

Sexual Dimorphism in Neuronal Number of the Posterodorsal Medial Amygdala Is Independent of Circulating Androgens and Regional Volume in Adult Rats

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ABSTRACT

The posterodorsal medial amygdala (MePD) in rodents integrates olfactory and pheromonal information, which, coupled with the appropriate hormonal signals, may facilitate or repress reproductive behavior in adulthood. MePD volume and neuronal soma size are greater in male rats than in females, and these sexual dimorphisms are maintained by adult circulating hormone levels. Castration of adult males causes these measures to shrink to the size seen in females 4 weeks later, whereas testosterone treatment of adult females for 4 weeks enlarges these measures to the size of males. We used stereological methods to count the number of cells in the MePD and found that, in addition to the sex difference in regional volume and soma size, males also have more MePD neurons than do females, yet these numbers are unaffected by the presence or absence of androgen in adults of either sex. Males also have more glial cells than do females, but, in contrast to the effects on neuronal number, the number of glial cells is affected by androgen in the right MePD of both sexes and, therefore, may contribute to regional volume changes in adulthood in that hemisphere. Thus, regional volume, neuronal size, and glial numbers vary in the MePD of adult rats in response to circulating androgens, but neuronal number does not. These results suggest that the sex difference in neuronal number in the rat MePD may be “organized” by androgens prior to adulthood, whereas regional volume, neuronal size, and glial numbers can be altered by androgens in adulthood. *J. Comp. Neurol.* 506:851–859, 2008. © 2007 Wiley-Liss, Inc.

Indexing terms: medial amygdala; adult hormone manipulation; neural plasticity; glia; brain morphology; stereology; testosterone; sexual dimorphism

In mammals, sex differences in brain morphology typically result from differential exposure to gonadal steroid hormones during critical periods in development, although steroids also exert effects on the brain during puberty and in adulthood. As an example, the medial amygdala is influenced by both perinatal and adult androgen manipulations (Mizukami et al., 1983; Malsbury and McKay, 1994; Cooke et al., 1999; Stefanova and Ovcharov, 2000). One quadrant of the medial amygdala, the posterodorsal aspect (MePD), is particularly sensitive to androgen manipulations in adult rodents. Adult levels of circulating androgen maintain the larger volume of the MePD in male rats compared with females. Castration of adult males, or testosterone (T) treatment of females, abolishes the sex difference by decreasing or increasing MePD volume in males or females, respectively (Cooke et al., 1999). Although many brain regions are sexually di-

morphic in volume, the MePD appears unusual in that it is sensitive to hormones in adulthood.

Gonadal steroids may activate androgen receptors (AR) and/or estrogen receptors (ER) to cause the sexual dimorphism in MePD volume. Systemic treatment of adult gonadectomized males with estradiol, but not dihydrotestosterone (DHT), maintains MePD volume in rats (Cooke et

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al., 2003), yet male rats with a dysfunctional AR exhibit MePD volume intermediate to that of control males and females (Morris et al., 2005), indicating that AR also contributes to the masculinization of the MePD. In adulthood, this region contains abundant neurons expressing AR and/or ER protein (Roselli, 1991; Li et al., 1997; Yokosuka et al., 1997) and mRNA (McAbee and DonCarlos, 1998; Shughrue et al., 1997), suggesting that gonadal hormones may act directly in the MePD to influence its morphology.

Sex differences in MePD volume may be caused by a number of integral anatomical components: cell size (soma and processes), cell number, vasculature, neuropil, and extracellular space. Although the size of neurons in the MePD is dimorphic (Bubenik and Brown, 1973; Cooke et al., 1999; Morris et al., 2005; Hermel et al., 2006) and, therefore, might account for the difference in MePD volume, previous work has shown that DHT treatment maintains neuronal size in the male MePD, without maintaining volume (Cooke et al., 2003). This dissociation of neuronal size and regional volume indicates that other components of the MePD are probably affected by circulating androgen to alter MePD volume. For example, no one has determined whether the sex difference in adult medial amygdala volume is accompanied by a sex difference in neuronal number, nor whether androgen manipulations in adulthood affect the number of neurons. This possibility is raised because T increases neurogenesis in the medial amygdala of castrated adult male meadow voles (Fowler et al., 2003) and prevents apoptosis in the preoptic area of developing male rats, resulting in a larger adult volume (Davis et al., 1996).

We report here that male rats have more neurons and glia in the MePD than do females. Although neuronal number is unaffected by hormone manipulations in adults of either sex, glial number is affected in the MePD of the right hemisphere only. These results further delineate the cellular mechanisms involved in the hormone-regulated plasticity of the rat MePD, indicating that a permanent sex difference in neuronal number is organized earlier in life and does not contribute to fluctuations in regional volume in adulthood.

MATERIALS AND METHODS

Animals

Sixty-day-old male and female Long Evans rats (Charles River, Wilmington, MA) were housed three per cage by gender in standard rat cages with food and water freely available. Males and females were housed in separate rooms. Lights were turned off at 1900 hours and on at 0700 hours. Animal care followed standards set by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at Michigan State University. After 1 week of acclimation, surgeries and hormone capsule implantations were performed during isoflurane inhalant anesthesia using aseptic procedures.

