resulting in the MePD of P25 males containing more astrocytes than P25 females. Finally, the male biased sex difference in astrocyte number in the MePD at P25 may not actually reflect sex differences in the rate of their proliferation, but rather in their survival. Thus, sex differences in the number of astrocytes at P25 could arise because of some trophic influence which keeps more astrocytes alive in males compared to females. One likely candidate is estrogen, a known regulator of cell survival in the brain (Tsukahara et al. 2008).

Sexual differentiation of MePD astrocytes after the prepubertal period

Comparing current results to previous findings based on rats from the same colony using the same methodologies, we find substantial increases in the number and size of astrocytes in the MePD from pubertal onset to adulthood (Fig 5). While the number of astrocytes increases between P25 and P110 in both hemispheres, this increase is much larger on the right, reflecting a near doubling in the number of astrocytes on this side (Fig 5B). Moreover, numbers increase significantly in all three genotypes (wt males, Tfm males and females), indicating that increases in the number of astrocytes during the pubertal-to-adult transition are largely independent of testicular hormones or AR. Interestingly, this increase in astrocyte number is accompanied by an increase in MePD regional volume as well (Fig 5A) so that the density of astrocytes in the MePD does not change appreciably from the peripubertal stage of development to adulthood (Table 1).

Because Tfm males, with dysfunctional ARs, have a masculine number of astrocytes at P25 but a feminine number by adulthood (Fig 5B), the apparent role of ARs during puberty is to maintain a sex difference in astrocyte number that gets established prepubertally. Hence, either the production of new astrocytes and/or their survival becomes partially dependent on AR once puberty begins. The idea that androgens act via ARs to maintain the number of MePD astrocytes is consistent with our finding that castration of adult male rats leads to a decrease in the number of astrocytes in the right MePD (Johnson et al. 2012). Ahmed and colleagues (2008) also found a hormone-dependent increase in the number of cells produced during early puberty in the medial amygdala of rats, consistent with the idea that androgens

act via ARs to enhance either astrocyte production and/or survival during puberty in the MePD.

Unlike astrocyte number, which is sexually dimorphic before the onset of puberty, we see no sex differences in any of our measures of astrocyte complexity at P25. Thus, sometime between P25 and P110, MePD astrocytes grow extensive processes, but only in wt males, and only in the left hemisphere, engendering the marked sex difference and asymmetry in the number of branch endings that is evident by adulthood (Fig 5C). A similar pattern is also seen for the other measures of astrocyte arbor complexity (average number of branch nodes per astrocyte and average number of primary branches per astrocyte; data not shown).

This marked increase in arbor complexity in the left MePD of wt males is not seen in TFM males, demonstrating that the sexually dimorphic growth of astrocyte arbors is normally AR-mediated in males (Johnson et al. 2008). In the right MePD, some astrocytes seem to have attained mature arbors by P25 in all three genotypes, as arbor complexity on this side is comparable in juveniles and adults (Johnson et al. 2008; Fig 5C). Achieving apparently adult arbors in the face of marked increases in their number during this same developmental period indicates that the mechanisms controlling the number of astrocytes versus growth of their processes during puberty are likely to be different, and that ARs influence these mechanisms differently on each side. 1) In the right hemisphere only, a *non-AR-mediated process* organizes a sex difference in astrocyte number in the MePD by P25, and then an *AR-dependent process* maintains this sex difference in the face of a general increase in astrocyte numbers in both sexes. 2) In the left hemisphere only, an AR-mediated process underlies the growth and elaboration of astrocyte arbors between P25 and maturity. Thus, both the number and complexity of MePD astrocytes undergo sexually dimorphic changes during the transition from juvenile to adulthood, which may be triggered by circulating testosterone. That both optical density of GFAP-immunoreactivity (Martinez et al. 2006) and complexity of astrocyte arbors decrease in the adult MePD of castrated animals (Johnson et al. 2012) confirms that MePD astrocytes are sensitive to circulating androgens. Whether this is also true during pubertal development has yet to be established.

The functional significance of changes in the number and complexity of astrocytes in the MePD during puberty is currently unclear. However, evidence suggests that masculinization of the medial amygdala by androgens during puberty facilitates the development and function of an olfactory-amygdala-hypothalamus pathway necessary for adult mating behaviors in males (Schulz et al. 2009; Zehr et al. 2006; Wood and Coolen 1997; Coolen and Wood 1999). In the hamster medial amygdala, puberty is a period of synaptic reorganization, including reduction in the number primary dendrites and density of terminal spines, which is accompanied by increases in the expression of synaptophysin and markers of excitatory synapses, namely glutamate transporter-2 and post-synaptic density 95 (Zehr et al. 2006; Cooke 2011). Perhaps the changes in male MePD astrocyte arbors during puberty enhance the formation and/or strength of synapses in the medial amygdala, two well established functions of astrocytes in other systems (Elmariash et al. 2005; Nishida and Okabe 2007; reviewed in Allen and Barres, 2005; see also Theodosis et al. 2006).

It is also possible that increases in astrocyte number have consequences for neuronal number. In the bird song system, sex differences in gliogenesis precede sex differences in neuron number, suggesting glia may control the number of developing neurons in this system by rescuing neurons from cell death (Nordeen and Nordeen 1996). The increase in astrocyte numbers in the MePD during puberty may well play a similar role, but stereological counts of MePD neurons during this stage are needed before such connections can be drawn between astrocytes and neurons in the MePD.

