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Review

Dendritic spines of the medial amygdala: plasticity, density, shape, and subcellular modulation by sex steroids

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Summary. The medial nucleus of the amygdala (MeA) is a complex component of the "extended amygdala" in rats. Its posterodorsal subnucleus (MePD) has a remarkable expression of gonadal hormone receptors, is sexually dimorphic or affected by sex steroids, and modulates various social behaviors. Dendritic spines show remarkable changes relevant for synaptic strength and plasticity. Adult males have more spines than females, the density of dendritic spines changes in the course of hours to a few days and is lower in proestrous and estrous phases of the ovarian cycle, or is affected by both sex steroid withdrawal and hormonal replacement therapy in the MePD. Males also have more thin spines than mushroom-like or stubby/wide ones. The presence of dendritic fillopodia and axonal protusions in the MePD neuropil of adult animals reinforces the evidence for local plasticity. Estrogen affects synaptic and cellular growth and neuroprotection in the MeA by regulating the activity of the cyclic AMP response element-binding protein (CREB)-related gene products, brain-derived neurotrophic factor (BDNF), the anti-apoptotic protein B-cell lymphoma-2 (Bcl-2) and the activity-regulated cytoskeleton-related protein (Arc). These effects on signal transduction cascades can also lead to local protein synthesis and/or rearrangement of the cytoskeleton and subsequent numerical/morphological alterations in dendritic spines. Various working hypotheses are raised from these experimental data and reveal the MePD as a relevant region to study the effects of sex steroids in the rat brain.

Key words: Amygdala/cytology, CREB, Gonadal hormones, Neural pathways/axonal network, Sexual dimorphism

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Introduction

The amygdaloid complex in the basal forebrain is composed of various nuclei and subnuclei with anatomical and functional particularities (Brodal, 1981; McDonald, 1998; Pitkänen, 2000; Rasia-Filho et al., 2000; de Olmos et al., 2004). Considerable efforts have been devoted to its study in rodents (e.g., rat, mouse, and hamster), monkeys, and humans. The medial nucleus of the amygdala (MeA) is a superficial and relatively large component of the "extended amygdala" in rats (Alheid et al., 1995; Alheid, 2003; de Olmos et al., 2004). The embryological development of the mouse MeA indicates that it is a "mosaic" formed by cells coming from a caudoventral pallidal subdivision, the ventral pallium, the commissural preoptic area, and the supraopto-paraventricular domain of the hypothalamus (García-López et al., 2008; Bupesh et al., 2011). These data did not support an exclusively striatal nature for the mice MeA (Swanson and Petrovich, 1998). The rat MeA can be further subdivided according to histological, connectional, neurochemical, and functional criteria in

the anterodorsal (MeAD), anteroventral (MeAV), posterodorsal (MePD), and posteroventral (MePV) subnuclei (Alheid et al., 1995; Petrovich et al., 2001; de Olmos et al., 2004; Carrillo et al., 2007; Dall'Oglio et al., 2008a,b). In coronal sections, the MePD shows three parallel vertically-oriented columns of aggregated cells that extend from the medial to the lateral border of this subnucleus, close to the optic tract (OT) and ventrally to the stria terminalis (ST; de Olmos et al., 2004). Surrounding the external borders of the MePD and the MePV there is a cell-sparse rim initially termed the "molecular layer", but rather formed by axons coming from outside the MeA subnuclei (Scalia and Winans, 1975; Nishizuka and Arai, 1983; de Olmos et al., 2004).

Neuroanatomical and functional differences of MeA subnuclei were revealed by different methodological approaches (Coolen et al., 1997; Newman, 1999; Petrovich et al., 2001; de Olmos et al., 2004; Rasia-Filho et al., 2004; Blake and Meredith, 2011). Previous results suggested that the MeA could have an anterior chemosensory information-sensitive part and a hormonally-sensitive posterior aspect (Gomez and Newman, 1991; Malsbury and McKay, 1994; Wood and Newman, 1995; Rasia-Filho et al., 1999). However, due to its axonal projections, the MePD appeared to be the most different component within the rat MeA, even when compared to the MePV (Canteras et al., 1995). The proposition of a "ventral" MeA, made by the MeAD and the MePV, received additional commentaries questioning its actual existence in rats (Dall'Oglio et al., 2008b). Therefore, as previously stated for the whole amygdaloid complex (Brodal, 1981; Swanson and Petrovich, 1998), the MeA is neither an anatomical nor a functional unit. The MeA can have subregion-specific features and this heterogeneity can affect the interpretation of the data from the whole MeA (see parallel comments in Rosa et al., 2011). This implies that the experimental data obtained from the whole MeA have to be considered as the "resultant" of all subnuclei contributions. Thus, remarkable results could be due to a great effect consistently found in one or more than one of its subnuclei. Otherwise, it is also possible that a significant effect in one subnucleus can be masked or diminished after mixing data from other subnuclei where the actions are less intense.