Animals were randomly assigned to one of four groups of $N = 10$ animals each. Females were ovariectomized (OVX) and implanted s.c. with two Silastic capsules (each having 20 mm effective release length, 30 mm total length, i.d. 0.062 inch; o.d. 0.125 inch) containing either crystalline testosterone (T) or nothing ("blank") and killed 28 days later. Capsules were incubated for 48 hr in

phosphate-buffered saline (pH 7.4) before implantation. Males were either castrated or subjected to sham surgery and then killed 28 days later.

At the end of the experiment, animals were injected IP with an overdose of sodium pentobarbital (120 mg/kg). Deep anesthesia was noted by lack of reflexes to tail and foot pinch as well as a lack of a corneal reflex. Blood was taken via cardiac puncture for radioimmunoassay. Animals were then perfused intracardially with 0.9% saline, followed by 10% neutral buffered formalin (~300 ml/animal). Gonadectomies and hormone implants were confirmed at sacrifice. Brains, spinal cords, preputial glands, seminal vesicles, and perineal muscles were removed; placed into the same fixative solution; and, after at least 1 month of fixation, trimmed and weighed.

Hormone assay

Plasma testosterone concentrations were measured in duplicate using the Coat-a-Count Total Testosterone Kit (Diagnostic Products Corp., Los Angeles, CA) and sample volumes of 50 μ l plasma. The lower limit of detectability was 0.1 ng/ml, and the intraassay coefficient of variation was 9.0%.

Histology

After at least 1 month postfixation in buffered formalin, the brains were placed overnight in 20% phosphate-buffered sucrose (pH 7.4) at 4°C prior to coronal sectioning on a freezing sliding microtome set to 40 μ m using Multi-Brain Technology (Neuroscience Associates). An embedding matrix ensured no lost sections and preserved hemispheric orientation. Brains were sectioned throughout the entire region of interest and alternate sections mounted onto gelatin-subbed slides with a random start to ensure that every section had an equal probability of being chosen for sampling. Mounted tissue was allowed to air dry, stained with thionin for Nissl substance, and coverslipped with Permount.

Stereological analysis

All analyses were conducted by an investigator blind to group status. The investigator first measured the regional volume of the MePD on both sides of the brain. MePD boundaries were determined as previously described (Morris et al., 2005) following nomenclature from a standard rat atlas (Paxinos and Watson, 2005) and using criteria from Hines et al. (1992) and Canteras et al. (1995). Furthermore, in defining the MePD borders, at least two cytoarchitectonic features (size, shape, distribution, orientation, staining intensity, packing density, and heterogeneity of cells) were used to distinguish it from the surrounding areas, which allowed the entire reference space to be accurately and consistently traced (Fig. 1).

StereoInvestigator (MicroBrightField, Colchester, VT) software was used to estimate the volume of the MePD and the average perikaryal size ($n = 8$ animals per group). A digital camera on a Zeiss Axioplan 2 compound microscope captured an image containing the area of interest from which the perimeter of the area was traced in successive sections throughout the rostrocaudal axis. Total volume of the MePD in each hemisphere was calculated by multiplying the sampling ratio (4) times section thickness (40 μ m) times the total two-dimensional area traced.

We estimated the size of neuronal cell bodies only and not apparent glia. For perikaryal estimates, sample re-

