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Inhibitory and Multisynaptic Spines, and Hemispherical Synaptic Specialization in the Posterodorsal Medial Amygdala of Male and Female Rats

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Abstract

The density of dendritic spines is sexually dimorphic and variable throughout the female estrous cycle in the rat posterodorsal medial amygdala (MePD), a relevant area for the modulation of reproductive behavior in rats. The local synaptic activity differs between hemispheres in prepubertal animals. Here, we used serial section transmission electron microscopy to produce three-dimensional reconstructions of dendritic shafts and spines to characterize synaptic contacts on MePD neurons of both hemispheres in adult males and in females along the estrous cycle. Pleomorphic spines and non-synaptic filopodia occur in the MePD. On average, 8.6% of dendritic spines received inputs from symmetric GABA-immunoreactive terminals, whereas 3.6% received two synaptic contacts on the spine head, neck or base. Presynaptic terminals in females right MePD had a higher density of synaptic vesicles and docked vesicles than the left MePD, suggesting a higher rate of synaptic vesicle release in the right MePD of female rats. In contrast, males did not show laterality in any of those parameters. The proportion of putative inhibitory synapses on dendritic shafts in the right MePD of females in proestrus was higher than in the left MePD, and higher than in the right MePD in males, or in females in diestrus or estrus. This work shows synaptic laterality depending on sex and the estrous cycle phases in mature MePD neurons. Most likely, sexual hormones effects are lateralized in this brain region, leading to higher synaptic

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Conflict of Interest

The authors declare no conflict of interest

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: JB, AARF, JM. Acquisition of data: JB, SM, ETI. Analysis and interpretation of data: JB, RSP, AARF, JM. Writing of the manuscript: JB, SM, RSP, BK, AARF, JM. Statistical analysis: AARF.

activity in the right than in the left hemisphere of females, mediating timely neuroendocrine and social/reproductive behavior.

Keywords

extended amygdala; 3D reconstructions; electron microscopy; laterality; inhibitory dendritic spines; multisynaptic dendritic spines

Introduction

Synaptic organization and plasticity has been the focus of intense investigation. One of the relevant brain areas of interest is the posterodorsal medial amygdala (MePD), a complex subcortical forebrain area in the extended amygdala (de Olmos et al. 2004) that participates in the interpretation of olfactory/vomeroneasal (Meredith and Westberry 2004) and genitosensorial information (Oberlander and Erskine 2008), and modulates social behaviors in both male and female rats (Newman 1999; Choi et al. 2005; Rasia-Filho et al. 2012a,b). The male MePD modulates mainly the intromission and ejaculatory behaviors (Coolen et al., 1996; Rasia-Filho et al., 2012b). In females, the MePD is involved in the regulation of hypothalamic neuroendocrine secretion of gonadotrophin releasing hormone and prolactin (Polston et al., 2001; Simerly, 2004), and sexual (Coolen et al., 1996; Pfaus and Heeb 1997) and maternal behavior (Sheehan et al., 2000). The MePD is highly sensitive to gonadal hormone actions (Simerly et al. 1990; Österlund et al. 1998; Cooke et al. 1999; Gréco et al. 2003), and the volume of the MePD (Hines et al. 1992; Cooke et al. 1999), its neuronal (Morris et al. 2008) and glial densities (Johnson et al. 2008), neuronal volume (Hermel et al. 2006), dendritic orientation (Dall'Oglio et al. 2008a,b), and synaptic contacts (Nishizuka and Arai 1983a) are all sexually dimorphic and/or change along the estrous cycle. In such a manner, the MePD is considered a relevant brain area to promote timely physiological changes in neuroendocrine secretion and behavioral display according to different stimuli and social demands perceived by the animal (Newman, 1999; Choi et al., 2005; Rasia-Filho et al., 2012a,b).

In young Sprague-Dawley rats, males have more neurons in the right MePD than females, with no difference between sexes in the left MePD. In these same animals, mini excitatory postsynaptic current (mEPSC) frequency and the number of excitatory synapses is 80% higher in the left MePD of males than in females, with no difference in the right MePD (Cooke and Woolley 2005). Also, the MePD of adult males shows a higher density of dendritic spines than in females in proestrus, estrus or metestrus, but not diestrus (Rasia-Filho et al. 2004, 2012a). Dendritic spines are the primary sites of excitatory synapses on neurons and are subcellular compartments integral to synaptic plasticity (Shepherd 1996; Nimchinsky et al. 2002; Tsay and Yuste 2004). The morphology of a spine can change rapidly through activity-dependent mechanisms (Fu et al., 2012; Lai et al., 2012). Changes in the morphology, number and distribution of dendritic spines impact the strength and integration of synaptic inputs, and circuit connectivity (Harris and Kater 1994; Tsay and Yuste 2004; Hayashi and Majewska 2005; Kitanishi et al. 2009). Typically, spines receive one excitatory contact in adults (Farb et al. 1992; Arellano et al. 2007). However, dendritic

spines also receive symmetric inhibitory synapses (Harris et al. 1992; Cooke and Woolley 2005; Kubota et al. 2007), or form synapses with two or more axonal boutons (Genoud et al. 2004; Stewart et al. 2005a,b; Popov and Stewart 2009). Prepubescent Sprague-Dawley rats receive structurally symmetric and asymmetric inputs on MePD dendritic spines (Cooke and Woolley 2005), but there are no available data with such details available for the MePD of adult animals. This is a relevant issue because excitatory synapses and number of spines in the MePD are related to the development of male-typical rough-and-tumble play (Cooke and Woolley, 2009), whereas adult castration reduces the local density of spines at the time of impairment of sexual behavior in rats (de Castilhos et al., 2008).

To better understand the MePD regulation on neuroendocrine and social/reproductive behavior, we studied the different dendritic spine shapes and their ultrastructural connections. We also asked whether the laterality found previously on prepubescent rats is maintained in adults, if this laterality changes throughout the female estrous cycle, and if inhibitory contacts on dendritic spines are present in adult male and female rats. Using three-dimensional reconstruction of serial sections imaged with transmission electron microscopy (TEM), we demonstrate GABAergic and multisynaptic contacts on dendritic spines, and synaptic laterality depending on sex and estrous cycle phase in adult rats. Males did not show laterality, but females in proestrus presented a higher ratio of inhibitory to excitatory synapses on dendritic shafts in the right MePD than in the left MePD. Also in females, presynaptic terminals in the right MePD had a higher density of synaptic vesicles and docked vesicles, indicators of higher synaptic activity, than in the left MePD.

Materials and Methods

Animals

Five 3-month-old male Wistar rats and 6 age-matched virgin females were housed at 21°C with free access to food and water on a 12 h light/dark cycle (lights on at 6 am). Females had their estrous cycle phase determined daily in the late afternoon by vaginal smears examined under a light microscope, following cytological criteria (Montes and Luke 1988; Singletary et al. 2005). The estrous cycle was monitored for 3 weeks before euthanasia, and only regularly cycling females in diestrus, proestrus or estrus were used in this study. For ultrastructural analysis and 3D reconstructions, two animals per group were utilized (males, females in diestrus, proestrus and estrus), an acceptable number when ultrastructural morphometry is used along with statistical analysis and 3D reconstruction of ultra-thin sections. The amount of subcellular structures were recorded, as different kind of vesicles and different forms of spines could account for minor biological variations. When variations were much over the limits of high or low numbers or densities, a new animal would be used for comparison. To confirm that symmetric contacts found on MePD dendritic spines were in fact GABAergic, another 3 male rats were used for immunolabeling of GABA after freeze substitution. Metestrus females were not included in our experiments because there were no changes in the density of dendritic spines (Rasia-Filho et al, 2004), cell body volume (Hermel et al., 2006) and GFAP reactivity (Martinez et al., 2006) between the phases of metestrus and estrus in the MePD.