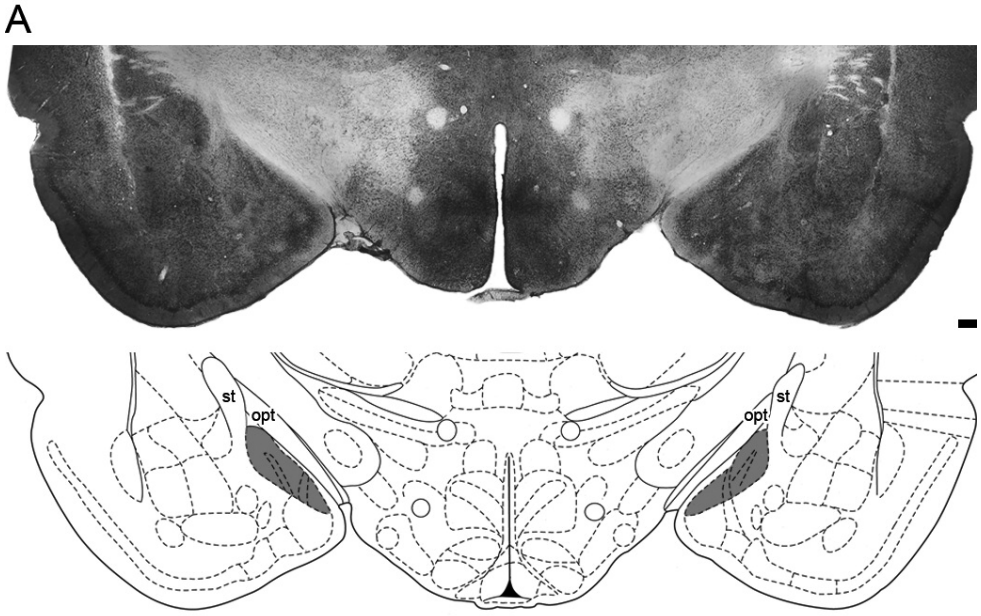
The MeA subnuclei are part of brain circuits sensitive to sex steroids where local cells show plastic changes according to the level of gonadal hormones in circulation (Gréco et al., 1998, 2001, 2003; Newman, 1999; Rasia-Filho et al., 2004, 2009, 2012; de Castilhos et al., 2008). The expression of receptors for testosterone, estradiol or progesterone is different in the MeA subnuclei (Simerly et al., 1990; Shughrue et al., 1997; De Vries and Simerly, 2002). The MePD presents one of the highest concentrations of sex steroid hormones receptors in the rat brain, resembling those found in the hypothalamic nuclei that control reproduction (Simerly et al., 1990; Shughrue et al., 1997). This suggests that males and females have to be studied apart and, in adult females, the phases of the estrous cycle have to be considered as a presumed source of variability in the results. If females had the experience of motherhood, the MePD differed from that of a virgin age-matched female in various respects (Rasia-Filho et al., 2004). These methodological procedures were achieved after many trials, based on many pioneering results, and they are helping us to conduct future work. In the present review, whenever possible, attention will be given to the MePD (Figure 1) because of its known plasticity.

