HHS Public Access

Author manuscript

J Comp Neurol. Author manuscript; available in PMC 2017 February 15.

Published in final edited form as:

J Comp Neurol. 2016 August 15; 524(12): 2492–2502. doi:10.1002/cne.23964.

Sex and Laterality Differences in Medial Amygdala Neurons and Astrocytes of Adult Mice

Daniel R. Pfau[*], **Nicholas J. Hobbs**, **S. Marc Breedlove**, and **Cynthia L. Jordan**

Neuroscience Program, Michigan State University, East Lansing, Michigan

Abstract

The posterodorsal aspect of the medial amygdala (MePD) in rats is sexually dimorphic, being larger and containing more and larger neurons in males than in females. It is also highly lateralized, with the right MePD larger than the left in both sexes, but with the smaller left MePD actually containing more and larger neurons than the larger right. Astrocytes are also strikingly sexually differentiated, with male-biased numbers and lateralized favoring the right in the rat MePD. However, comparable information is scant for mice where genetic tools offer greater experimental power. Hence, we examined the MePD from adult male and female C57Bl/6^{J} mice. We now report that the MePD is larger in males than in females, with the MePD in males containing more astrocytes and neurons than in females. However, we did not find sex differences in astrocyte complexity or overall glial number nor effects of laterality in either measure. While the mouse MePD is generally less lateralized than in rats, we did find that the sex difference in astrocyte number is only on the right because of a significant lateralization in females, with significantly fewer astrocytes on the right than the left but only in females. A sex difference in neuronal soma size favoring males was also evident, but only on the left. Sex differences in the number of neurons and astrocytes common to both rodent species may represent core morphological features that critically underlie the expression of sex-specific behaviors that depend on the MePD.

Keywords

glia; sexual dimorphism; GFAP; olfaction; vomeronasal; Nissl

Sex differences in brain organization are thought to be essential for the expression of sex-specific behaviors (Keverne, 2008), with such behaviors often elicited in rodents by detection of olfactory and pheromonal cues (Clancy et al., 1984). Such cues are sent to the posterodorsal aspect of the medial amygdala (MePD) (Keverne, 2008; Cadiz-Moretti et al.,

[*]CORRESPONDENCE TO: Daniel Pfau, Neuroscience Program, 293 Farm Lane, Rm 108 Giltner Hall, Michigan State University, East Lansing, MI 48824-1101. Daniel.Prism@gmail.com.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR ROLES

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: CLJ, SMB. Acquisition of data: DRP. Analysis and interpretation of data: DRP, CLJ. Drafting of the article: DRP, CLJ. Critical revision of the article for important intellectual content: DRP, CLJ, SMB, NJH. Statistical analysis: DRP, CLJ. Obtained funding: CLJ, SMB. Administrative, technical, and material support: CLJ, NJH. Study supervision: CLJ.

2014). The MePD is profoundly sexually differentiated in rats, with adult males having a much larger MePD than adult females (Cooke et al., 1999). In mice, efferent connections of the MePD terminate in regions specific to reproductive behavior, including the bed nucleus of the stria terminalis, medial preoptic area, and ventromedial hypothalamus (DiBenedictis et al., 2014; Gutierrez-Castellanos et al., 2014). Thus, it seems likely that the sexually dimorphic MePD plays a role in gating pheromonal cues to elicit sex-specific sociosexual behaviors (Halem et al., 1999; Meredith and Fewell, 2001; Rasia-Filho et al., 2012).

Substantial work has been done to understand the cellular basis of this sex difference in regional volume of the MePD in rats. Morphometric analyses reveal that the rat MePD contains more neurons and astrocytes in males than in females (Johnson et al., 2008; Morris et al., 2008a). MePD neurons and astrocytes are also sexually differentiated in size, with larger neuronal somata and more extensive astrocytic processes in males than females (Cooke et al., 1999, 2003; Morris et al., 2008a; Johnson et al., 2012). Interestingly, the MePD is also highly lateralized in rats, with both males and females having more MePD neurons in the left hemisphere than in the right (Morris et al., 2008a). The number of astrocytes and the complexity of their arbors is also highly lateralized, with more MePD astrocytes in the right hemisphere and more complex astrocytes on the left side (Johnson et al., 2008, 2012). Consequently, sex differences in astrocytes are also highly lateralized with the sex difference in astrocyte number only on the right side, and sex difference in arbor complexity only on the left (Johnson et al., 2008). This wealth of lateralization interacting with sex differences raises questions about whether the MePD on each side functions differently in sex-specific ways in rats, as functional magnetic resonance imaging (fMRI) data suggests to be the case in the human amygdala (Cahill et al., 2004; Kilpatrick et al., 2006). To lead to later studies examining the functional significance of these sex and laterality differences in the MePD, we turn to mice. The current study lays the groundwork for using genetically engineered mice to address questions about sex-specific brain circuitry and laterality and their relevance to sex-specific behaviors.

Information about sex differences in the MePD of mice is scant. Published evidence thus far indicates that the MePD in BALB/c mice is larger in males than females, but lateralized in volume *only* in females (Morris et al., 2008b). In the current study we characterized the cellular morphology of the MePD in adult male and female C57Bl/6^{J} mice, the strain most commonly used in genetic manipulations. We measured the size and number of neurons in Nissl-stained sections and the number and complexity of astrocytes in sections stained for glial fibrillary acidic protein (GFAP), an astrocytic marker. We find that the MePD in C57Bl/6^{J} mice is both sexually dimorphic and lateralized but the details of these differences differ from rats. The MePD in male mice is larger in regional volume than in females and contains a greater number of neurons and astrocytes. However, we found no sex differences in the complexity of astrocytic arbors. As in rats, there was evidence of laterality in the mouse MePD, although it is not as marked as in rats. Ultimately, these data may help to understand the underpinnings of sex-specific social behavior.

MATERIALS AND METHODS

Animals

C57Bl/6J (RRID:IMSR_JAX:000664) male (n = 15) and female (n = 15) mice were purchased as adults (6–8 weeks) from Jackson Laboratory (Bar Harbor, ME). After at least a week of acclimation, mice were overdosed with sodium pentobarbital (210 mg/kg) and perfused transcardially with 0.9% saline followed by phosphate buffered (0.1M, ph 7.4) 4% paraformaldehyde. Brains were harvested and postfixed 2 hours in the same paraformaldehyde solution followed by cryoprotection in 20% sucrose in 0.1M phosphate buffer for 48 hours (at 4°C). Brains were scored along the right dorsal surface of the cortex to mark side and then cross-sectioned at 30 μm on a freezing sliding microtome. Two adjacent series were collected and stored at –20°C in de Olmos cyroprotectant until stained (de Olmos et al., 1978). All animal procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University.