All experimental procedures were approved by the Animal Ethics Committee of the São Paulo University, School of Medicine at Ribeirão Preto (Protocol No. 045/2008), and are in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85–23, reviewed 1985).

Transmission electron microscopy

The animals were anesthetized with intraperitoneal ketamine and xylazine (80 mg/kg and 10 mg/kg, respectively) and fixed with rapid transcardiac perfusion of 600 ml of 2% paraformaldehyde, 2% glutaraldehyde and 0.05% of CaCl_2 in 0.1 M cacodylate buffer, pH 7.4, at room temperature (RT) for ultrastructural synaptic preservation. Rats were decapitated and heads maintained in the same fixative solution for 4 h at RT, followed by removal of the brain, rinsing, separating the brain hemispheres, and sectioning into 100- μm -thick coronal slices using a Vibratome VT 1000S (Leica Microsystems, Germany). Slices were postfixed in 4% glutaraldehyde in cacodylate buffer for 2 h, then in 1% osmium tetroxide in cacodylate buffer for 1 h, rinsed with 0.1 M sodium acetate buffer, pH 5.0, and placed in 0.4% uranyl acetate in sodium acetate buffer under gentle agitation overnight at 4°C (Tao-Cheng et al. 2007). After rinsing and dehydrating in a graded series of ethanol solutions and in propylene oxide, samples were infiltrated in Embed 812 resin (Electron Microscopy Sciences - EMS, USA), and polymerized between Aclar sheets (Ted Pella, USA) at 60°C for 48 h. The MePD was dissected following the coordinates of the Paxinos and Watson rat brain atlas (1998; approximately 3.0 – 3.3 mm posterior to the bregma, ventral to the stria terminalis and lateral to the optic tract; Rasia-Filho et al. 2004; de Castilhos et al. 2006; Arpini et al. 2010), and glued to a resin chuck for sectioning. Semithin sections (0.5 μm) were stained on glass slides with toluidine blue to visualize and trim the MePD under light microscopy. Ultrathin (60 – 70 nm) serial sections were mounted on copper single slot grids (SynapTek™ 1 × 2 mm slot, Ted Pella) coated with Formvar (Ted Pella).

Morphological analysis

The ultrastructure of synaptic contacts on dendritic shafts and dendritic spines was analyzed in an area of 450 to 900 μm^2 containing 300 – 400 synapses per group in both hemispheres. Pictures were taken from 5 different sequences across the MePD block, each one separated by 20 μm . Using TEM (Jeol 1010, Japan; or Zeiss EM 10, Germany), pictures of the neuropil were taken randomly, at magnifications of 12,000 × or 20,000 ×.

Synapses were identified by the presence of a pre- and postsynaptic membranes apposition, synaptic cleft, pre- and postsynaptic densities, and clustering of synaptic vesicles near the presynaptic membrane. Synaptic terminals were morphologically classified as inhibitory when their PSD was less evident or symmetric, and more than 40% of synaptic vesicles were small, flattened, and electron-lucent (Fig 1H), and as excitatory if the postsynaptic density (PSD) was prominent (characterized as asymmetric in relation to the presynaptic terminal) and with over 80% of round and small (40 – 60 nm) electron-lucent synaptic vesicles (Gray, 1959; Andersen et al. 1987; Dall'Oglio et al. 2013; Fig 1I). Synaptic vesicles with lengths twice as much as their width were considered flattened (Uchizono 1965; Matsuda et al. 2004; Dall'Oglio et al. 2013). Docked vesicles were the ones with no

separation between vesicle and presynaptic membrane (Fig 1J). The presynaptic area, synaptic vesicles diameter and number of presynaptic vesicles were measured/counted using Image J software (<http://rsbweb.nih.gov/ij/>; NIH, EUA).

For 3D reconstructions, the same neuropil area of every serial section was photographed, and series of 200 to 450 digital images were aligned. Eight dendritic shafts, parent dendritic spines and synaptic contacts were identified, manually outlined and three-dimensionally reconstructed with the aid of the Reconstruct software (Reconstruct 1.0.6.1; available from <http://synapses.clm.utexas.edu/>; Fiala and Harris 2001; Fiala 2005). Different morphological features of dendritic spines (presence and length of a neck, number of protrusions, head diameter and shape) allowed their classification as thin, mushroom-like, stubby or wide (Stuart et al. 1999; Fiala and Harris, 1999; Arellano et al., 2007; Brusco et al., 2010; Fig 1A).

Antibody Characterization

The primary rabbit polyclonal anti-GABA antibody (A2052; Sigma, Germany) was produced using GABA-BSA as the immunogen, and was isolated from antiserum by immunospecific methods of purification. Antigen specific affinity isolation removes essentially all rabbit serum proteins, including immunoglobulins, which do not specifically bind to GABA. Anti-GABA showed positive binding with GABA, and GABA-KLH in a dot blot assay, and negative binding with BSA (manufacture's datasheet). This antibody was previously used on TEM in a similar experiment (Lu et al., 2009), and it is listed in the JCN antibody database V.12 (ref # 2328 to 2333 and 2340 to 2351). In our experiments, the GABA polyclonal antibody stained as predicted. Gold particles were seen just in symmetric synapses and not in asymmetric terminals (Fig 4A – 4C), and the same terminals were identically labeled in serial-sections (Fig 4D).

Immunogold electron microscopy

Wistar rats were perfused with a solution of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.12 M phosphate buffer. Brains were immersed for 2 h in the same fixative solution at 4°C, and rinsed in 4% glucose. Coronal sections (100 µm-thick) were cut with a VT 1000S Vibratome (Leica Microsystems). The MePD region was dissected, and samples rinsed in increasing concentrations of glycerol in phosphate buffer (10%, 20% and 30%) for 30 min each, and left overnight in 30% glycerol.

MePD samples were rapidly submerged in Freon cooled by liquid nitrogen, and then immersed in 1.5% uranyl acetate in methanol for 24 h at –90°C in an automatic freeze-substitution system unit (Leica EM-AFS, Leica Microsystems). The temperature was increased in steps of 4°C/h from –90°C to –45°C, and samples were rinsed in methanol, and infiltrated with Lowicryl HM20 resin (EMS) using increasing concentrations of Lowicryl (60%, 75% and 100%) in methanol for 48 h at –45°C. Blocks were polymerized with UV light (360 nm) at an initial temperature of –45°C, increasing 1°C/h until RT (Moreira et al. 1996, 1998; Zhao et al. 1998). Blocks were trimmed for semithin (500 nm) and ultrathin (70 nm) sections; and placed on nickel grids (200 hexagonal mesh SynapTek™, EMS) or single slot grids (SynapTek™, 1 × 2 mm, EMS) coated with Formvar (EMS) for serial sections.