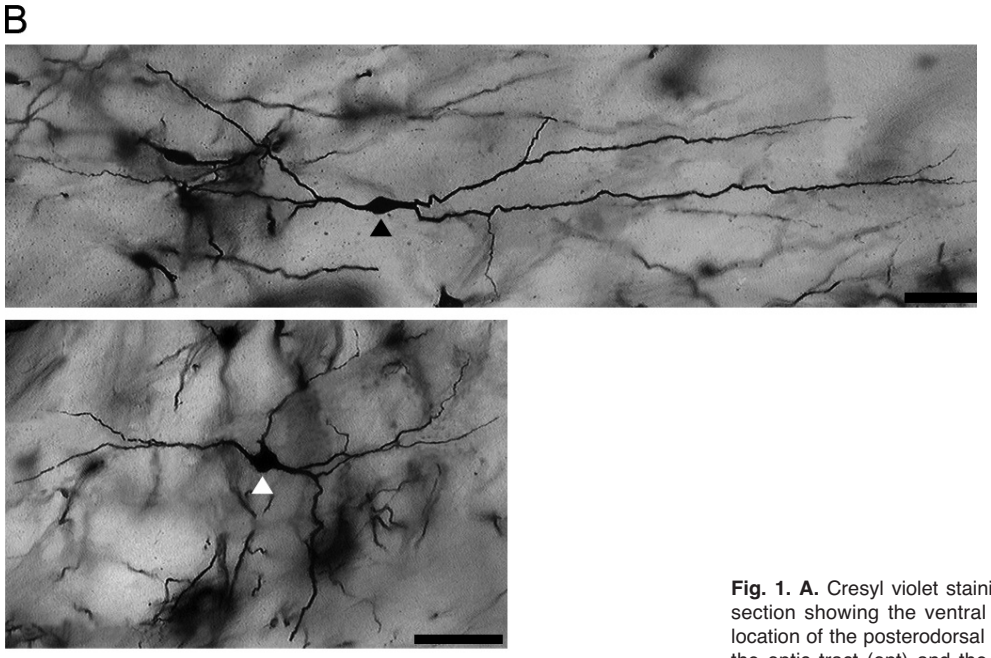
The posterodorsal medial amygdala

The basic morphological description of the MePD was provided from prepubertal Sprague Dawley rats (Cooke et al., 2007). In these animals, the MePD volume was greater in males in both left and right hemispheres (mean ± standard error of the mean, 0.242±0.03 and 0.258±0.026 mm^3, respectively) than females (0.203±0.01 and 0.216±0.023 mm^3, respectively), which represents a statistically significant sex difference close to 15±5 %. The neuronal density was estimated using the optical disector in Nissl-stained coronal sections. In the left and right MePD of males there were 101,744±7,686 and 110,168±7,905 cells/mm^3, whereas females had 110,306±10,223 and 107,208±6,930 cells/mm^3, respectively. By the relative neuronal density and total volume of the MePD, males had more neurons in the right, but not in the left, MePD than females. That is, for the left and right MePD, values were 24,611±3,858 and 28,550±4,525 for males and 22,411±2,934 and 23,079±2,355 for females, respectively. From electron micrograph images the neuropil of the left MePD of males, compared to females, showed significantly higher values, mainly due to the volume occupied by dendritic shafts (41% vs. 32%) and glial cells (15% vs. 13%). On the other hand, more axons filled the left MePD in females than in males (50% vs. 40%). Dendritic spines and synapses accounted for 1% each in both sexes. That is, genetic and/or early epigenetic-mediated sex differences affect the rat MePD prior to the pubertal "activational" period of gonadal hormone action.

In Sprague-Dawley rats (7 months of age) the total number of Nissl-stained cells in the whole MeA (including the surrounding "molecular layer") was estimated using the optical fractionator method in coronal sections (Chareyron et al., 2011). According to these authors, the MeA has (mean ± standard deviation) 188,742±19,688 neurons and 103,936±9,036 glia cells. No sex difference or lateralization was found in the data obtained from 2 males and 2 females.

On the other hand, Morris et al. (2008) studied specifically the MePD of young adult Long Evans rats. They used the thionin technique in coronal sections and counted cells by the optical fractionator method. The MePD volume was greater in the right hemisphere than in the left and males had higher values than females. For the left and right MePD of males the results were (mean