GFAP immunohistochemistry and hematoxylin counterstain

One series of sections was brought to room temperature and thoroughly rinsed in phosphate-buffered saline (0.1M, pH 7.4) containing 0.3% Triton and 0.1% gelatin (PBS-GT). This same solution was used throughout as the vehicle for immunoreagents and rinses. Following a published protocol for GFAP immunostaining of astrocytes in rats (Johnson et al., 2008), sections were incubated in 0.5% sodium borohydride in PBS-GT (15 minutes), blocked for nonspecific avidin-biotin binding (Avidin and Biotin blocking kit, SP-2001, Vector Laboratories, Burlingame, CA), followed by an overnight incubation at 4°C in monoclonal mouse anti-GFAP (EMD Millipore, Bedford, MA; Cat# MAB360, RRID:AB_2109815, 1:50,000 dilution). Sections were then incubated in biotinylated horse antimouse secondary antibody (Vector Laboratories, Cat# BA-2001, RRID:AB_2336180,, 1:2,500 dilution) followed by incubation in half-strength avidin-biotin complex solution (Elite Avidin Biotin complex kit, PK-6100, Vector Laboratories) and reacted for 5 minutes using 0.025% diaminobenzidine (DAB, Sigma, St. Louis, MO) with 0.0125% H_2O_2 in Tris buffer (0.05M, pH 7.2), to visualize horse radish peroxidase. After rinsing to quench the reaction, sections were mounted onto gel-subbed slides and allowed to dry before counterstaining with Harris's Hematoxylin solution (Sigma, #010M4354) followed by alcohol dehydration, clearing, and coverslipping with Permount (Fisher Scientific, Pittsburgh, PA).

Antibody characterization

A full list of antibodies used can be found in Table 1. Staining was absent when the primary antiserum was omitted. Staining for GFAP reliably led to the visualization of morphology resembling that of astrocytes, as previously reported by our lab (Johnson et al., 2012). In western blots of mouse brain lysate, the primary antiserum recognizes a single band at ~51 kDa, corresponding to GFAP (manufacturer information).

Nissl stain

The other series of sections were brought to room temperate, rinsed in PBS-GT, and mounted onto gelsubbed slides. Once dry, sections were stained with thionine (Morris et al., 2008a), dehydrated, cleared, and coverslipped with Permount.

Stereological analysis

A Zeiss Axioplan II microscope equipped with an Optronics MicroFire digital video camera was used for the stereological analysis using StereoInvestigator software (v. 7.0, MBF Bioscience, Williston, VT). The total number of neurons, glia, and astrocytes were determined in the MePD of each hemisphere. At low magnification, boundaries of the MePD were traced in each section. The MePD was identified based on a mouse atlas (Paxinos and Franklin, 2004) and previously defined parameters within our laboratory (Morris et al., 2008b). Landmarks visible through Nissl and hematoxylin staining included the optic tract, shape of the stria terminalis, and posteroventral medial amygdala (Fig. 1A–D). Once the contours were traced, astrocytes were counted using a 40× Zeiss plan-neofluar objective (n.a. = 0.75) in GFAP-stained sections and neurons and overall glia counted in Nissl-stained sections using a 100× Zeiss plan-neofluar oil-immersion objective (n.a. = 1.3). Higher magnification was required to resolve cellular characteristics that distinguish neurons from glia in Nissl-stained sections, as detailed below. Slides were coded, making the observer blind to the sex of the animal.

The optical fractionator probe was used to perform unbiased counts of the number of neurons and overall glial cells within the MePD in Nissl-stained sections. Neurons were identified based on having a cytoplasmic shell surrounding a distinct nucleus and nucleolus, while glial cells, which include astrocytes, were identified based on the absence of an apparent cytoplasmic compartment but with a distinct nucleus and often lobed nucleoli (Fig. 1E). Astrocytes were counted using the optical fractionator probe in GFAP-labeled sections and identified based on having at least three GFAP-labeled fibers emanating from the cell body surrounding a distinct hematoxylin-stained nucleus (Fig. 1F). These methods provided estimates of the total number of neurons, overall glial cells, and astrocytes, along with regional volumes and cell densities within the MePD. The average cell number of adjacent sections was included in the StereoInvestigator optical fractionator analysis when only one section was missing in a hemisphere. If more sections were damaged or missing, the brain was excluded from the analysis. However, average volumes of sections adjacent to missing tissue cannot be included in the program's algorithm, the Cavalieri estimator, in this way, accounting for the lower reported sample sizes for volume measures.

Neurolucida software (v. 7.0, MBF Bioscience) was used to trace and estimate the complexity of astrocyte arbors. In six randomly selected sections containing the MePD from each animal, the complete astrocytic arbor of two astrocytes randomly selected by an observer blind to sex were traced using the 100× objective, with 12 astrocytes sampled from each hemisphere. Based on these traces, the average number of primary processes, average number of branch points, average number of branch endings, and average branch length were calculated for each animal and hemisphere.

To measure the size of neuronal somata, neurons were traced in five randomly selected sections. The optical fractionator probe was used to pick two random sites within each contour (total of 10 cells per hemisphere) and the soma of the closest neuron was traced using the nucleator probe and the 100× objective.

Statistical analysis

Separate mixed design analyses of variance (ANOVAs) were conducted for each dependent variable (MePD volume, number of astrocytes, primary processes, branch points, branch endings, branch length, number of neurons and overall glial cells, and neuronal soma size), with left and right hemispheres as the repeated measure and sex as the between-group measure. If no significant difference in laterality was found, the ANOVA was collapsed across sides, yielding a oneway ANOVA with sex being the only factor. Post-hoc least-square difference tests were performed to isolate sources of significant variance. Results are reported as means ± SEM (standard error of the mean) with an alpha level of 0.05. To examine the relationship between cellular components of the MePD and its volume, correlation analyses were performed. To increase power by reducing the number of tests run, all measurements of astrocyte complexity were put through dimension reduction with principal component analysis. Separate analyses were run for each hemisphere and sex and revealed one factor that strongly loaded every complexity measure. Using this complexity factor a total of 10 correlations were run with a familywise error rate of 0.1 and an alpha level of 0.01. In addition, the rostrocaudal extent of the MePD was examined for density changes in astrocytes, overall glia or neurons by grouping sections into four bins. Separate ANOVA analysis was used to analyze differences across the four rostral to caudal areas for each hemisphere between the sexes.