Incubations for the immunogold labeling of GABA were done at RT. Initially, grids were placed for 10 min with 0.2% sodium borohydride and 50 mM glycine in 0.02 M Tris buffer containing 0.01% triton X-100, pH 7.4 (Tris-triton buffer). Samples were blocked for 30 min with 10% goat serum in Tris-triton buffer, incubated for 2 h with the primary antibody (rabbit polyclonal anti-GABA antibody; A2052; Sigma, Germany) diluted 1:1500 in 1% goat serum, rinsed with Tris-triton buffer, incubated 1 h with secondary gold-tagged antibody (10 nm goat-anti-rabbit IgG antibody; 25108, EMS) diluted 1:50 in 1% goat serum, and rinsed in Tris-triton buffer and distilled water. Negative controls, with the omission of primary antibody, were included in every experiment. After two days, grids were photographed in a TEM (Jeol 1010) at 20,000 X. Main attention was given to the neuropil, and serial-section pictures were taken as described above.

Statistical analysis

Ultrastructural data were analyzed using the weighted least squares transformation and compared between brain hemispheres of the studied groups (males and cycling females) using the repeated measure ANOVA test followed by the Tukey-Kramer test in the Statistical Analysis System Software 9.2 (Statistical Analysis System Institute Inc., EUA) and the Statistical Package for the Social Sciences 18 (SPSS; IBM Corporation, EUA). The statistical level of significance was set at $p = 0.05$.

Results

Pleomorphic spines and non-synaptic filopodia occur in the MePD

Presynaptic axons made excitatory and inhibitory contacts on dendritic shafts and dendritic spines. Thin, mushroom-like, stubby, wide, or ramified dendritic spines (Fig 1A – 1G; Fiala and Harris 1999; Brusco et al. 2010) and spines with a protruding spinule (Fig 2E; Spacek and Harris 2004) were found sparsely distributed and in clusters in the neuropil of the MePD (Fig 1A). Glial processes were frequently seen surrounding axo-spinous contacts. Synaptic contacts typically occurred on the spine head, albeit, less frequently (1%), there were synapses on the spine neck or the spine base (Fig 1D, 3). Some filopodia did not show apparent synaptic contacts (Fig 2B; Bhatt et al. 2009).

MePD dendritic spines receive inhibitory and multisynaptic contacts

In the MePD of both brain hemispheres in males, and females along the estrous cycle, synaptic contacts were more commonly found on dendritic shafts (76%) than on dendritic spines (24%; axo-somatic synapses were not analyzed). On dendritic shafts, most synaptic contacts were asymmetric and classified as excitatory (72%). Predominantly, dendritic spines showed just one putative excitatory contact, but $8.6 \pm 4.6\%$ (mean $\pm$ SD) of the synapses on spines were symmetric (Fig 2A, 3B), with no difference between hemispheres, sex or phase of the estrous cycle ($p > 0.05$).

Among all dendritic spines studied, 3.6% were multisynaptic. More frequently we found two asymmetric synapses on dendritic spines classified as mushroom-like (Fig 3A), although single spines forming two symmetric synapses (Fig 3B), or one asymmetric and one symmetric synapse (Fig 2D) were also commonly found.

Symmetric synapses on dendritic spines are GABAergic

The immunogold labeling of freeze-substituted ultrathin sections with an antibody against the neurotransmitter GABA showed symmetric terminals filled with gold particles, and asymmetric terminals completely free of particles. GABA positive terminals made synapses on both dendritic shafts and dendritic spines (Fig 4).

The right MePD of females in all estrous cycle phases shows ultrastructural indicators of higher synaptic activity than the left MePD

The density of synaptic vesicles (number of vesicles per μm^2 of presynaptic terminal area) was statistically different between MePD hemispheres [$F(1,11) = 75.8$, $p < 0.01$], but not when sex and estrous cycle phase were compared [$F(3,11) = 0.85$, $p = 0.53$]. However, the interaction of these two factors, MePD hemispheres and sex/estrous cycle phases, showed statistical difference [$F(3,11) = 8.35$, $p = 0.03$]. The density of presynaptic vesicles was twice as high in the right MePD of females in diestrus, proestrus and estrus than in the left MePD of the same groups, or the right and left MePD of males ($p < 0.05$ in all cases). Males did not show differences between MePD hemispheres. These differences were found on both excitatory and inhibitory terminals on dendritic branches and dendritic spines (Fig 5A).

A significant difference was also identified in the density of docked synaptic vesicles in the presynaptic terminal between MePD hemispheres [$F(1,11) = 129.3$, $p < 0.001$], but it was not different if comparing sex and estrous cycle phases [$F(3,11) = 3.72$, $p = 0.12$]. However, the interaction of sex/estrous cycle phases and brain hemisphere was statistically different [$F(3,11) = 14.24$, $p = 0.01$]. Docked synaptic vesicles were more frequent in synapses in the right MePD of females in diestrus, proestrus and estrus than in the left MePD of the same groups, or the right and left MePD of males ($p < 0.01$ in all cases). Males did not show a difference between hemispheres (Fig 5B).

The right MePD of females in proestrus shows more inhibitory synapses on dendritic shafts

Statistical analysis of the proportion of putative excitatory and inhibitory synaptic contacts on dendritic shafts showed a significant difference between MePD hemispheres [$F(1,11) = 10.6$, $p = 0.031$], sex and estrous cycle phases [$F(3,11) = 8.76$, $p = 0.031$] and the interaction of these factors [$F(3,11) = 8.09$, $p = 0.035$]. Post hoc comparison revealed more putative inhibitory contacts on dendritic shafts in the right MePD of proestrus females than in males, females in diestrus or estrus, or in the left MePD of the same animals ($p < 0.05$ in all cases; Fig 5C). No significant difference was found between the proportion of excitatory and inhibitory synapses on dendritic spines between hemispheres or experimental groups.

Gap junctions and axonal protrusions in the MePD neuropil

TEM also revealed gap junctions between glial cells in the MePD neuropil (Fig 6A). No such structures could be found on MePD neurons, either because these junctions are rare, have a restricted distribution, or have a modified structure that cannot be identified readily. Puncta adherens were evident between axon terminals and neuronal bodies or dendritic shafts (Fig 6B). We also observed collateral protrusions along axonal fibers and axons

making synapses “en passant” in the MePD neuropil. These findings and the invagination of portions of the pre- and postsynaptic terminals are shown in Fig 6.

Discussion

This work is the first to show detailed 3D reconstruction of dendritic spines and their synaptic patterns in the adult rat MePD. We found inhibitory and multiple synapses on MePD dendritic spines in adult male and female rats. We also show synaptic sexual dimorphism and estrous cycle specific lateralization in a brain region critical to social behaviors. The right MePD of females in all estrous cycle phases presented a higher density of synaptic vesicles and docked vesicles than the left MePD. We also show that the vast majority of synaptic contacts are made on dendritic shafts, where the ratio of putative excitatory to inhibitory synapses is lower in the right MePD of proestrus females than in the right MePD of females in other estrous cycle phases, and in males.