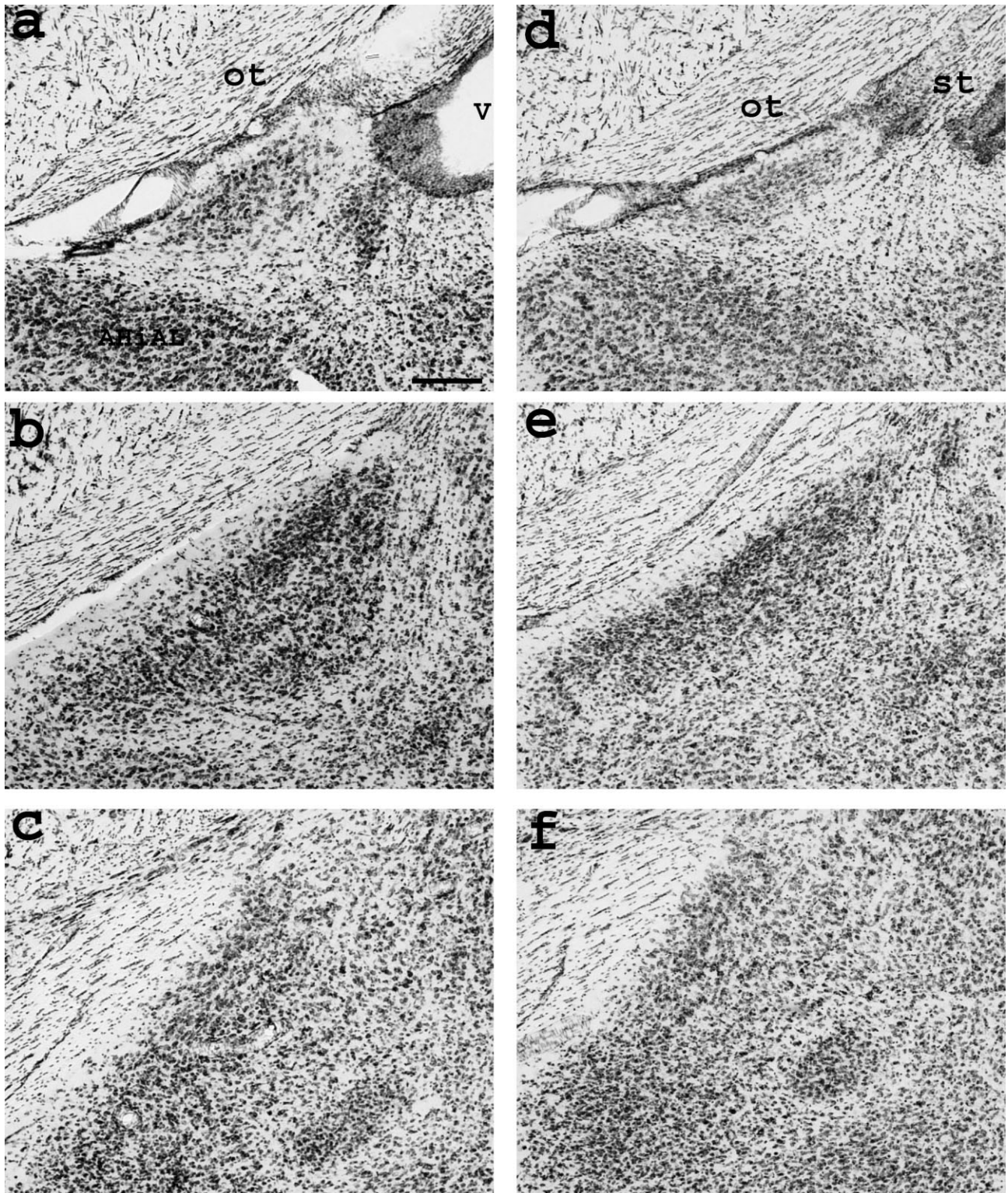


Fig. 1. Boundaries of the rat posterodorsal medial amygdala (MePD). Photomicrographs of coronal sections of the MePD in an adult, gonadally intact male rat (left column) and an adult, ovariectomized female rat without androgen treatment (right column). The caudalmost portion of the MePD appears as a capsule ventral to the optic tract (ot) surrounded by the relatively soma-sparse area of the stria terminalis (st), at a rostrocaudal level intersecting the lateral ventricle (v) and the anterolateral part of the amygdalohippocampal transition area (AHIAL). At this extreme end, the MePD is of a similar

size in males (a) and females (d). More rostrally, the MePD in males (b) has a triangular or wedge-shaped profile, with a slight reduction in cell density in the center, which gives the appearance of an inverted letter "v." In females (e), the nucleus is considerably smaller and has a less cuneate appearance. The rostral end of the MePD is still adjacent to the ventral-lateral aspect of the ot and is more prominent in males (c) than in females (f). In both sexes, the ovoid intercalated nucleus of the amygdala is visible to the right of the MePD at this level. Scale bar = 250 μ m.