RESULTS

MePD volume

Comparable to the sex difference in MePD volume in rats, the MePD in C57BL/6^{J} mice was also sexually differentiated (female MePD ranging from 0.09 to 0.19 mm^3 and males from 0.12 to 0.24 mm^3), with males having a significantly larger MePD than females ($P = 0.026$, $d = 1.08$; Fig. 2A) in GFAP-hematoxylin-stained sections. However, there was no significant effect of hemisphere ($P = 0.087$) nor a significant interaction between hemisphere and sex ($P = 0.596$). We found a comparable sex difference in volume in Nissl-stained sections ($P = 0.035$, $d = 1.33$; Fig. 2B). Interestingly, volume of the MePD as outlined in Nissl-stained sections *was* significantly lateralized, with the left MePD larger than the right ($P = 0.031$; $d = 0.44$). This lateralization did not interact with sex ($P = 0.454$), suggesting that the MePD in both males and females contributes to this lateralization.

Number of neurons and overall glial cells and size of neuronal somata in Nissl-stained MePD

The number of neurons in Nissl-stained sections was significantly greater in males than in females ($P = 0.024$, $d = 1.1$; Fig. 3A). Such counts, however, did not reveal a significant effect of hemisphere ($P = 0.652$) nor did sex and hemisphere interact significantly ($P =$

0.111). In contrast, we found no significant differences in the number of MePD glia stained with thionine (sex: $P = 0.608$; hemisphere: $P = 0.066$; interaction: $P = 0.938$; Fig. 3B).

The size of neuronal somata in the MePD was not affected by sex ($P = 0.074$) or hemisphere ($P = 0.872$), but these two factors did significantly interact ($P = 0.016$). Post-hoc analysis revealed that MePD neurons were significantly larger in males than in females on the left ($P = 0.014$; $d = 1.2$; Fig. 3C) but not the right ($P = 0.36$).

MePD astrocyte number and complexity

The number of MePD astrocytes was significantly larger in males than in females ($n = 13$; $P = 0.033$, $d = 0.9$), but there were no significant differences between the two sides ($P = 0.80$; Fig. 4A). We did, however, find that sex and hemisphere interacted significantly ($P = 0.015$). Post-hoc analysis revealed that the MePD in females contains more astrocytes on the left than the right ($P = 0.030$, $d = 0.2$) while males displayed no such laterality ($P = 0.144$). Consequently, the sex difference in astrocyte number was significant only on the right ($P = 0.018$, $d = 1.0$), although a similar trend was also present on the left ($P = 0.068$). The density of astrocytes did not depend on sex ($P = 0.49$) or hemisphere ($P = 0.386$), nor did these two factors interact ($P = 0.468$).

We found no significant differences in any of our measures of astrocyte complexity (Fig. 4B, Table 2). Neither the number of primary branches (Fig. 4B), number of branch nodes, branch endings, nor branch length (average length / branch or total) (Table 2) varied with sex or hemisphere (P values ranged from 0.191 to 0.893); neither did these two factors interact (P values ranged from 0.672 to 0.930).

Correlations and rostrocaudal analysis

We found a positive correlation between astrocyte number and volume in GFAP-stained sections but the relationship was significant only in males on the right side along with a trend on the male left (right: $P = 0.008$; $r^2 = 0.7833$ versus male left: $P = 0.018$; $r^2 = 0.7037$; female right: $P = 0.514$; $r^2 = 0.583$ versus female left: $P = 0.793$; $r^2 = 0.278$). Astrocyte complexity also approached being significantly correlated with MePD volume but only on the left in males ($P = 0.027$; $r^2 = 0.6586$) and only on the right in females ($P = 0.037$; $r^2 = 0.5412$). While we also found a positive trend between soma size and MePD regional volume in females ($P = 0.019$; $r^2 = 0.0232$), the correlation score suggests the model does not fit well to the data. The number of neurons did not significantly correlate with MePD volume in Nissl-stained sections in both males (left: $P = 0.188$; $r^2 = 0.499$ versus right: $P = 0.292$; $r^2 = 0.352$) and females (left: $P = .956$; $r^2 = 0.1138$ versus right: $P = 0.756$; $r^2 = 0.3342$). Together, these data suggest that differences in the number of neurons and astrocytes may contribute differences in MePD volume, but the strength of these relationships is not strong. No significant findings in our measures of the rostrocaudal analysis differed from those seen in our overall means and so these data were not included here.

DISCUSSION

Anatomical sex differences in select brain nuclei are thought to underlie sex-specific reproductive behavior. One such example, the MePD, receives vomeronasal signals that are processed and relayed to brain regions tied to sexual reproduction (Scalia and Winans, 1975; Martinez-Marcos, 2009). Previous reports of robust and widespread sex differences in MePD neurons and glia of rats (Johnson et al., 2008, 2012) prompted the current study in mice, where such information is largely lacking. We examined the MePD in normal adult male and female C57Bl/6^{J} mice, staining alternate brain sections from the same animals for either Nissl or GFAP to learn about possible sex differences in MePD neurons and overall glia, in a fashion similar to previous studies with rats. We now report sex differences in the volume of the MePD in C57Bl/6^{J} mice, with males having a larger MePD than females, consistent with previous findings in rats (Cooke et al., 1999; Morris et al., 2008a), Balb/c mice (Morris et al., 2008b), and a wild monogamous rodent species (*Peromyscus californicus*; Campi et al., 2013). Volumes appear nearly identical to the closely related *Mus musculus* Balb/c strain, while the larger *P. californicus*, a separate species, has a slightly bigger MePD (0.14 to 0.28 mm^3), which nonetheless is sexually differentiated even after accounting for whole brain size, necessary due to high genetic variation in wild-caught animals (Campi et al., 2013). In short, a sex difference in MePD volume has now been reported in two different inbred strains of mice and two other rodent species, arguing for a conserved and significant role in sex-specific behavior.

While these volume differences are of interest, the primary goal of our study was to elucidate possible cellular attributes with the MePD that might contribute to such volumetric sex differences. To that end, we find the MePD has more neurons and astrocytes in males than in females, with MePD neurons also having larger somata in males than in females. These results mirror previous findings in rats (Johnson et al., 2008, 2012; Morris et al., 2008a). We did not, however, find evidence of sex differences in astrocyte *complexity* in the mouse MePD, as we previously reported for rats (Johnson et al., 2008). Also of note, the molecular layer, included in the rat atlas and detected in the *P. californicus* (Paxinos and Watson, 2005; Campi et al., 2013), is not visible in the mouse MePD (Fig. 1A–H) and thus does not contribute to our measures of sex differences in the current study. Our findings are consistent with the idea that sex differences in the morphology of the MePD may critically mediate sex differences in chemosensory-dependent behavior. Our characterization of the mouse MePD sets the stage for future studies using cre-lox technology to better understand the cellular mechanisms leading to the expression of sexually differentiated behavior.