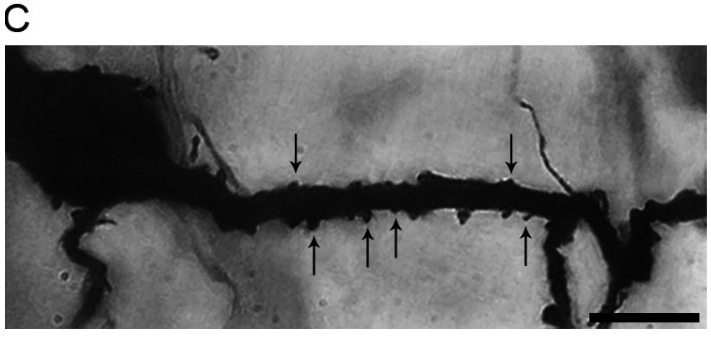


Fig. 1. A. Cresyl violet staining and schematic drawing of a coronal section showing the ventral aspect of the Wistar rat brain and the location of the posterodorsal medial amygdala (colored gray) close to the optic tract (opt) and the stria terminalis (st) approximately 3.30 mm posterior to the bregma (based on Paxinos and Watson, 1988). **B.** Reconstructed digitized microscopic image showing representative multipolar neurons from the rat MePD. Golgi-impregnated multipolar neurons were classified as bitufted (black arrow head) or stellate (white arrow head) ones. **C.** Photomicrograph showing Golgi-impregnated pleomorphic spines (arrows) protruding from a proximal dendrite. Only background contrast was adjusted (Photoshop CS3 10.0, USA). Reprinted from Arpini M., Menezes I.C., Dall'Oglio A. and Rasia-Filho A.A. "The density of Golgi-impregnated dendritic spines from adult rat posterodorsal medial amygdala neurons displays no evidence of hemispheric or dorsal/ventral differences". Neurosci. Lett. 469, 209-213. Copyright (2010) with permission from Elsevier. Bars: A, 1 mm; B, 50 μm; C, 10 μm.

± standard error of the mean) 0.29±0.01 and 0.325±0.015 mm^3, whereas females had 0.18±0.01 and 0.18±0.01, respectively (approximate data based on results presented in Figure 3A, page 855). The number of neurons was higher in the left MePD than in the right MePD and males have more neurons than females. Approximate values for the number of neurons in the left and right MePD of males were 29,000±2,500 and 26,000±2,500, and females had 21,000±2,500 and 16,000±2,000, respectively (based on data presented in Figure 4A, page 856). The number of glial cells is greater in the right than the left MePD and males have more glial cells in both hemispheres compared to females (left and right male MePD, approximately 16,000±1,000 and 24,000±2,000; in females 12,500±2,000 and 13,000±1,000, respectively; Figure 4B, page 856). That is, in the young adult rat, the left MePD is smaller in volume but contains more neurons than the right MePD, which has more glial cells. Johnson et al. (2008) confirmed these MePD volume data and found that astrocytes were more numerous on the right than in the left MePD and in males than in females. In addition, the left MePD had more complex astrocytes compared to those in the right, and the left MePD astrocytes of males had more primary branches, total number of branches, and branch points, and longer branches than females, although females had a greater astrocytic density than males.

Therefore, the rat MePD is an interesting area to study neural gonadal steroid actions. As demonstrated by the Golgi method, the neuronal population in the adult rat MePD of both sexes is comprised of multipolar cells classified as bitufted (not "bipolar", as per Ramón y Cajal's classical description; cf. Rasia-Filho et al., 1999) or stellate neurons (de Olmos et al., 1985; Gomez and Newman, 1991; McDonald, 1992; Rasia-Filho et al., 1999; Cooke et al., 2007; Marcuzzo et al., 2007; Dall'Oglio et al., 2008a). Representative images of these neurons are shown in Figure 1 (see additionally Rasia-Filho et al., 1999, 2004; Dall'Oglio et al., 2008a; de Castilhos et al., 2008). Bitufted neurons are characterized by two dendritic shafts that emerge from a rather fusiform or round soma, whereas stellate neurons have three or more primary dendrites (Rasia-Filho et al., 1999; to compare general morphology with neurochemically distinct MePD neurons, see a recent elaboration in Rasia-Filho et al., 2012). The dendritic trees are rectilinear or sinuous, branch sparingly, show preferred spatial localizations and extend over a wide range of path lengths (Alheid et al., 1985; Rasia-Filho et al., 1999; Dall'Oglio et al., 2008a). There is no clear morphological evidence for striatum-like medium spiny stellate neurons in the MePD of adult Wistar rats (for comparison, see Bennur et al., 2007; Marcuzzo et al., 2007; Dall'Oglio et al 2008a), but other way to classify neurons in the caudal MePD of rats was already reported (Akhmadeev, 2008). In mice, some neurons in the posterior part of the MeA resemble pyramidal neurons from the piriform cortex (Bian et al., 2008). In both mice and rats the local axons project with different orientations but emerge or leave the MePD via the ST, a bidirectional pathway of this subnucleus (Valverde, 1962; Cooke and Simerly, 1995; see also below).