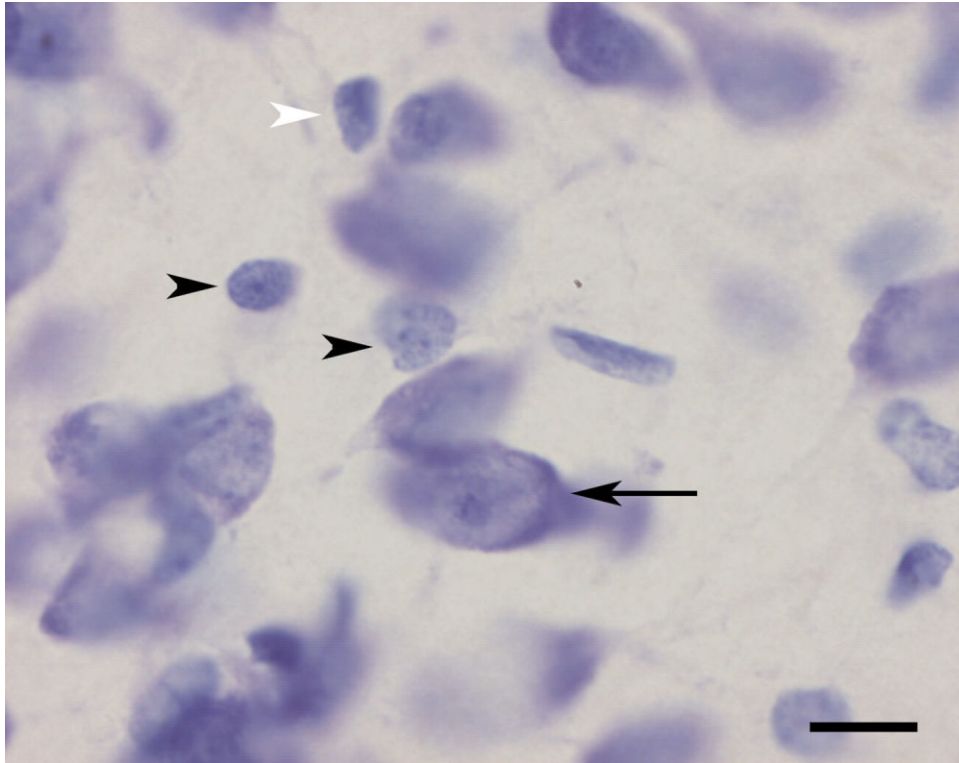


Fig. 2. Classification of cell types. Typical cells viewed with a $\times 100$ objective in a sham male adult rat MePD as revealed by thionin stain for Nissl. Arrow indicates a neuron; black arrowheads indicate glia (oligodendrocyte or astrocyte); white arrowhead indicates a microglia. Scale bar = 10 μm .

gions were randomly selected by the StereoInvestigator software, which positioned points within the MePD without bias for location or appearance. On average, the two-dimensional profile areas of 10 perikarya from each section were measured throughout the rostrocaudal extent of the MePD (sampling 55–95 neurons per hemisphere) and were traced with a $\times 100$ Plan-NeoFluar, 1.3 N.A. oil-immersion objective. Neuronal perikarya measures were averaged within each hemisphere, yielding a mean soma size (area in square micrometers) per hemisphere for each animal.

Neurons were identified by the presence of a distinct Nissl-stained cytoplasm and nucleolus (Fig. 2). Glia were identified by the presence of many darkly stained heterochromatic bodies in the nucleus that were interspersed with strands of thionin-stained material. Such presumptive glial nuclei were generally smaller and were not surrounded by a distinct cytoplasmic shell. The smallest cells that also had a very darkly stained nucleus with several large stained bodies within the nucleus were categorized as microglia. Cells that could not be classified with confidence into these three categories were designated as “unknown.”

The optical fractionator method was used for estimating number of cells (West et al., 1991). This method estimates total number of objects (Nobj) from the sum of objects sampled (ΣN) in a known fraction of the reference space. The calculation then is: $N_{obj} = (\Sigma N)(1/ssf)(1/asf)(1/tsf)$, where section sampling fraction (ssf) is the number of sections sampled divided by the entire number of sections

through the reference space, area sampling fraction (asf) is the total area sampled by the array of counting frames divided by the total area of all sampled sections, and thickness sampling fraction (tsf) is the height of the detector divided by the average total section thickness.

Cells in the MePD were counted by the optical fractionator method (West et al., 1991) with a Plan-NeoFluar $\times 100$ oil-immersion (1.3 N.A.) objective, which allowed us to distinguish in most cases neurons from glia and to ensure discrimination of discrete objects. Sampling parameters were set to allow a coefficient of error ($m = 1$; Gundersen, 1999) of no more than 0.10 for each animal, and percentage of total observed variance resulting from interanimal variance to be at least double that of variance from sampling error ($n = 5$ per group). A pilot study indicated that sampling boxes with the following measures were appropriate: height, 5 μm ; guard height, 1.5 μm minimum; sampling frame area, 625 μm^2 ; x-y spacing 125 μm . An average of 272 cells was counted in each side of each brain. Average thickness of stained sections for each brain was estimated by measuring section thickness in every third counting frame and was used to adjust cell counts for that brain. Overall average section thickness was 8 μm . DIC optics used in a pilot study indicated that potential slicing artifact was limited to about 1 μm , which left approximately 5 μm of depth for sampling cell counts. Neuronal nucleoli and glial nuclei were used as unique counting points. Adobe Photoshop 7.0 was used to generate figures containing photomicrographs. No processing of images occurred except for resizing and brightness.

TABLE 1. Average Weights of the Body, Preputial Glands, Seminal Vesicles, and Bulbocavernosus/Llevator Ani Muscles (BC/LA) for Each Group \pm SEM¹

Subject	Body weight(g)	Preputial glands (g)	Seminal vesicles (g)	BC/LA (g)	T (ng/ml)
Male sham	462 \pm 14	0.21 \pm 0.01	1.99 \pm 0.14	1.65 \pm 0.05	2.68 \pm 0.43
Malecastrate	382 \pm 9.1	0.12 \pm 0.02	0.30 \pm 0.03	0.75 \pm 0.05	ND
OVX female + T	319 \pm 8.8	0.24 \pm 0.01			1.79 \pm 0.28
OVX female + B	297 \pm 13	0.12 \pm 0.01			ND

¹Plasma testosterone (T) levels were detectable only in sham males and OVX females given T, which were not significantly different from one another. ND, below limit of detection of 0.1 ng/ml. For each measure within each sex, hormone manipulation (castration in males, T treatment in females) had a significant effect (*t*-tests, all *P*s < 0.05), with the exception of body weight in females.