Because astrocytes are no longer viewed as just the “glue” between neurons, but rather as active regulators of neuronal function and structure (He and Sun, 2007), the sex difference in astrocyte number in the mouse MePD may play an important role in the formation and/or function of sexually differentiated circuits that engage the MePD. Because MePD astrocytes in male rats express androgen receptors (Johnson et al., 2012), gonadal testosterone may act directly on astrocytes. Astrocytes may then indirectly provide neurotrophic support to developing neurons of the MePD, helping to establish sex differences in the adult number of neurons, as has been suggested for songbirds (Nordeen and Nordeen, 1996; Jordan, 1999). Astrocytes also critically mediate the formation and function of synapses (Ullian et al.,

2001; Eroglu and Barres, 2010) and are implicated in the hormone-dependent regression of synapses underlying female receptivity (Garcia-Segura et al., 1999; Garcia-Segura and Melcangi, 2006). Moreover, sex discrimination is perturbed in knockout models lacking the glial-specific glutamate transporter (Grosjean et al., 2008), further suggesting that astrocytes may well confer sexually differentiated function in the MePD. Indeed, our correlation data suggests that differences in the number of astrocytes may contribute to differences in MePD volume in males only. In the female brain, it seems likely that all cell types contribute in some way to volume differences rather than relying on a single cell population. Taken together, this indicates that the sex difference in the mouse MePD volume is a product of sex differences in astrocyte number.

We find that the larger MePD in adult C57BL/6^{J} male mice contains significantly more neurons than the smaller MePD of females. While it is tempting to conclude that more neurons lead to larger brain regions, our correlational analyses suggest this relationship is by no means assured. For example, while the MePD in rats is both larger and contains more neurons overall in males than in females, this relationship breaks down when one considers which side of the rat brain is being examined. MePD volume is larger on the right but contains more neurons on the left in both sexes in rats, dissociating regional volume from neuronal number (Morris et al., 2008b). Likewise, in the current study volume of the MePD was lateralized in both sexes in Nissl-stained MePD, being larger on the left than on the right. However, the number of neurons was not lateralized, indicating that for the MePD of both rats and mice, more neurons are sometimes packed into less volume on one side versus the other (i.e., neuronal density in the MePD is not equivalent across sides for either C57Bl/6^{J} mice or rats). We also found that neuronal soma size is sexually differentiated, but only on the left side, where males have larger neuronal somata than females. Hence, soma size may contribute to laterality differences in MePD volume, but only in males. Clearly, another common theme for both rats and mice is that the MePD is lateralized, evident at the level of volume and at the level of its individual cellular constituents, neurons and overall glia. An obvious ancillary theme is that sex differences in the cellular makeup of the MePD is sometimes relevant to only one side, such as the sex differences in astrocyte number and soma size only on one side but not the other. Figure 5 is a schematic showing this complex interplay between sex differences and lateralization for regional volume, neurons, and astrocytes in the MePD of rats and mice. Note that fewer measures are lateralized for the MePD of C57BL/6^{J} mice than rats, and astrocyte complexity only varies within the rat brain.

When estimating the number of overall glia in Nissl-stained MePD, we found no sex difference in their number, despite finding a significant sex difference in astrocyte number. This may indicate that oligodendrocytes and/or microglia do not vary by sex, possibly diluting out the sex difference in astrocytes (which are included in our counts of total glia from Nissl-stained MePD). It is also possible the individual glial types have opposing sex differences. However, using Nissl-stained material for estimating the number of overall glia may be less accurate, because it relies on more subtle differences in staining to sort out glia from neurons, compared to cell-specific labels, such as the antiserum directed against GFAP to specifically mark astrocytes. As a result, there may have been errors in the classification process for counting overall glial cells. Indeed, our analysis of astrocytes in GFAP-stained sections unveiled a robust sex difference in this type of glial cell that would not have been

detected if our analyses relied solely on Nissl staining. Future analyses with specific cell markers may yet reveal sex differences in other glial cell types within the MePD.

Although MePD astrocytes in mice are sexually differentiated in number, as in rats, we did not find sex differences in the complexity of their arbors, unlike our previous findings in rats (Johnson et al., 2008). Because the same approach used in rats did not reveal a sex difference in arbor complexity in mice, astrocyte complexity may not critically mediate sexually differentiated functions of the MePD in all rodents. It is also possible that ovarian hormones regulate the length of MePD astrocytes (Martinez et al., 2006), leading to sex differences in complexity at one point in the cycle but not at other times. We did not control for fluctuations in ovarian hormones in the current study.

It is also of interest that results based on Nissl staining reveal significant laterality differences in MePD volume for both males and females that is not detected when the MePD was stained for GFAP and DNA (hematoxylin). In females, the lateralization appears to emerge because of less variance in the estimate of volume for the right MePD. Interestingly, for males, Nissl staining leads to an apparent increased size of the left MePD, suggesting that the apparent boundaries of the MePD depend on the kind of stain used, although the effect in this case is selective to males only on the left side. These results are a reminder that the ability to detect anatomical differences will likely depend on the visualization method used.

An important question is raised by these observations: What are the mechanisms involved in establishing such sex and laterality differences in the mouse MePD? Based on current evidence, the sex difference in MePD volume and neuronal soma size in both rats and mice appears largely a function of adult hormone levels (Cooke et al., 1999; Morris et al., 2008a,b), whereas the number of MePD neurons in rats appears fixed by adulthood, and is not affected by changes in adult gonadal hormone levels (Morris et al., 2008a). On the other hand, while the number and complexity of astrocytes in the rat MePD are each sensitive to changes in adult gonadal hormones, those sex differences are not eliminated when testosterone levels are equalized in adult males and females (Johnson et al., 2012), suggesting that sex differences in astrocyte number and complexity may develop under the control of gonadal hormones earlier in life, as is likely the case for MePD neuronal number. Our work in rats also shows that such sex differences in MePD astrocytes critically depend on functional androgen receptors (ARs) since MePD astrocytes in male rats with defective ARs are completely feminized (Johnson et al., 2008). While there is no information to date about whether ARs also play a critical role in determining adult sex differences in neuron number in the MePD, existing evidence in rats indicates that both MePD volume and neuronal soma size do depend on ARs, but only partially (Morris et al., 2008a), so sex differences in these particular attributes of the rat MePD also reflect the influence of other factors. MePD astrocytes in rats show somewhat the same theme; while the sex difference in astrocyte number is established before the onset of puberty, independent of ARs, maintaining the sex difference in number after the onset of puberty depends exclusively on AR (Johnson et al., 2013). On the other hand, adult sex differences in astrocyte complexity emerge sometime after puberty begins, when most of the adult astrocytic arbor develops, and depends exclusively on ARs (Johnson et al., 2013). Moreover, because both adult neurons

and astrocytes in rats express ARs (Wood et al., 1992; Johnson et al., 2012), both represent candidate sites of androgen action for driving morphological sex differences in the MePD. Important caveats include that we do not yet know whether neurons and astrocytes express AR in the mouse MePD, and if so, when during the lifespan do MePD neurons and astrocytes begin to express ARs.