MePD dendritic spines receive inhibitory and multiple synapses

Beaulieu et al. (1992) found that 25% of GABAergic synapses in the visual cortex of adult macaque monkeys occur on dendritic spines. Inhibitory contacts on dendritic spines were in fact found in the cerebral cortex (Harris et al. 1992; Kubota et al. 2007) and in the MePD of prepubescent Sprague-Dawley rats (Cooke and Woolley, 2005). While the proportion of spines (53/616; 8.6%) expressing GABAergic synapses in the adult MePD is small compared with non-GABAergic synapses, their absolute number predicts an important role in circuit function. In both MePD brain hemispheres of adult males and females, 24% of the 2,569 chemical synapses examined were on dendritic spines. Since the total MePD area of an adult Wistar male rat is approximately 0.56 mm³, and our data show 4.76 synapses/μm², we estimate that there is a total of 163 million synapses, including 39 million synaptic contacts on dendritic spines, in the MePD.

Besides the presence of different types of intrinsic GABAergic neurons in the MePD (Bian, 2013), most synapses in the MeA are made with axons coming from other brain regions (Nishizuka and Arai, 1983a). Considering that there are approximately 3 million spines receiving GABAergic synapses in the MePD, the present work indicate a significant contribution of inhibitory projections to dendritic spines in regulating the overall excitability of this region (Marcuzzo et al. 2007). Indeed, GABAergic synapses on spines are well positioned to selectively regulate excitatory inputs (Dehay et al. 1991; Knott et al. 2002) and modulate synaptic plasticity (Chalifoux and Carter 2011). In the MePD, they participate in the elaboration of vomeronasal/pheromonal stimuli that regulate social behavior (Meredith and Westberry 2004; Pereno et al., 2011).

Multisynaptic dendritic spines are found in other brain regions (Nikonenko et al. 2003; Genoud et al. 2004; Stewart et al. 2005a, 2005b; Popov and Stewart 2009). In the MePD, 3.6% or approximately one million dendritic spines are multisynaptic, similar to the levels observed in the dentate gyrus (Popov and Stewart 2009). Although we found multiple synapses on thin spines, they were much more common on mushroom spines, which are considered mature spines with larger synaptic currents (Kasai et al. 2003; Hayashi and Majewska 2005). These inputs likely underlie a number of functions since they occur as two

excitatory, two inhibitory or mixed contacts. MePD multisynaptic spines probably play a role in more complex synaptic integration and activity-dependent plasticity, but the signaling properties of these spines still depend on the identification of their specific excitatory and inhibitory afferents and the specific synaptic receptors (Wyllie et al., 2013).

Dendritic filopodia, spinules on dendritic spines, and axonal protrusions are indicators of plasticity in the mature MePD

These three features indicate high plasticity in the MePD of adult rats. Their ultrastructure here described expands previous data obtained with confocal microscopy (Brusco et al. 2010). Dendritic filopodia without synapses are believed to be precursors of mature spines (Knott et al. 2006), or vestiges of synaptic loss on a pre-existing spine due to reduced network activity (Segal 2010). Spinules on dendritic spines also reflect an enhanced plastic state; they are rapidly formed after synaptic stimulation (Applegate and Landfield 1988; Tao-Cheng et al. 2009) or by induction of long-term potentiation (Schuster et al. 1990), and may be involved in intercellular signaling between spines and the adjacent neuropil (Spacek and Harris 2004). Axonal protrusions and varicosities, possibly making “en passant” synapses, were first observed in the MePD of adult rats using confocal microscopy (Brusco et al. 2010). Here we show the ultrastructure of these presynaptic elements, which are similar to those found in the accessory olfactory bulb (Larriva-Sahd 2008) and the auditory cortex (Szentágothai, 1978). Axonal varicosities make GABAergic synaptic contacts in the MePD, and these “en passant” synapses can affect multiple neurons at the same time, thereby enhancing local inhibition in locally divergent circuits. It remains to be established whether they can alter the functioning of local cells for the rapid display and/or disinhibition of social behaviors.

Females display MePD laterality in synaptic activity

Ultrastructural findings suggest that MePD synaptic activity is higher in the right than in the left hemisphere of females in diestrus, proestrus and estrus. Previous reports identified hemispherical lateralization in the MePD. For example, in young adult Long Evans rats, the MePD is larger in the right than in the left hemisphere, which has more neurons in males than in females (Morris et al. 2008). In young Sprague-Dawley rats, males also have more neurons in the right MePD than females, with no difference between sexes in the left MePD. However, mEPSC frequency and the number of excitatory synapses are higher in the left MePD of males than in females, with no difference in the right MePD (Cooke and Woolley 2005). No evidence for hemispheric difference was found in the density of dendritic spines of Golgi-impregnated MePD neurons of adult male and diestrus female Wistar rats (Arpini et al. 2010).

One possible reason for this hemispheric specialization is that the left medial amygdala (MeA) is specialized for chemosensory and/or steroid negative feedback regulation of the secretion of hypothalamic luteinizing hormone (discussed in Cooke and Woolley 2005). MePD laterality could be modulated by differences in hormone or aromatase exposure during development, or by differential sensitivity to sex steroids across brain hemispheres. The result would be differences in production or effective action of estrogens between hemispheres, modulating axon targeting and even synaptic activity in females. In this sense,

aromatase activity is lateralized in the MeA, it is higher in the left MeA at gestational day 22, higher in the right MeA at postnatal day 6, and equivalent in both hemispheres at postnatal day 15 (von Ziegler and Lichtensteiger 1992). Steroid-dependent plasticity of adult MePD occurs on synapses already organized into male and female lateralized phenotypes (Cooke and Woolley 2005; see also Nishizuka and Arai, 1981) although morphological changes continue to occur in the MeA during puberty (Zehr et al. 2006) to reach the complex ongoing scenario of cellular and synaptic organization of the adult rat MePD (Rasia-Filho et al. 2012b).

The proportion of inhibitory synapses increases on dendritic shafts during proestrus

We show that synapses on dendritic shafts exhibit phasic shifts throughout the estrus cycle, with less excitatory and more inhibitory contacts in proestrus, specifically in the right MePD. In the MePD, dendritic spine density decreases when females transit from diestrus to proestrus, coincident with a reduction in synapsin reactivity (Rasia-Filho et al. 2004; Oberlander and Erskine 2008; Rasia-Filho et al. 2012a). These spines receive mainly excitatory synapses, and the reduced density of spines would decrease the excitatory inputs to MePD neurons during proestrus.