Statistical analysis

Results are expressed as mean \pm SEM. A three-way mixed-design analysis of variance (ANOVA), with left and right sides as a repeated measure, and two independent factors [sex and androgen status (high in gonadally intact males and T-treated females, low in castrate males and blank-treated females)], was conducted for each dependent variable (MePD volume, average perikaryal area, neuronal number, glial number). One statistically significant interaction was detected for each of two measures (soma size and glial number), prompting followup two-way ANOVAs to confirm trends detected by the three-way ANOVA. *t*-Tests comparing sham-operated males and blank-treated females were used to assess previously reported sex differences in MePD regional volume and soma size (Cooke et al., 1999; Morris et al., 2005). *t*-Tests were also used to assess the well-documented effects of T on the periphery to confirm androgen manipulations. For all analyses, a value of 0.05 was used as the significance criterion, with N representing the number of animals in each group.

RESULTS

Bioassays of T treatment

Testosterone manipulations for 28 days had gross effects in both males and females (Table 1). Castration of males significantly decreased the average weight of the preputial glands, seminal vesicles, bulbocavernosus/levator ani muscles, and overall body weight (all *P*s < 0.05). T implants in females significantly increased the average weight of preputial glands (*P* < 0.05), but not overall body weight (*P* = 0.17). As expected, circulating T was beneath the detection limit in castrated males and blank-treated females. Insofar as T levels did not differ significantly between sham-castrated males and T-treated females (*P* = 0.10), the treatment appeared to provide physiologically relevant androgen stimulation to females.

Regional volume

As in previous studies, the volume of the MePD was greater in males than in females and, in both males and females, was greater in the right hemisphere than in the left and was affected by adult androgen levels (Fig. 3A). The three-way ANOVA revealed these differences as significant main effects of sex (male greater than female; *P* < 0.0001), of androgen (greater in the presence of androgen than absence of androgen; *P* < 0.003), and of hemisphere

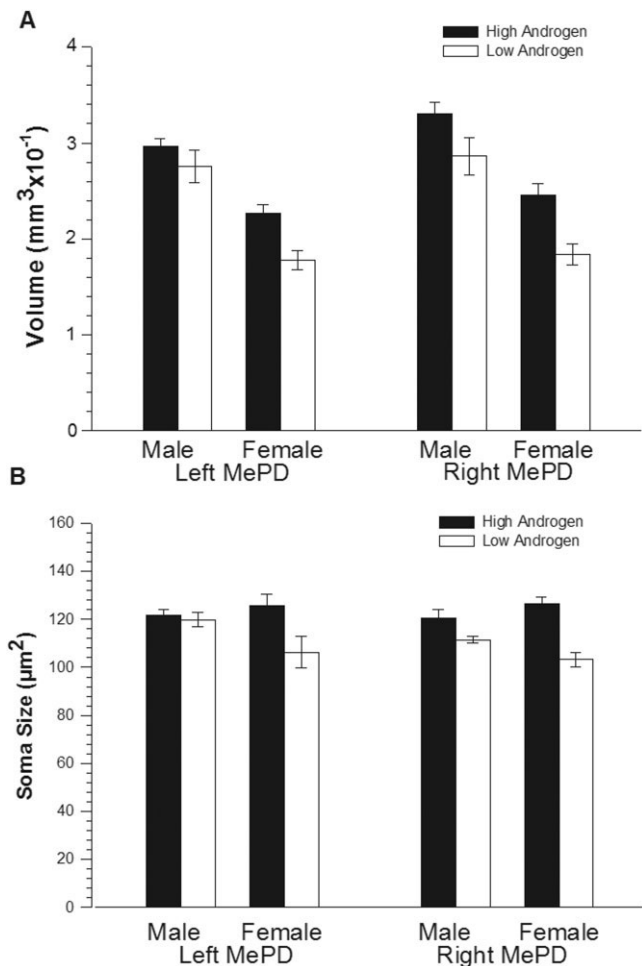


Fig. 3. Androgen regulates medial amygdala volume and soma size in adult rats. **A:** As seen in previous studies, manipulations of androgen in adult rats alter the regional volume of the posterodorsal medial amygdala (MePD). Males were either castrated (low androgen) or subjected to sham surgery (high androgen), whereas females were ovariectomized and given capsules containing either testosterone (high androgen) or nothing (low androgen). Three-way ANOVA revealed a sex difference (males > females; main effect of sex; *P* < 0.0001), asymmetry (right > left; main effect of hemisphere; *P* < 0.005) and a response to androgen manipulation (main effect of hormone; *P* < 0.0003), with no significant interactions. **B:** Neuronal soma size in the rat MePD also responded to androgen manipulations (main effect of androgen; *P* = 0.0005), but there was no evidence of asymmetry (*P* > 0.2). Although there was not a significant sex difference overall, there was an interaction of sex and androgen status (*P* = 0.02), because soma size was more responsive in females than in males. The previously reported sex difference in MePD soma size in gonadally intact animals is represented here by the larger somata in sham males compared with females receiving no androgen.

(right greater than left; *P* < 0.005), with no significant interaction terms. Hence no post hoc comparisons for regional volume were called for, although examination of the data suggests that the left MePD of males may be less responsive to androgen than the right (Fig. 3A). We have twice reported a sex difference in MePD volume in gonadally intact rats. A post hoc comparison of the closest comparison groups available from this study (sham males and untreated ovariectomized females) also revealed volumet-

ric differences in both the left and the right MePD (*t*-tests; $P_s < 0.05$), presumably a result of higher circulating androgen in the males than in the females (Table 1).