While the sex differences in mouse and rat brains likely have a function in producing sex-typical responses to olfac-tory stimuli, the function of lateralization in the mouse and rat brain is less apparent. Interestingly, information passes from nostril to hemisphere on the ipsilateral side in both humans and mice, restricting both stimulation and brain processing to one hemisphere (Gordon and Sperry, 1969; Kucharski et al., 1986). The conserved motif of MePD laterality in rodents likely provides unique physiology to each nostril and its associated hemisphere. Indeed, several mammals show lateralized functions for nostril use (Siniscalchi et al., 2011, 2015). Discrimination between odors and detection of novel stimuli is a well-documented function of the right nostril and likely the right hemisphere (Zatorre and Jones-Gotman, 1990; Savic and Berglund, 2000). Arousing stimuli detected on the right have greater control over homeostasis and behavioral responses, indicating this hemisphere may also have greater control over physiological responses to odors (Siniscalchi et al., 2015). In addition, as stimuli are repeated over time, the right-nostril dominance in the detection of novel stimuli is replaced by left-nostril recognition of routine olfactory investigations (Siniscalchi et al., 2011). This may be even more specialized depending on the stimulus presented. For example, in rats, removing the left olfactory bulb but not the right leads to deficiencies in behavioral responses to a stressed conspecific (Dantzer et al., 1990). Laterality and sex differences in laterality within the MePD may represent individual and sex-specific differences in the processing of novel, familiar, and/or arousing stimuli.

In sum, we found several sexually differentiated attributes of the mouse MePD, including volume, the number of neurons and astrocytes, and in the size of neuronal somata. Some sex differences were evident only on one side, while others were present on both sides, indicating a possible contribution of laterality to sex-typical and olfactory-mediated behaviors. The current data demonstrate that the MePD in C57Bl/6^{J} mice is broadly sexually differentiated, implicating its role in sex-specific behavior triggered by the detection and discrimination of sex-specific chemosensory cues. These findings provide strong rationale for future studies using genetically engineered mice to identify and understand the cellular mechanisms behind morphological sex differences in the brain and their role in the expression of sex-specific behavior.

ACKNOWLEDGMENTS

The authors thank Dr. Deborah Kashy and Dr. Casey Henley for help with statistical analysis, and Kate Mills and Diane Redenius for technical support.

LITERATURE CITED

Cadiz-Moretti B, Otero-Garcia M, Martinez-Garcia F, Lanuza E. Afferent projections to the different medial amygdala subdivisions: a retrograde tracing study in the mouse. Brain Struct Funct. 2014 [Epub ahead of print].

Cahill L, Uncapher M, Kilpatrick L, Alkire MT, Turner J. Sex-related hemispheric lateralization of amygdala function in emotionally influenced memory: an FMRI investigation. Learn Mem. 2004; 11:261–266. [PubMed: 15169855]

Campi KL, Jameson CE, Trainor BC. Sexual dimorphism in the brain of the monogamous California mouse (Peromyscus californicus). Brain Behav Evol. 2013; 81:236–249. [PubMed: 23881046]

Clancy AN, Coquelin A, Macrides F, Gorski RA, Noble EP. Sexual behavior and aggression in male mice: involvement of the vomeronasal system. J Neurosci. 1984; 4:2222–2229. [PubMed: 6541245]

Cooke BM, Tabibnia G, Breedlove SM. A brain sexual dimorphism controlled by adult circulating androgens. Proc Natl Acad Sci U S A. 1999; 96:7538–7540. [PubMed: 10377450]

Cooke BM, Breedlove SM, Jordan CL. Both estrogen receptors and androgen receptors contribute to testosterone-induced changes in the morphology of the medial amygdala and sexual arousal in male rats. Horm Behav. 2003; 43:336–346. [PubMed: 12694644]

Dantzer R, Tazi A, Bluthé R-M. Cerebral lateralization of olfactory-mediated affective processes in rats. Behav Brain Res. 1990; 40:53–60. [PubMed: 2278657]

de Olmos J, Hardy H, Heimer L. The afferent connections of the main and the accessory olfactory bulb formations in the rat: an experimental HRP-study. J Comp Neurol. 1978; 181:213–244. [PubMed: 690266]

DiBenedictis BT, Helfand AI, Baum MJ, Cherry JA. A quantitative comparison of the efferent projections of the anterior and posterior subdivisions of the medial amygdala in female mice. Brain Res. 2014; 1543:101–108. [PubMed: 24262912]

Eroglu C, Barres BA. Regulation of synaptic connectivity by glia. Nature. 2010; 468:223–231. [PubMed: 21068831]

Garcia-Segura LM, Melcangi RC. Steroids and glial cell function. Glia. 2006; 54:485–498. [PubMed: 16906540]

Garcia-Segura LM, Naftolin F, Hutchison JB, Azcoitia I, Chowen JA. Role of astroglia in estrogen regulation of synaptic plasticity and brain repair. J Neurobiol. 1999; 40:574–584. [PubMed: 10453057]

Gordon H, Sperry R. Lateralization of olfactory perception in the surgically separated hemispheres of man. Neuropsychologia. 1969; 7:111–120.

Grosjean Y, Grillet M, Augustin H, Ferveur JF, Featherstone DE. A glial amino-acid transporter controls synapse strength and courtship in Drosophila. Nat Neurosci. 2008; 11:54–61. [PubMed: 18066061]

Gutierrez-Castellanos N, Pardo-Bellver C, Martinez-Garcia F, Lanuza E. The vomeronasal cortex — afferent and efferent projections of the posteromedial cortical nucleus of the amygdala in mice. Eur J Neurosci. 2014; 39:141–158. [PubMed: 24188795]

Halem HA, Cherry JA, Baum MJ. Vomeronasal neuroepithelium and forebrain Fos responses to male pheromones in male and female mice. J Neurobiol. 1999; 39:249–263. [PubMed: 10235679]

He F, Sun YE. Glial cells more than support cells? Int J Biochem Cell Biol. 2007; 39:661–665. [PubMed: 17141551]

Johnson RT, Breedlove SM, Jordan CL. Sex differences and laterality in astrocyte number and complexity in the adult rat medial amygdala. J Comp Neurol. 2008; 511:599–609. [PubMed: 18853427]

Johnson RT, Schneider A, DonCarlos LL, Breedlove SM, Jordan CL. Astrocytes in the rat medial amygdala are responsive to adult androgens. J Comp Neurol. 2012; 520:2531–2544. [PubMed: 22581688]