Synapses on dendritic shafts have been shown to be stronger than spine synapses (Ivenshitz and Segal 2010). In the rat MeA, the number of estrogen-dependent shaft synapses is mainly implicated in the process of sexual differentiation of local neuronal networks (Nishizuka and Arai 1982). Furthermore, the release of estrogen and progesterone during proestrus may act differently on sites that send projections to different MePD hemispheres, such as the bed nucleus of the stria terminalis (Dong and Swanson 2006). It is reasonable to think that this synaptic reorganization in females modulates phasic physiological events related to neuroendocrine secretion and sexual behavior along the estrous cycle (see details in Rasia-Filho et al. 2012a,b). As an example, among other projections, it is possible that enhanced inhibition of the MePD during proestrus may suppress its inhibition of the ventrolateral portion of the ventromedial nucleus of the hypothalamus (Petrovich et al. 2001), which is involved in lordosis behavior (Nelson 2000), and blocks the MePD-mediated inhibition of GABAergic interneurons of the medial pre-optic area, responsible for pro-receptive behavior (Pfaus and Heeb 1997; Rasia-Filho et al. 2009; 2012a,b). In this way, shaft synapses can play a role in more consistent and tonic activity whereas contacts on plastic spines can serve for modulation of phasic, normally changeable inputs to the adult female rat MePD (Rasia-Filho et al. 2004; 2009).

Altogether, our results reveal the ultrastructural complexity of the adult MePD neuropil, including variable synaptic inputs, excitatory and inhibitory contacts on dendritic shafts, and compartmentalized inhibitory contacts and multisynaptic dendritic spines. These new quantitative analyses combined with the TEM reconstructions and immune-based confirmation of GABAergic synapses represent a comprehensive characterization of sexual dimorphism and estrous cycle specific lateralization in this brain region critical to sexual behavior, and they provide qualitative and quantitative characterization of the poorly understood inhibitory synapses on dendritic spines. Considering other research fields, these data can also provide insights for the study of the synaptic reorganization that involves the

MeA neurons after seizure induction in male and female rats (Okada et al. 1993; Moriyama et al. 2013).

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References

- Applegate MD, Landfield PW. Synaptic vesicle redistribution during hippocampal frequency potentiation and depression in young and aged rats. *J Neurosci.* 1988; 8(4):1096–1111. [PubMed: 3357011]
- Arpini M, Menezes IC, Dall'Oglio A, Rasia-Filho AA. The density of Golgi-impregnated dendritic spines from adult rat posterodorsal medial amygdalaneurons displays no evidence of hemispheric or dorsal/ventral differences. *Neurosci Lett.* 2010; 469(2):209–213. [PubMed: 19962427]
- Arellano JJ, Espinosa A, Fairén A, Yuste R, DeFelipe J. Non-synaptic dendritic spines in neocortex. *Neuroscience.* 2007; 145(2):464–469. [PubMed: 17240073]
- Beaulieu C, Kisvárdy Z, Somogyi P, Cynader M, Cowey A. Quantitative distribution of GABA-immunopositive and -immunonegative neurons and synapses in the monkey striate cortex (area 17). *Cereb Cortex.* 1992; 2(4):295–309. [PubMed: 1330121]
- Bhatt DH, Zhang S, Gan W-B. Dendritic spine dynamics. *Annu Rev Physiol.* 2009; 71:261–282. [PubMed: 19575680]
- Bian X. Physiological and morphological characterization of GABAergic neurons in the medial amygdala. *Brain Res.* 2013; 1509:8–19. [PubMed: 23524192]
- Brusco J, Dall'Oglio A, Rocha LB, Rossi MA, Moreira JE, Rasia-Filho AA. Descriptive findings on the morphology of dendritic spines in the rat medial amygdala. *Neurosci Lett.* 2010; 483(2):152–156. [PubMed: 20691759]
- Chalifoux JR, Carter AG. GABAB receptor modulation of voltage-sensitive calcium channels in spines and dendrites. *J Neurosci.* 2011; 31(11):4221–4232. [PubMed: 21411663]
- Choi GB, Dong HW, Murphy AJ, Valenzuela DM, Yancopoulos GD, Swanson LW, Anderson DJ. Lhx6 delineates a pathway mediating innate reproductive behaviors from the amygdala to the hypothalamus. *Neuron.* 2005; 46(4):647–660. [PubMed: 15944132]
- Cooke BM, Tabibnia G, Breedlove SM. A brain sexual dimorphism controlled by adult circulating androgens. *Proc Natl Acad Sci USA.* 1999; 96(13):7538–7540. [PubMed: 10377450]
- Cooke BM, Woolley CS. Sexually dimorphic synaptic organization of the medial amygdala. *J Neurosci.* 2005; 25(46):10759–10767. [PubMed: 16291949]
- Cooke BM, Woolley CS. Effects of prepubertal gonadectomy on a male-typical behavior and excitatory synaptic transmission in the amygdala. *Dev Neurobiol.* 2009; 69(2–3):141–152. [PubMed: 19067323]
- Coolen LM, Peters HJPW, Veening JG. Fos immunoreactivity in the rat brain following consummatory elements of sexual behavior: a sex comparison. *Brain Res.* 1996; 738(1):67–82. [PubMed: 8949929]
- Dall'Oglio A, Gehlen G, Achaval M, Rasia-Filho AA. Dendritic branching features of posterodorsal medial amygdala neurons of adult male and female rats: Further data based on the Golgi method. *Neurosci Lett.* 2008a; 430(2):151–156. [PubMed: 18078715]
- Dall'Oglio A, Gehlen G, Achaval M, Rasia-Filho AA. Dendritic branching features of Golgi-impregnated neurons from the "ventral" medial amygdala subnuclei of adult male and female rats. *Neurosci Lett.* 2008b; 439:287–292. [PubMed: 18538473]