Neuronal soma size

Also as in previous studies, there was no laterality of MePD soma size, but this measure was affected by adult hormone levels in both sexes (Fig. 3B). The ANOVA revealed these effects as a significant main effect of androgen ($P < 0.001$), with no significant main effect of laterality nor any significant interaction of any factors with laterality. There was no significant main effect of sex, indicating that adult androgen status is more important than sex in determining soma size. There was a significant sex-by-androgen interaction ($P = 0.02$), because androgen status had a greater effect in females than in males. We previously reported sex differences in MePD soma size in gonadally intact rats and in the present study found that the somata are larger in sham males than untreated females in both the left and the right MePD ($P_s < 0.05$, *t*-tests), presumably a result of higher circulating androgen in the males than in the females. The absence of laterality in MePD soma size, despite the robust asymmetry in regional volume, cannot be attributed to asymmetry in soma size. Sham males have also been reported to have equal-sized neurons across hemispheres, despite asymmetry in regional volume.

Neuron number

Males have more MePD neurons than do females (main effect of sex $P < 0.002$), and there are more neurons in the left hemisphere than the right in both sexes (main effect of side; $P < 0.0001$; Fig. 4A). However, these sex differences and laterality in neuronal number are unaffected by adult androgen status, as there was no main effect of androgen status ($P > 0.5$) nor any statistically significant interaction terms ($P_s > 0.13$). Thus, the left MePD is smaller in volume than the right MePD, yet the left contains more neurons than the right, and this dissociation is seen in both sexes. Furthermore, the absence of androgenic effects on neuronal number indicates that other factors must underlie the effects of androgen on regional volume in the MePD of adult rats and is another dissociation of neuronal number and regional volume.

Glial number

Males have more MePD glial cells than do females, in both hemispheres (main effect of sex; $P < 0.003$; and no significant interaction of sex with any other factor). The number of glial cells is greater on the right than on the left (main effect of hemisphere; $P < 0.001$), and there was no significant main effect of androgen status ($P > 0.4$). However, there was a significant interaction of androgen status and hemisphere ($P < 0.03$), which prompted a followup analysis of the left and right hemispheres separately (Fig. 4B). Two-way ANOVA (sex and androgen status as independent factors) of glial numbers in the left hemisphere revealed a marginally significant sex difference ($P = 0.06$) but no effect of androgen. In contrast, in the right hemisphere, there was both a sex difference (main effect; $P < 0.003$) and an effect of androgen ($P < 0.01$), with no interaction. Thus high androgen is associated with a greater number of glial cells in the MePD in the right hemisphere only, and this effect is seen in both sexes.

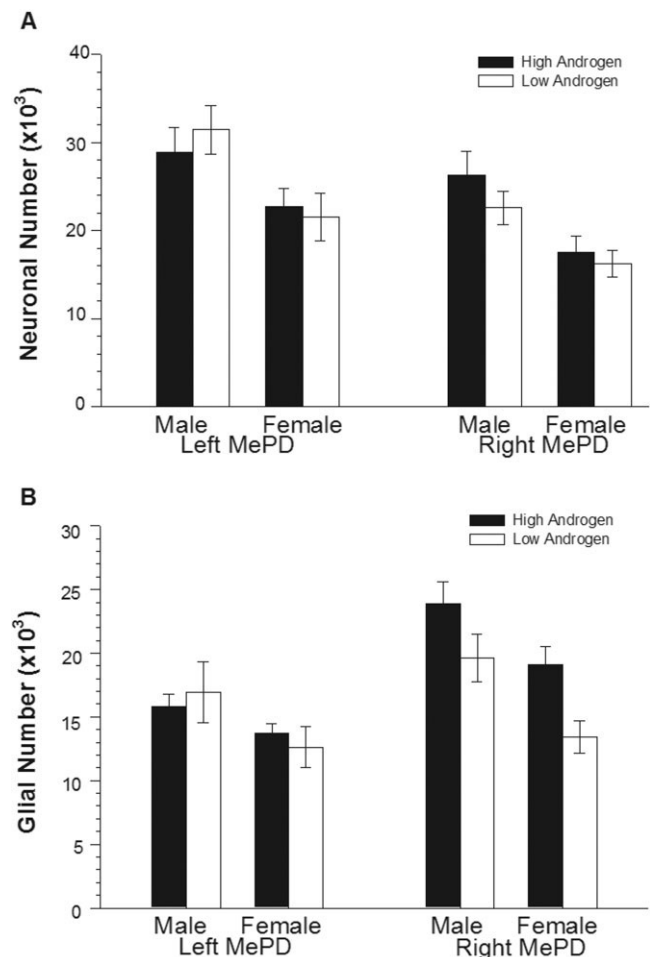


Fig. 4. Androgen has no effect on neuronal number in the adult rat MePD. **A:** Males have more MePD neurons than females (main effect of sex; $P < 0.002$), and there are more neurons in the left MePD than the right (main effect of hemisphere; $P < 0.0001$), but there was no effect of androgen manipulations in either sex or in either hemisphere (no significant interactions; $P_s > 0.14$). **B:** Males also have more glial cells in the MePD than do females (main effect of sex; $P < 0.003$), and there are more glia in the right MePD than the left (main effect of hemisphere; $P < 0.001$). However, there was an interaction of androgen status and hemisphere ($P < 0.03$). Subsequent separate analyses of the left and right MePD revealed that androgen is associated with more glia in both sexes, but only in the right MePD.