Johnson RT, Breedlove SM, Jordan CL. Androgen receptors mediate masculinization of astrocytes in the rat posterodorsal medial amygdala during puberty. J Comp Neurol. 2013; 521:2298–2309. [PubMed: 23239016]

Jordan CL. Glia as mediators of steroid hormone action on the nervous system: an overview. J Neurobiol. 1999; 40:434–445. [PubMed: 10453047]

Keverne EB. Visualisation of the vomeronasal pheromone response system. Bioessays. 2008; 30:802–805. [PubMed: 18693258]

Kilpatrick LA, Zald DH, Pardo JV, Cahill LF. Sex-related differences in amygdala functional connectivity during resting conditions. Neuroimage. 2006; 30:452–461. [PubMed: 16326115]

Kucharski D, Johanson IB, Hall W. Unilateral olfactory conditioning in 6-day-old rat pups. Behav Neural Biol. 1986; 46:472–490. [PubMed: 3814049]

Martinez FG, Hermel EE, Xavier LL, Viola GG, Riboldi J, Rasia-Filho AA, Achaval M. Gonadal hormone regulation of glial fibrillary acidic protein immunoreactivity in the medial amygdala subnuclei across the estrous cycle and in castrated and treated female rats. Brain Res. 2006; 1108:117–126. [PubMed: 16842763]

Martinez-Marcos A. On the organization of olfactory and vomeronasal cortices. Prog Neurobiol. 2009; 87:21–30. [PubMed: 18929620]

Meredith M, Fewell G. Vomeronasal organ: electrical stimulation activates Fos in mating pathways and in GnRH neurons. Brain Res. 2001; 922:87–94. [PubMed: 11730705]

Morris JA, Jordan CL, Breedlove SM. Sexual dimorphism in neuronal number of the posterodorsal medial amygdala is independent of circulating androgens and regional volume in adult rats. J Comp Neurol. 2008a; 506:851–859. [PubMed: 18076082]

Morris JA, Jordan CL, King ZA, Northcutt KV, Breedlove SM. Sexual dimorphism and steroid responsiveness of the posterodorsal medial amygdala in adult mice. Brain Res. 2008b; 1190:115–121. [PubMed: 18054901]

Nordeen EJ, Nordeen KW. Sex difference among non-neuronal cells precedes sexually dimorphic neuron growth and survival in an avian song control nucleus. J Neurobiol. 1996; 30:531–542. [PubMed: 8844516]

Paxinos, G.; Franklin, KB. The mouse brain in stereotaxic coordinates. Gulf Professional Publishing; Houston, TX: 2004.

Rasia-Filho AA, Haas D, de Oliveira AP, de Castilhos J, Frey R, Stein D, Lazzari VM, Back F, Pires GN, Pavesi E, Winkelmann-Duarte EC, Giovenardi M. Morphological and functional features of the sex steroid-responsive posterodorsal medial amygdala of adult rats. Mini Rev Med Chem. 2012; 12:1090–1106. [PubMed: 22827219]

Savic I, Berglund H. Right-nostril dominance in discrimination of unfamiliar, but not familiar, odours. Chem Senses. 2000; 25:517–523. [PubMed: 11015323]

Scalia F, Winans SS. The differential projections of the olfactory bulb and accessory olfactory bulb in mammals. J Comp Neurol. 1975; 161:31–55. [PubMed: 1133226]

Siniscalchi M, Sasso R, Pepe AM, Dimatteo S, Vallortigara G, Quaranta A. Sniffing with the right nostril: lateralization of response to odour stimuli by dogs. Anim Behav. 2011; 82:399–404.

Siniscalchi M, Padalino B, Aube L, Quaranta A. Right-nostril use during sniffing at arousing stimuli produces higher cardiac activity in jumper horses. Laterality. 2015; 20:483–500. [PubMed: 25635853]

Ullian EM, Sapperstein SK, Christopherson KS, Barres BA. Control of synapse number by glia. Science. 2001; 291:657–661. [PubMed: 11158678]

Wood RI, Brabec RK, Swann JM, Newman SW. Androgen and estrogen concentrating neurons in chemosensory pathways of the male Syrian hamster brain. Brain Res. 1992; 596:89–98. [PubMed: 1468005]

Zatorre RJ, Jones-Gotman M. Right-nostril advantage for discrimination of odors. Percept Psychophys. 1990; 47:526–531. [PubMed: 2367173]