- Dall'Oglio A, Xavier LL, Hilbig A, Ferme D, Moreira JE, Achaval M, Rasia-Filho AA. Cellular components of the human medial amygdaloid nucleus. *J Comp Neurol.* 2013; 521(3):589–611. [PubMed: 22806548]
- Dehay C, Douglas RJ, Martin KA, Nelson C. Excitation by geniculocortical synapses is not “vetoed” at the level of dendritic spines in cat visual cortex. *J Physiol.* 1991; 440:723–734. [PubMed: 1804984]
- de Castilhos J, Marcuzzo S, Forti CD, Frey RM, Stein D, Achaval M, Rasia-Filho AA. Further studies on the rat posterodorsal medial amygdala: Dendritic spine density and effect of 8-OH-DPAT microinjection on male sexual behavior. *Brain Res Bull.* 2006; 69(2):131–139. [PubMed: 16533661]
- de Olmos, JS.; Beltramino, CA.; Alheid, G. Amygdala and extended amygdala of the rat: a cytoarchitectonical, fibroarchitectonical, and chemoarchitectonical survey. In: Paxinos, G., editor. *The Rat Nervous System*. San Diego: Elsevier Academic Press; 2004. p. 509–603.
- Dong HW, Swanson LW. Projections from bed nuclei of the stria terminalis, dorsomedial nucleus: implications for cerebral hemisphere integration of neuroendocrine, autonomic, and drinking responses. *J Comp Neurol.* 2006; 494(1):75–107. [PubMed: 16304681]
- Farb C, Aoki C, Milner T, Kaneko T, LeDoux J. Glutamate immunoreactive terminals in the lateral amygdaloid nucleus: a possible substrate for emotional memory. *Brain Res.* 1992; 593(2):145–158. [PubMed: 1360318]
- Fiala, JC.; Harris, KM. Dendrite structure. In: Stuart, G.; Sprutson, N.; Häusser, M., editors. *Dendrites*. New York: Oxford University Press; 1999. p. 1–34.
- Fiala JC, Harris KM. Extending unbiased stereology of brain ultrastructure to three-dimensional volumes. *J Am Med Inform Assoc.* 2001; 8(1):1–16. [PubMed: 11141509]
- Fiala JC. Reconstruct: a free editor for serial section microscopy. *J Microsc.* 2005; 218(Pt1):52–61. [PubMed: 15817063]
- Flügge G, Pfender D, Rudolph S, Jarry H, Fushs E. 5HT1A-receptor binding in the brain of cyclic and ovariectomized female rats. *J Neuroendocrinol.* 1999; 11(4):243–249.
- Fu M, Yu X, Lu J, Zuo Y. Repetitive motor learning induces coordinated formation of clustered dendritic spines *in vivo*. *Nature.* 2012; 483:92–95. [PubMed: 22343892]
- Genoud C, Knott GW, Sakata K, Lu B, Welker E. Altered synapse formation in the adult somatosensory cortex of brain-derived neurotrophic factor heterozygote mice. *J Neurosci.* 2004; 24(10):2394–2340. [PubMed: 15014114]
- Gray EG. Electron microscopy of synaptic contacts on dendrite spines of the cerebral cortex. *Nature.* 1959; 183(4675):1592–1593. [PubMed: 13666826]
- Gréco B, Blasberg ME, Kosinski EC, Blaustein JD. Response of ERalpha-IR and ERbeta-IR cells in the forebrain of female rats to mating stimuli. *Horm Behav.* 2003; 43(2):444–453. [PubMed: 12788290]
- Harris KM, Jensen FE, Tsao B. Three-dimensional structure of dendritic spines and synapses in rat hippocampus at postnatal day 15 and adult ages: Implications for the maturation of synaptic physiology and long-term potentiation. *J Neurosci.* 1992; 12(7):2687–2705.
- Harris KM, Kater SB. Dendritic spines: Cellular specializations imparting both stability and flexibility to synaptic function. *Ann Rev Neurosci.* 1994; 17:341–371. [PubMed: 8210179]
- Hayashi Y, Majewska AK. Dendritic spine geometry: functional implication and regulation. *Neuron.* 2005; 46(4):529–532. [PubMed: 15944122]
- Hermel EE, Ilha J, Xavier LL, Rasia-Filho AA, Achaval M. Influence of sex and estrous cycle, but not laterality, on the neuronal somatic volume of the posterodorsal medial amygdala of rats. *Neurosci Lett.* 2006; 405(1–2):153–158. [PubMed: 16860473]
- Heuser, JE.; Reese, TS. Structure of the synapse. In: Kandel, ER., editor. *Handbook of Physiology. The Nervous System*. Baltimore: American Physiological Society; 1977. p. 261–294.
- Hines M, Allen LS, Gorski RA. Sex differences in subregions of the medial nucleus of the amygdala and the bed nucleus of the stria terminalis of the rat. *Brain Res.* 1992; 579(2):321–326. [PubMed: 1352729]
- Ivenshitz M, Segal M. Neuronal density determines network connectivity and spontaneous activity in cultured hippocampus. *J Neurophysiol.* 2010; 104(2):1052–1060. [PubMed: 20554850]

- 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(5):599–609. [PubMed: 18853427]
- Kasai H, Matsuzaki M, Noguchi J, Yasumatsu N, Nakahara H. Structure-stability-function relationships of dendritic spines. *Trends Neurosci*. 2003; 26(7):360–368. [PubMed: 12850432]
- Kitanishi T, Ikegaya Y, Matsuki N, Yamada MK. Experience-dependent, rapid structural changes in hippocampal pyramidal cell spines. *Cereb Cortex*. 2009; 19(11):2572–2578. [PubMed: 19240139]
- Knott GW, Quairiaux C, Genoud C, Welker E. Formation of dendritic spines with GABAergic synapses induced by whisker stimulation in adult mice. *Neuron*. 2002; 34(2):265–273. [PubMed: 11970868]
- Knott GW, Holtmaat A, Wilbrecht L, Welker E, Svoboda K. Spine growth precedes synapse formation in the adult neocortex in vivo. *Nat Neurosci*. 2006; 9(9):1117–1124. [PubMed: 16892056]
- Kubota Y, Hatada S, Kondo S, Karube F, Kawaguchi Y. Neocortical inhibitory terminals innervate dendritic spines targeted by thalamocortical afferents. *J Neurosci*. 2007; 27(5):1139–1150. [PubMed: 17267569]
- Lai CS, Franke TF, Gan WB. Opposite effects of fear conditioning and extinction on dendritic spine remodelling. *Nature*. 2012; 483:87–91. [PubMed: 22343895]
- Larriva-Sahd J. The accessory olfactory bulb in the adult rat: a cytological study of its cell types, neuropil, neuronal modules, and interactions with the main olfactory system. *J Comp Neurol*. 2008; 510(3):309–350. [PubMed: 18634021]
- Lu H, Esquivel AV, Bower JM. 3D Electron microscopic reconstruction of segments of rat cerebellar purkinje cell dendrites receiving ascending and parallel fiber granule cell synaptic inputs. *J Comp Neurol*. 2009; 514:583–594. [PubMed: 19363797]
- Marcuzzo S, Dall'Oglio A, Ribeiro MF, Achaval M, Rasia-Filho AA. Dendritic spines in the posterodorsal medial amygdala after restraint stress and ageing in rats. *Neurosci. Lett*. 2007; 424:16–21. [PubMed: 17709204]
- 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 amygdale subnuclei across the estrous cycle and in castrated and treated female rats. *Brain Res*. 2006; 1108:117–126. [PubMed: 16842763]
- Matsuda S, Kobayashi Y, Ishizuka N. A quantitative analysis of the laminar distribution of synaptic boutons in field CA3 of the rat hippocampus. *Neurosci Res*. 2004; 49(2):241–252. [PubMed: 15140566]
- Meredith M, Westberry JM. Distinctive responses in the medial amygdala to same-species and different-species pheromones. *J Neurosci*. 2004; 24(25):5719–5725. [PubMed: 15215294]
- Montes GS, Luque EH. Effects of ovarian steroids on vaginal smears in the rat. *Acta Anat (Basel)*. 1988; 133:192–199. [PubMed: 3227778]
- Moreira JE, Reese TS, Kachar B. Freeze-substitution as a preparative technique for immunoelectronmicroscopy: Evaluation by atomic force microscopy. *Microscopy Res Tech*. 1996; 33(3):251–261.
- Moreira JE, Dodane V, Reese TS. Immunoelectronmicroscopy of soluble proteins and membrane proteins with a sensitive post embedding method. *J Histochem Cytochem*. 1998; 46(7):847–854. [PubMed: 9632744]
- Moriyama C, Galic MA, Mychasiuk R, Pittman QJ, Perrot TS, Currie RW, Esser MJ. Prenatal transport stress, postnatal maternal behavior, and offspring sex differently affect seizure susceptibility in young rats. *Epilepsy Behav*. 2013; 29:19–27. [PubMed: 23920381]
- 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*. 2008; 506(5):851–859. [PubMed: 18076082]
- Nelson, RJ. An introduction to behavioral endocrinology. Sunderland: Sinauer Associates; 2000.
- Newman SW. The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network. *Ann N Y Acad Sci*. 1999; 877:242–257. [PubMed: 10415653]