Continued investigation may reveal whether changes in MePD astrocytes lead to alterations in the function of this brain region and may also shed light on how basic cellular processes underlying the normal function and structure of the medial amygdala might go awry. Abnormal amygdala growth and/or function are present in several disorders including autism (Nordahl et al. 2012), schizophrenia (Pinkham et al. 2011) and depression (Hamidi et al. 2004). Further investigation of both human and rodent models is needed to understand how gonadal hormones influence the cellular anatomy of the amygdala during critical periods in development to determine its function.

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Role of Authors All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: RTJ, CLJ, SMB. Acquisition of data: RTJ. Statistical analysis: RTJ. Drafting of the manuscript: RTJ. Critical revision of the manuscript for important intellectual content: RTJ, CLJ, SMB. Obtained funding: RTJ, CLJ, SMB. Administrative, technical, and material support: CLJ, SMB.

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Figure 1.

Astrocytes in the MePD of prepubertal (25 day old) male rats have arbors that range in complexity from relatively simple (A) to relatively complex (B). Astrocytes (arrows) were identified based on GFAP immunoreactivity and hematoxylin staining of their nuclei. An arrowhead identifies a blood vessel. Scale bar = 10 microns.

Figure 2.

Photomicrograph depicting a complex astrocyte in the juvenile rat 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 any one time in the photomicrograph. Complex astrocytes frequently demonstrated multiple branches that emerged as the plane of focus was moved through the tissue, resulting in the fuller view provided by the tracing. Scale bar = 10 microns.

Figure 3.

Sex differences in the volume of MePD and the number of MePD astrocytes develop independent of functional androgen receptors (ARs) before puberty. (A) At P25, overall volume of the MePD is larger in wildtype (wt) males and testicular feminization mutant (Tfm) males than in wt females. (B) The juvenile MePD is also lateralized in volume, but only for wt and Tfm males, with MePD volume larger on the right than the left, indicating that laterality of the juvenile MePD is a masculine trait established independent of AR. Moreover, the right MePD is also sexually differentiated in volume, with wt males having a larger MePD than females, and Tfm males having an even greater MePD volume than wt males. In contrast, left MePD volume differs only between Tfm males and females. (C) The number of astrocytes is sexually differentiated by P25, with both wt and Tfm males having more astrocytes overall than females, indicating that this sex difference in number develops independent of functional AR. (D) Sex differences in astrocyte number are evident on both sides of the brain, although in the left MePD, only wt males have significantly more astrocytes than females. The number of astrocytes is also lateralized, with the right MePD containing significantly more astrocytes than the left for all three groups. Values are means of N = 9 rats/group ($\pm$ standard error of the mean, SEM).

Figure 4.

The length and complexity of astrocyte branches is neither lateralized nor sexually differentiated in the MePD at the start of puberty. Estimated number of primary branches (A), branch points (B), branch endings (C), and average branch length (D) of astrocytes in the MePD of wt male, Tfm male, and female rats at 25 days of age. Values are means of $N = 5$ rats/ group ($\pm$ SEM).

Figure 5.

The MePD undergoes a period of hemisphere-specific growth during the pubertal-to-adult transition including increases in regional volume (A) increases in the number of astrocytes (B) and increases in the complexity of astrocyte arbors (C). Comparison of MePD volumes in prepubertal (P25) and adult brains (P110; from Johnson 2008) reveals that while MePD volume increases in all three genotypes on both sides of the brain, the right side undergoes a dramatic enlargement in wt males that creates a sex difference in adult animals. Tfm males experience reduced growth in the right MePD during this same period suggesting both AR-dependent and -independent mechanisms contribute to adult MePD volume. The number of MePD astrocytes also increases during the pubertal-to-adult transition, in step with the increases in MePD volume. Comparison of astrocyte numbers in the MePD of prepubertal (P25) and adult brains (P110; from Johnson 2008) reveals that astrocyte numbers increase substantially during this period on both sides of the brain, although the increase is greater on the right than the left. Moreover, on the right, while sex differences in astrocyte number are present at each age, the number of astrocytes is fully masculinized in Tfm males at P25, but fully feminine by P110, suggesting that both AR-dependent and -independent mechanisms determine the adult sex differences in MePD astrocyte number. Apparently AR plays no role in initially establishing the sex difference in astrocyte numbers in the right MePD, but is required for maintaining this sex difference into adulthood by contributing to their proliferation and/or survival. MePD astrocytes also show extensive growth of their branches, but only in males and only on the left side of the brain. Females and Tfm males exhibit little to no increase. These data suggests that astrocytes in the left MePD extend processes in response to the pubertal rise in testicular androgens that work through ARs. It is noteworthy that MePD astrocytes on the right exhibit little such growth in all three genotypes during the same developmental period. Values are means of $N = 8-10$ rats/group ($\pm$ SEM).

Table 1

Mean number of astrocytes per cubic micron $\times 10^5$ in the rat MePD

	Males		TTMs Males		Females	
	Postnatal Day 25		Postnatal Day 25		Postnatal Day 25	
	Left MePD	Right MePD	Left MePD	Right MePD	Left MePD	Right MePD
Mean	4.39	4.14	3.66	3.5	4.01	4.28
S.E.M.	0.21	0.17	0.16	0.15	0.14	0.23

	Postnatal Day 100		Postnatal Day 100		Postnatal Day 100	
	Left MePD	Right MePD	Left MePD	Right MePD	Left MePD	Right MePD
Mean	3.16	3.52	3.4	3.43	3.65	4.24
S.E.M.	0.26	0.21	0.22	0.22	0.18	0.21