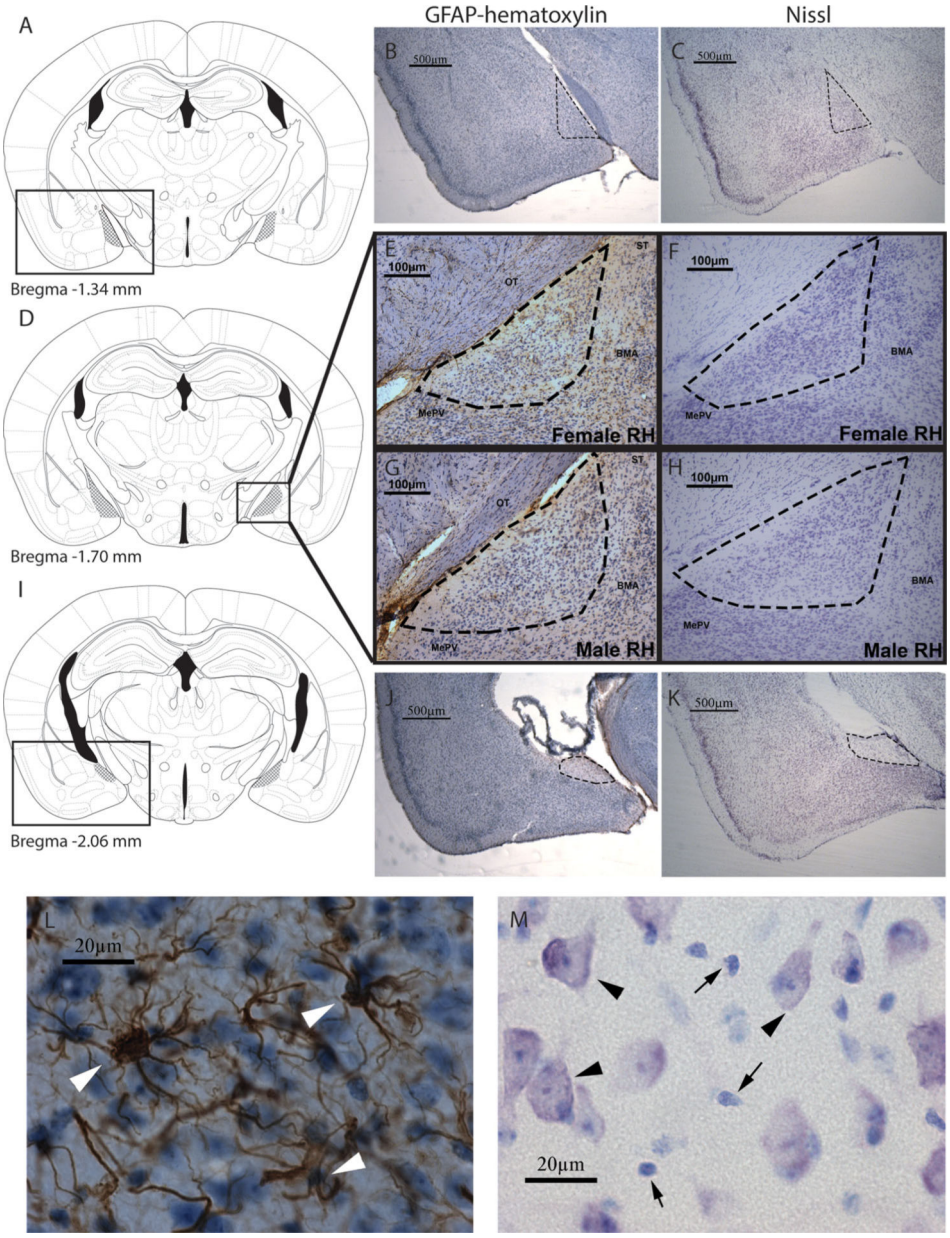

Figure 1.

The adult MePD in adult male and female mice was stained for the astrocytic protein, glial fibrillary acidic protein (GFAP) and counterstained with hematoxylin, or stained for Nissl substance using thionin. The most rostral portions **(A)** are easily discernible in GFAP +hematoxylin **(B)** and Nissl **(C)**-stained tissues. Marked sex differences in the cross-sectional area of the MePD are revealed in representative sections from middle portion of the MePD **(D–H)**. The caudal-most portion of the MePD **(I)** is also clearly visible in sections stained for either GFAP **(J)** or Nissl **(K)**. GFAP+hematoxylin-stained tissue **(L)** was used to analyze astrocyte (white arrowheads) number and process complexity of astrocytes. Nissl-stained tissue **(M)** was used to quantify total glia (black arrows) and neurons (black arrowheads) in the MePD of adult male and female mice.

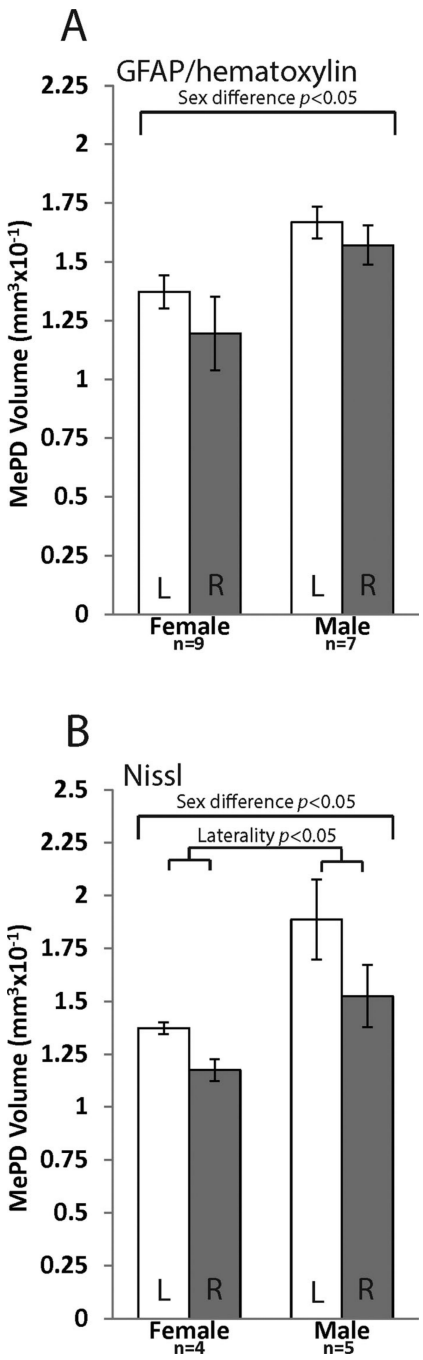

Figure 2.

Regional volume of the MePD in adult C57Bl/6^{J} mice is sexually dimorphic. Males have a larger MePD than females as defined by either **(A)** GFAP (counterstained with hematoxylin) or **(B)** Nissl staining. The MePD is also significantly larger on the left than the right when collapsed across sex in Nissl-stained sections.

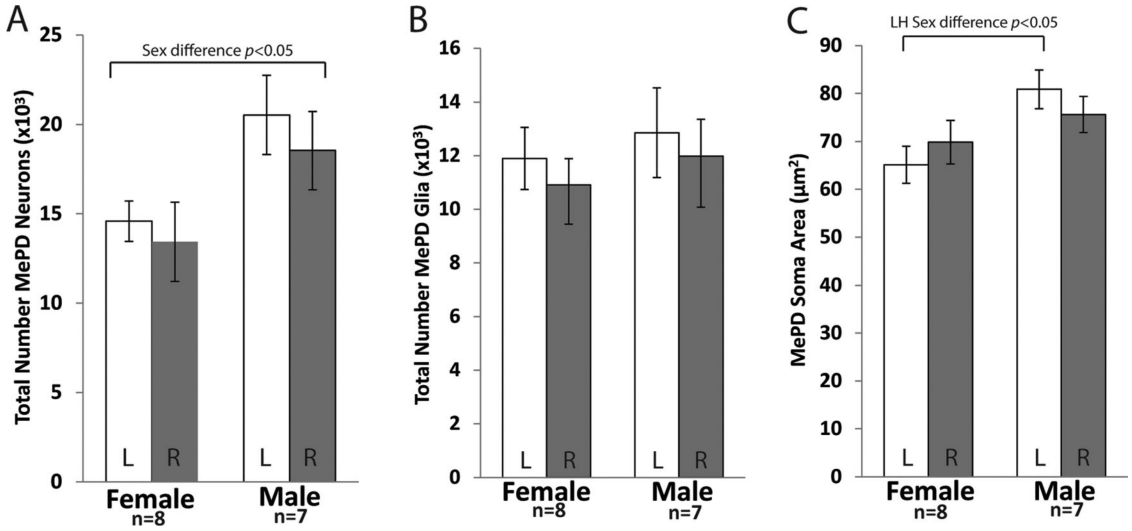

Figure 3.