- Nikonenko I, Jourdain P, Muller D. Presynaptic remodeling contributes to activity-dependent synaptogenesis. *J Neurosci.* 2003; 23(24):8498–8505. [PubMed: 13679418]
- Nimchinsky EA, Sabatini BL, Svoboda K. Structure and function of dendritic spines. *Annu Rev Physiol.* 2002; 64:313–353. [PubMed: 11826272]
- Nishizuka M, Arai Y. Sexual dimorphism in synaptic organization in the amygdala and its dependence on neonatal hormone environment. *Brain Res.* 1981; 212:31–38. [PubMed: 7225863]
- Nishizuka M, Arai Y. Synapse formation in response to estrogen in the medial amygdala developing in the eye. *Proc Natl Acad Sci USA.* 1982; 79(22):7024–7026. [PubMed: 6960361]
- Nishizuka M, Arai Y. Intrinsic connections in the medial amygdala as revealed by complete deafferentation. *Neurosci Lett.* 1983a; 35(3):247–251. [PubMed: 6843897]
- Nishizuka M, Arai Y. Regional difference in sexually dimorphic synaptic organization of the medial amygdala. *Exp Brain Res.* 1983b; 49(3):462–465. [PubMed: 6641844]
- Oberlander JG, Erskine MS. Receipt of vaginal-cervical stimulation modifies synapsin content in limbic areas of the female rat. *Neuroscience.* 2008; 153(3):581–593. [PubMed: 18407423]
- Okada R, Nishizuka M, Iizuka R, Arai Y. Persistence of reorganized synaptic connectivity in the amygdala of kindled rats. *Brain Res Bull.* 1993; 31(6):631–635. [PubMed: 8518954]
- Österlund M, Kuiper GG, Gustafsson J-A, Hurd YL. Differential distribution and regulation of estrogen receptor-alpha and -beta mRNA within the female rat brain. *Brain Res Mol Brain Res.* 1998; 54(1):175–180. [PubMed: 9526077]
- Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates.* San Diego: Academic Press; 1998.
- Pelletier G, Leclerc R, Puviani R, Polak JM. Electron immunocytochemistry in vasoactive intestinal peptide (VIP) in the rat brain. *Brain Res.* 1981; 210(1–2):356–360. [PubMed: 7013904]
- Petrovich GD, Canteras NS, Swanson LW. Combinatorial amygdalar inputs to hippocampal domains and hypothalamic behavior systems. *Brain Res Brain Res Rev.* 2001; 38(1–2):247–289. [PubMed: 11750934]
- Pereno GL, Balaszczuk V, Beltramino CA. Detection of conspecific pheromones elicits fos expression in GABA and calcium-binding cells of the rat vomeronasal system-medial extended amygdala. *J Physiol Biochem.* 2011; 67(1):71–85. [PubMed: 20938761]
- Pfaus JG, Heeb MM. Implications of immediate-early gene induction in the brain following sexual stimulation of female and male rodents. *Brain Res Bull.* 1997; 44(4):397–407. [PubMed: 9370204]
- Polston EV, Heitz M, Barnes W, Cardamone K, Erskine MS. NMDA-mediated activation of the medial amygdala initiates a downstream neuroendocrine memory responsible for pseudopregnancy in the female rat. *J Neurosci.* 2001; 21(11):4104–4110. [PubMed: 11356898]
- Popov VI, Stewart MG. Complexity of contacts between synaptic boutons and dendritic spines in adult rat hippocampus: Three-dimensional reconstructions from serial ultrathin sections in vivo. *Synapse.* 2009; 63(5):369–377. [PubMed: 19173264]
- Rasia-Filho AA, Fabian C, Rigoti K, Achaval M. Influence of sex, estrous cycle and motherhood in dendritic spine density in the rat medial amygdala revealed by the Golgi method. *Neuroscience.* 2004; 126(4):839–847. [PubMed: 15207319]
- Rasia-Filho, AA.; Brusco, J.; Moreira, JE. Spine plasticity in the rat medial amygdala. In: Baylog, LR., editor. *Dendritic Spines: Biochemistry, Modeling and Properties.* Hauppauge: Nova Science Publishers; 2009. p. 67-90.
- Rasia-Filho AA, Dalpian F, Menezes IC, Brusco J, Moreira JE, Cohen RS. Dendritic spines of the medial amygdala: plasticity, density, shape, and subcellular modulation by sex steroids. *Histol Histopathol.* 2012a; 27(8):985–1011. [PubMed: 22763872]
- 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.* 2012b; 12(11):1090–1106. [PubMed: 22827219]
- Schuster T, Krug M, Wenzel J. Spinules in axospinous synapses of the rat dentate gyrus: changes in density following long-term potentiation. *Brain Res.* 1990; 523(1):171–174. [PubMed: 2207688]
- Segal M. Dendritic spines, synaptic plasticity and neuronal survival: activity shapes dendritic spines to enhance neuronal viability. *Eur J Neurosci.* 2010; 31(12):2178–2184. [PubMed: 20550565]