We considered the density of glia by treatment group, using the cell counts and regional volume measures reported above. These indicated that the right MePD contains a higher density of glia, and the left MePD contains a higher density of neurons, in all treatment groups. Overall, there does not appear to be a significant sex difference in neuronal density, but OVX females that receive no T have a higher density of glia than sham males. This result seems to confirm the report that female rats display a greater density of glial fibrillary acidic protein (GFAP) staining than males in the MePD (Rasia-Filho et al., 2002).

Other cell types

Our sampling parameters were not designed to obtain reliable counts of rarely encountered microglia or "un-

known" cells, but the mean estimates for the number of microglia suggest that, if there is a difference, it also favors males (sham males = $3,428 \pm 427$, castrated males = $5,118 \pm 576$, T-treated females = $2,720 \pm 323$, blank-treated females = $2,605 \pm 260$). Likewise, the estimated number of unknown cells was only slightly greater in males than in females (sham males = $8,148 \pm 1,491$, castrated males = $11,833 \pm 1,296$, T-treated females = $8,134 \pm 1,184$, blank-treated females = $6,678 \pm 1,424$). Because the mean estimates of microglia and unidentified cells are greater in males than in females, the sex differences in numbers of neurons and glia favoring males cannot be accounted for by misclassification of cells.

DISCUSSION

The adult rat MePD shows a remarkable level of plasticity that is regulated by gonadal hormones. We found that the adult female rat MePD responds to T treatment with increases in volume and neuronal size in both hemispheres and number of glia in the right hemisphere. The male MePD also responds to gonadal hormones: adult castration leads to reductions in regional volume and neuronal size in both hemispheres and the number of glia in the right hemisphere. Unlike the other measures, however, the number of neurons in males and females was not affected by changes in adult androgen. These results limit hypotheses about cellular mechanisms underlying androgen's effects on MePD regional volume in adult rats and how hormonally induced plasticity of this nucleus may affect reproductive behavior. In turn, adult behaviors that induce hormonal variations, such as exposure to an estrous female and social conflict, may influence the regional volume and neuronal size in this nucleus but are unlikely to affect neuronal number.

Because adult hormone manipulations can abolish sex differences in MePD volume (Cooke et al., 1999), one might have expected that the number of neurons was equivalent in the two sexes and that adult fluctuations in volume might reflect changes in neuropil that simply alter the density of neurons within the nucleus. However, we found that males in fact have more MePD neurons than do females in adulthood, no matter what the androgen status of the animals. Thus castration of adult males causes the MePD to shrink in volume, without any loss of neurons, whereas androgen treatment of females enlarges MePD volume, without adding to neuronal number. Change in regional volume cannot be explained by changes in neuronal number, so other factors must contribute to volumetric changes. We found evidence that androgen affects glial number, but only in the right hemisphere. In the present study, changes in MePD soma size generally reflected changes in volume, so soma size may contribute to some of the volume changes we detected. However, the persistent asymmetry in MePD volume stands in contrast to a lack of any asymmetry in MePD neuronal somata size. Furthermore, in previous studies, treatment with the various metabolites of testosterone can dissociate soma size from volume (Cooke et al., 2003). By elimination, these results together suggest that changes in synaptic neuropil, including the dendrites of neurons and processes of glia, probably underlie the bulk of change in MePD volume in adult rats. Reports of hormone-induced changes in MePD dendritic structure lend credence to this idea (Gomez and Newman, 1991; Rasia-Filho et al., 2005; Hermel

et al., 2006). Cooke et al. (2007) report a sex difference in the proportion of MePD volume occupied by dendritic processes in prepubertal rats, consistent with this idea. They also found more neurons in males than in females in the right MePD of prepubertal animals but no sex difference in neuronal number on the left. Thus the sex difference in the left MePD may arise during or after puberty, which would suggest a dramatic reorganization during this period of ontogeny.

There is also an interesting and pervasive dissociation of regional volume and neuronal number in these data. The MePD has a larger volume on the right than the left, yet there are more neurons on the left than on the right. We found this pattern of results in both sexes, and androgen manipulations had no effect on neuronal number. Again, one might have expected that the hemisphere containing a larger MePD would also contain more MePD neurons. It seems clear that the several components that contribute to the volume of a brain nucleus (neuronal number, glial number, neuronal soma size, etc.) are independent, at least in the rat MePD.