The number and size of MePD neurons is sexually dimorphic in adult C57Bl/6J mice. Male mice have significantly more MePD neurons than females **(A)**. However, number of overall glial cells in Nissl-stained MePD is not different across sex or side despite a modest trend towards a left-bias in total glial cell number overall **(B)**. MePD neurons in males also have significantly larger somata than in females but only in the left hemisphere **(C)**.

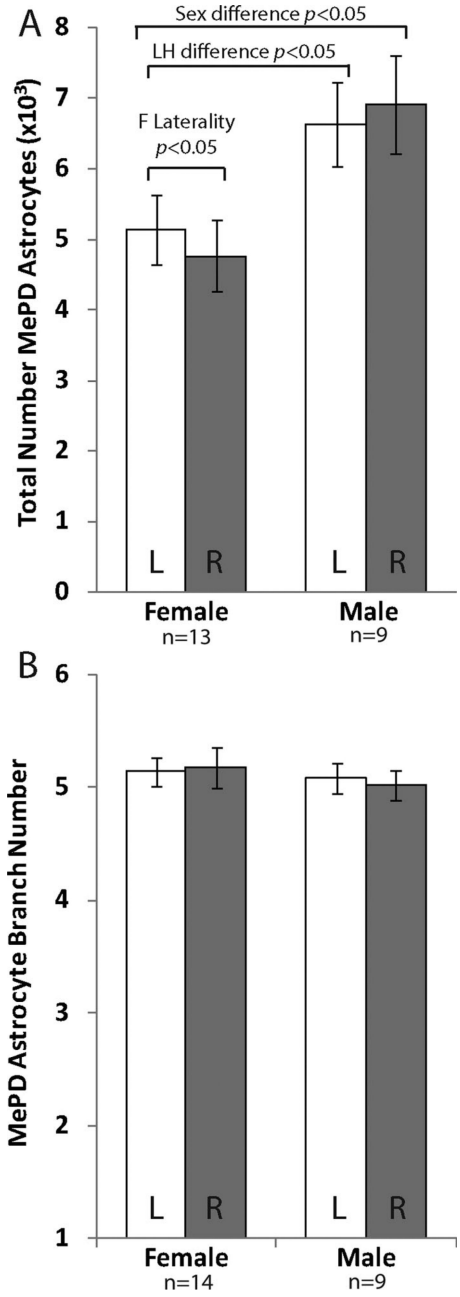

Figure 4.

The number of astrocytes in the MePD of adult C57Bl/6J mice is both sexually differentiated and lateralized. Males have more astrocytes than females in both hemispheres, whereas the number of MePD astrocytes is lateralized only in females, with more astrocytes on the left than the right **(A)**. Astrocytes have the same number of branches on both sides of the MePD and are not different in males and females **(B)**. Other measures of astrocyte complexity in these same animals are also neither sexually differentiated nor lateralized (Table 2).

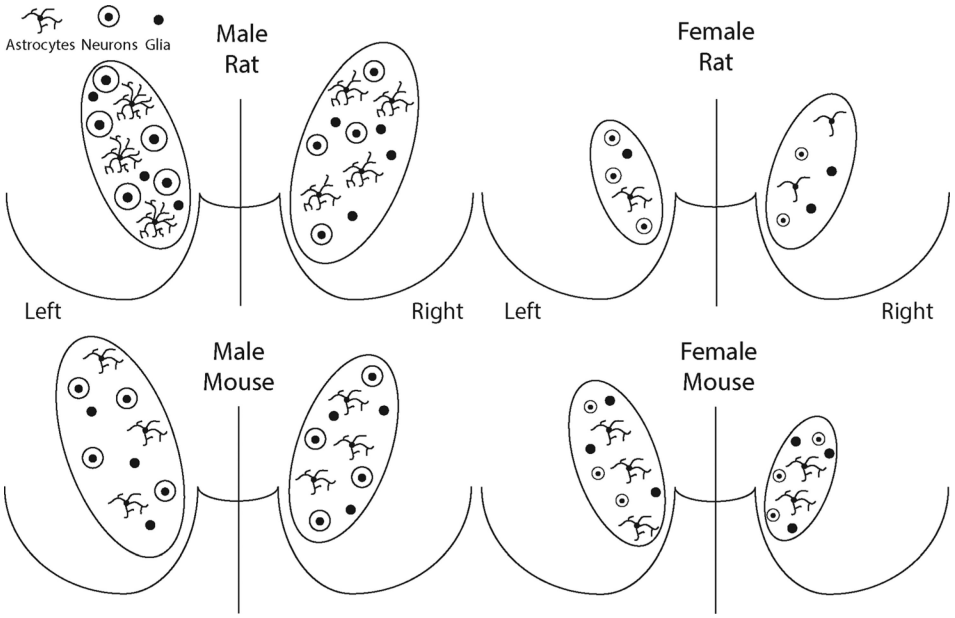

Figure 5.

Schematic summarizing sex and laterality differences in MePD cytoarchitecture in adult rats and mice. Neuron and glial cell number was measured in Nissl-stained sections, whereas number and arbor complexity of astrocytes were measured in GFAP-stained sections. Schematic is based on data reported in Cooke et al., 1999, 2003; Johnson et al., 2008, 2012, 2013; Morris et al., 2008a,b.

TABLE 1

List of Antibodies Used for GFAP Immimohistochemistry

Antigen	Description of immunogen	Source, host species, cat. #, clone or lot #, RRID	Concentration used
Glial fibrillary acidic protein clone GA5	Purified porcine spinal cord glial fibrillary acidic protein	EMD Millipore, mouse monoclonal, Cat# MAB360, RRID:AB_2109815	0.02 μl/ml
Antimouse IgG	Mouse IgG (H+L), rat absorbed, biotinylated	Vector Laboratories, horse polyclonal, Cat# BA-2001, RRID:AB_2336180	0.4 μl/ml

TABLE 2

Astrocyte Complexity in the MePD of C57BI/6J Mice Displays Neither Significant Sex Nor Laterality Differences

	Female C57Bl/6^{J} mice (N = 14)		Male C57Bl/6^{J} mice (N = 9)	
Hemisphere	***Left***	***Right***	***Left***	***Right***
Branch #	5.14 ± 0.12	5.18 ± 0.17	5.08 ± 0.13	5.02 ± 0.14
Node #	9.15 ± 0.73	8.74 ± 0.64	10.03 ± 0.96	8.86 ± 0.82
# Endings	14.46 ± 0.82	14.14 ± 0.80	16.22 ± 1.63	14.05 ± 0.96
Avg. Length	39.58 ± 2.21	39.09 ± 2.02	43.36 ± 2.65	41.96 ± 2.31
Total Length	199.10 ± 12.0	200.76 ± 15.0	216.84 ± 18.9	203.80 ± 14.97

Values are mean ± SEM.