- Sheehan TP, Cirrito J, Numan MJ, Numan M. Using c-Fos immunocytochemistry to identify forbrain regions that may inhibit maternal behavior in rats. *Behav Neurosci.* 2000; 114(2):337–352. [PubMed: 10832795]
- Shepherd GM. The dendritic spine: a multifunctional integrative unit. *J Neurophysiol.* 1996; 75(6): 2197–2210. [PubMed: 8793734]
- Simerly, RB. Anatomical substrates of hypothalamic integration. In: Paxinos, G., editor. *The Rat Nervous System*. San Diego: Academic Press; 2004. p. 335-368.
- Simerly RB, Chang C, Muramatsu M, Swanson LW. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: An in situ hybridization study. *J Comp Neurol.* 1990; 294(1):76–95. [PubMed: 2324335]
- Singletary SJ, Kirsch AJ, Watson J, Karim BO, Huso DL, Hurn PD, Murphy SJ. Lack or correlation of vaginal impedance measurements with hormone levels in the rat. *Contemp Top Lab Anim Sci.* 2005; 44(6):37–42. [PubMed: 16370578]
- Spacek J, Harris KM. Trans-endocytosis via spinules in adult rat hippocampus. *J Neurosci.* 2004; 24(17):4233–4241. [PubMed: 15115819]
- Stewart MG, Davies HA, Sandi C, Kraev IV, Rogachevsky VV, Peddie CJ, Rodriguez JJ, Cordero MI, Donohue HS, Gabbott PL, Popov VI. Stress suppresses and learning induces plasticity in CA3 of rat hippocampus: A three-dimensional ultrastructural study of thorny excrescences and their postsynaptic densities. *Neuroscience.* 2005a; 131(1):43–54. [PubMed: 15680690]
- Stewart MG, Medvedev NI, Popov VI, Schoepfer R, Davies HA, Murphy K, Dallerac GM, Kraev IV, Rodriguez JJ. Chemically induced long-term potentiation increases the number of perforated and complex postsynaptic densities but does not alter dendritic spine volume in CA1 of adult mouse hippocampal slices. *Eur J Neurosci.* 2005b; 21(12):3368–3378. [PubMed: 16026474]
- Stuart, G.; Spruston, N.; Häusser, M. *Dendrites*. Oxford: Oxford University Press; 1999.
- Szentágothai J. The Ferrier Lecture, 1977. The neuron network of the cerebral cortex: a functional interpretation. *Proc R Soc Lond B Biol Sci.* 1978; 201(1144):219–248. [PubMed: 27801]
- Tao-Cheng JH, Gallant PE, Brightman MW, Dosemeci A, Reese TS. Structural changes at synapses after delayed perfusion fixation in different regions of the mouse brain. *J Comp Neurol.* 2007; 501(5):731–740. [PubMed: 17299754]
- Tao-Cheng JH, Dosemeci A, Gallant PE, Miller S, Galbraith JA, Winters CA, Azzam R, Reese TS. Rapid turnover of spinules at synaptic terminals. *Neuroscience.* 2009; 160(1):42–50. [PubMed: 19248820]
- Tsay D, Yuste R. On the electrical function of dendritic spines. *Trends Neurosci.* 2004; 27:77–83. [PubMed: 15102486]
- Uchizono K. Characteristics of excitatory and inhibitory synapses in the central nervous system of the cat. *Nature.* 1965; 207(997):642–643. [PubMed: 5883646]
- von Ziegler NI, Lichtensteiger W. Asymmetry of brain aromatase activity: region- and sex-specific developmental patterns. *Neuroendocrinology.* 1992; 55(5):512–518. [PubMed: 1584336]
- Wyllie DJA, Livesey MR, Hardingham GE. Influence of GluN2 subunit identity on NMDA receptor function. *Neuropharmacology.* 2013; 74:4–17. [PubMed: 23376022]
- Zehr JL, Todd BJ, Schulz KM, McCarthy MM, Sisk CL. Dendritic pruning of the medial amygdala during pubertal development of the male Syrian hamster. *J Neurobiol.* 2006; 66(6):578–590. [PubMed: 16555234]
- Zhao HM, Wenthold RJ, Petralia RS. Glutamate receptor targeting to synaptic populations on Purkinje cells is developmentally regulated. *J Neurosci.* 1998; 18(14):5517–5528. [PubMed: 9651232]

Figure 1.

MePD dendritic spines. (A) Tridimensional views of the same dendritic shaft and dendritic spines reconstructed by electron micrographs from 420 serial sections of 60–70 nm. Spines are mushroom-like (m), stubby (s) and thin (t); the numbers indicate the same spine in different views. The asterisk points to a filopodium with no synaptic contact. Scale bars: 5 μ m. (B, C) Thin spines reconstructed from 6 to 7 serial sections. (D) Stubby and (E) wide spines reconstructed from 5 serial sections. (F, G)

Mushroom-like spines reconstructed from 6 to 8 serial sections. Synaptic contacts are in dark gray on the reconstructed structures. Scale bars: 250 nm. Examples of synapses classified as (H) symmetric or inhibitory (arrowhead) and (I) asymmetric structures. Scale bars: 250 nm. Examples of synapses classified as (H) symmetric or inhibitory (arrowhead) and (I) asymmetric

or excitatory (arrow). Scale bars: 500 nm. **(J)** Highlighted area in **I** to point examples of docked vesicles (arrows). Scale bar: 250 nm.

Figure 2.

Synaptic contacts on MePD dendritic spines. (A) Electron micrographs of sections 2 to 7 utilized for the reconstruction of a dendritic spine receiving one symmetric contact (arrowheads). Scale bar: 200 nm. (B) Filopodium (arrowheads) reconstructed from 6 serial sections and electron micrographs of section 3, 4 and 5. (C) Ramified dendritic spine (s_1 and s_2) reconstructed from 13 serial sections and electron micrographs of section 6, 8 and 10 utilized for the reconstruction. (B, C) Parent dendrites (d) and spines (s). (D) Dendritic spine receiving one asymmetric (arrows) and one symmetric (arrowhead) synaptic contact reconstructed from 8 serial sections (electron micrographs of sections 2 and 6 shown). Asymmetric and symmetric synaptic

contacts are represented in dark gray in the reconstructed spines. **(E)** Spine with a protruding spinule (arrow) reconstructed from 9 serial sections. Scale bar: 250 nm.

Figure 3.

(A) Electron micrographs of sections 1 to 6 utilized for the reconstruction of a dendritic spine receiving two asymmetric contacts (arrows). The first axon terminal (a_1), in light green, makes synapses with the spine (s) and the parent dendrite; the second axon (a_2), in orange, makes a synapse in this same spine; the third axon (a_3), in purple, contacts the parent dendrite. Asymmetric synaptic contacts are in red on the reconstructed spine. (B) Electron micrographs of sections 1 to 5 utilized for the reconstruction of a dendritic spine (s) receiving two symmetric contacts (arrowheads) from different axon terminals (a_1 and a_2). The reconstructions show the terminals in purple (a_1) and pink (a_2). Symmetric synaptic contacts are in green. Scale bar: 250 nm.

Figure 4.

Immunolabeling for GABA in the MePD of adult rats. **(A)** Examples of GABA negative (•) and positive terminals (*; filled with 10 nm gold particles) showing the characteristic GABA neurotransmitter reactivity on TEM. **(B)** Dendritic spine receiving one asymmetric synapse (arrow) from an axon terminal not reactive to GABA. **(C)** Dendritic spines (s) receiving one asymmetric synaptic contact (arrow) from a GABA negative terminal and one symmetric contact (arrowhead) from a GABA positive terminal. **(D)** Serial sections showing a dendritic spine (s) receiving one GABA positive (arrowheads) and one GABA negative (arrows) synaptic contact. Scale bars: 250 nm.

Figure 5.

(A) Mean $\pm$ SD of the total number of synaptic vesicles per μm^2 of presynaptic terminal area in the right and left MePD of males and females in diestrus, proestrus and estrus. The density of synaptic vesicles is higher in the right MePD of females in diestrus, proestrus and estrus than in the left MePD of the same groups, or in the right and left MePD of males (* = $p < 0.05$). (B) Mean $\pm$ SD of the density of docked synaptic vesicles in the right and left MePD of males and females in diestrus, proestrus and estrus. Docked synaptic vesicles are more frequent in synapses in the right MePD of females in diestrus, proestrus and estrus than in the left MePD of the same groups, or in the right and left MePD of males (* = $p < 0.01$). (C) Mean $\pm$ SD of the proportion of inhibitory synapses on dendritic shafts of MePD neurons. The right MePD of females in proestrus shows more inhibitory

synapses than the left MePD of these same animals and the right MePD of males, females in diestrus, proestrus and estrus (* = $p < 0.01$).

Figure 6.

(A) Gap junction (arrow) between two glial cells. Scale bar: 250 nm. (B) Puncta adherens (arrow) joining an axon terminal to a dendritic shaft. (C) Invagination of a pre- and (D) a postsynaptic terminal towards the opposite terminal (empty arrowheads). Scale bars: 500 nm. (E) Axon protrusion highlighted in gray, and (F) axonal varicosity making synapses *en passant* (highlighted in gray). Scale bars: 250 nm.