The sex difference in neuronal number within the MePD is unaffected by adult androgen manipulations, so presumably T or its metabolites affect neuronal number earlier in life. Cooke and Woolley (2005) showed that, in pre-pubertal rats at 25–29 days of age (when circulating androgens are equivalent in males and females), soma size, cell density, and neuronal number in the left MePD were not sexually differentiated, but volume was, being significantly larger in males. If the male amygdala contains more neurons only after puberty, it may be that rising levels of androgens promote increases in neuronal size and number in pubertal males. Though speculative, this would explain why, in humans, amygdalar volume increases faster in boys than in girls between ages 4 and 18 (Giedd et al., 1997; Merke et al., 2003). Furthermore, neurogenesis has been reported in the amygdala of young adult male monkeys (Bernier et al., 2002). Conversely, if female rats have fewer neurons after puberty than before, then androgens may prevent apoptosis in male rats to create the adult sex difference in neuronal number. In any case, future studies could ask when the sex difference in neuronal number arises in the developing MePD and whether androgen organizes this characteristic.

There is also the question of how such differences in neuronal number emerge. What is the cellular mechanism involved? Studies of cell number in the MePD during ontogeny might indicate whether the sex difference in neuronal number arises because of sex differences in neurogenesis, neuronal migration, neuronal differentiation, and/or apoptosis. Although we saw no change in neuronal number in the adult MePD, this does not address whether neurogenesis or apoptosis occurs in adults. If neurogenesis and apoptosis are ongoing and in balance in adults, our counts of the net number of neurons would not detect those processes. Although castration can affect neuronal proliferation in the MePD of adult male meadow voles (Fowler et al., 2003), there may be species differences in neurogenesis, neurodegeneration, or both.

Overall, the present findings add to the literature indicating that morphologic lateral asymmetry is common in the amygdala (Cooke et al., 2003; Morris et al., 2005). Although androgen receptor distribution has been found to be asymmetrical in adult rat hippocampus (Xiao and Jordan, 2002), no such laterality was reported in the me-

dial amygdala of mice (Lu et al., 1998). The human amygdala has also been reported to be asymmetric in terms of both structure (Murphy, 1986) and function (Phillips et al., 2001; Canli et al., 2002; McClure et al., 2004; Cahill et al., 2004; Hamann et al., 2004).

For glial number, only the right side of the MePD is sensitive to hormones. T-treated females had more glia than blank-treated females only in the right MePD. Conversely, castrated males have fewer glia than gonadally intact males only in the right MePD. These results suggest that the right MePD is more sensitive than the left to androgen's effects on glial number. Whether this increased sensitivity in the right hemisphere is due to asymmetry in steroid receptors, metabolic enzymes (such as aromatase), or some other factor has yet to be addressed.

Our results indicate that male rats have more glial cells in the MePD than do female rats. However, visualizing astrocytes in the rat MePD by GFAP immunoreactivity, which is expressed by most (but not all) astrocytes (Eng et al., 2000), reveals a sex difference in the opposite direction, with females showing a greater density of GFAP staining than do males (Rasia-Filho et al., 2002). GFAP marks only astrocytes, and our counts undoubtedly include both astrocytes and oligodendrocytes, so our findings of a higher glial density in the female MePD could account for the sex difference in GFAP staining seen by Rasia-Filho et al. (2002). On the other hand, it is possible that, despite the fewer glia found in females compared with males, the glia in females may have longer or more branched processes than those in males. In fetal rat hypothalamic cultures, astrocytic process elaboration, but not number, is increased by estradiol (Garcia-Segura et al., 1989). Other studies have shown that GFAP immunoreactivity in the dentate gyrus of the hippocampus and the MePD correlates with estrogen levels across the female's estrous cycle (Luquin et al., 1993; Martinez et al., 2006). It will be important in future studies to count the number of GFAP-expressing cells in the MePD both to validate the identification of glia in this study and to probe the relative contributions of astrocytes vs. other glia to our counts, as well as any responses to steroid manipulations in adulthood. Although astrocytes have been shown to express steroid receptors in some parts of the rat brain (Lorenz et al., 2005; Tabori et al., 2005), we do not know based on the current data which type of glia is increased in response to androgen treatment of adult females, nor do we know whether androgen is acting directly on glial cells or glial precursors, or acting on some other cell type, including neurons, to increase glial numbers indirectly in the right MePD. Finally, it is possible that the T treatment did not actually increase the number of glia in the right MePD but somehow altered glia morphology to make the glia more detectable in our Nissl-stained preparation. Thus any conclusions about the mechanisms of change in the number of glia found in the MePD must remain tentative.

Our androgen manipulations did not affect MePD volume as dramatically as in a previous study (Cooke et al., 1999), where mean volumes in gonadally intact males were not significantly larger than in females given androgen. In the present study, the sex difference in volume, seen as a main effect of sex in ANOVA, persisted even when controlling for androgen (Fig. 3A). It is possible that providing more androgen and/or extending the androgen treatment (Cooke et al. provided 30 days of T, and we provided 28) might have more closely duplicated the re-

sults of the previous study. In any case, post hoc tests of MePD volume in the right hemisphere (the more androgen-responsive side of the brain) show no significant difference between castrated males and females given androgen. Therefore, both studies demonstrate that the sex difference in MePD volume can be eliminated by manipulations of androgen in adulthood.

This plasticity of the adult MePD in rats continues to offer a model system for studying structure/function relationships in hormone-sensitive brain areas. Future studies should address directly whether adult hormone manipulations affect dendritic and/or synaptic structures in the MePD, the identity of glial cells affected, and the contributions each of these changes to alterations in behavior of both sexes.

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