Sex steroid actions in the MePD

The MePD neurons and glial cells in both sexes are normally affected by sex steroids, as summarized elsewhere (Rasia-Filho et al., 2009, 2012). They are also sensitive to changes in plasma levels of these hormones after experimental manipulations, such as castration and replacement therapies (Rasia-Filho et al., 2004; Cunningham et al., 2007; de Castilhos et al., 2008). By in situ hybridization, high concentrations of mRNA for androgen receptors (AR) were found in the MePD (Simerly et al., 1990). Functionally, pheromonal stimulation increased the number of AR-immunoreactive cells in MePD neurons of male hamsters (Blake and Meredith, 2011). In addition, a complete overlap was found between the immunoreactivity of the immediate early gene protein Fos, an indicator of cellular activation, and ARs in MePD neurons of males that ejaculated (Gréco et al., 1996). Local estrogen receptor (ER)-α (ER-α) increased density was associated with a single ejaculation or sexual satiety in male rats (Phillips-Farfán et al., 2007). In the MePD of female rats there were high concentrations of both ER-α and ER-ß (Simerly et al., 1990; Shughrue et al., 1997; Gréco et al., 1998, 2001, 2003). Different regional distribution of these ERs occurs in the dorsal and in the ventral parts of this subnucleus, but both ERs can also be co-localized in the same neurons (Gréco et al., 2001, 2003).

The replacement treatment with estradiol to castrated females decreased the number of MePD cells immunoreactive to ER-α and those co-expressing ER-α and ER-ß (Gréco et al., 2001). In castrated and hormone-primed female rats, Fos-ir following mating occurred in cells co-expressing ER-α or both ER-α/ER-ß in the dorsal MePD (Gréco et al., 2003). This same Fos detection occurred in cells only co-expressing ER-ß in the ventral MePD (Gréco et al., 2003). Progesterone receptors also showed complex dynamics in the female MePD along the estrous cycle or after castration (Gréco et al., 2001; Isgor et al., 2002). In addition, some MePD cells co-express ER-ß and progesterone receptors (Gréco et al., 2001). These data impose another level of complexity on the local effects of sex steroids in normal cyclic conditions or along the reorganizational period of the MePD following castration. For the latter, it is assumed that these effects can occur by two not mutually exclusive possibilities, i.e., after locally mediated/direct cellular actions, or indirectly via synaptic-induced changes mediated by other hormonally-responsive nuclei in interconnected brain circuits (Rasia-Filho et al., 1999, 2012). These propositions were already discussed by other authors (Nishizuka and Arai, 1982; Gomez and Newman, 1991; Lorenzo et al., 1992; Yoshida et al., 1994; Cooke and Woolley, 2009).

Furthermore, the rat MePD assembles coexisting subpopulations of neurons (Bupesh et al., 2011) with no obvious general morphological characteristic that identify them (Nabekura et al., 1986; Gomez and Newman, 1991; Rasia-Filho et al., 1999), but with functional particularities (Coolen et al., 1996; Gréco et al., 1998, 2003; Choi et al., 2005). Cells expressing the transcription factor Lhx6 constitute around 80% of all neurons in the MePD, as seen with confocal microscopy (Choi et al., 2005). This homogeneity for the MePD neurons would explain the low morphological variability (soma size, volume, and dendritic spine density) in male rats as described previously using the Golgi method (Rasia-Filho et al., 2004; de Castilhos et al., 2006, 2008; Arpini et al., 2010). Moreover, various morphological parameters in the rat MePD are sexually dimorphic or affected by sex steroids. Differences between males and females include: the volume of this subnucleus (Hines et al., 1992), the number of neurons and glial cells (Johnson et al., 2008; Morris et al., 2008), the neuronal somatic volume (Hermel et al., 2006a), the spatial orientation of the dendritic branches (Dall'Oglio et al., 2008a), the density of dendritic spines (Rasia-Filho et al., 2004; de Castilhos et al., 2008), the synaptic

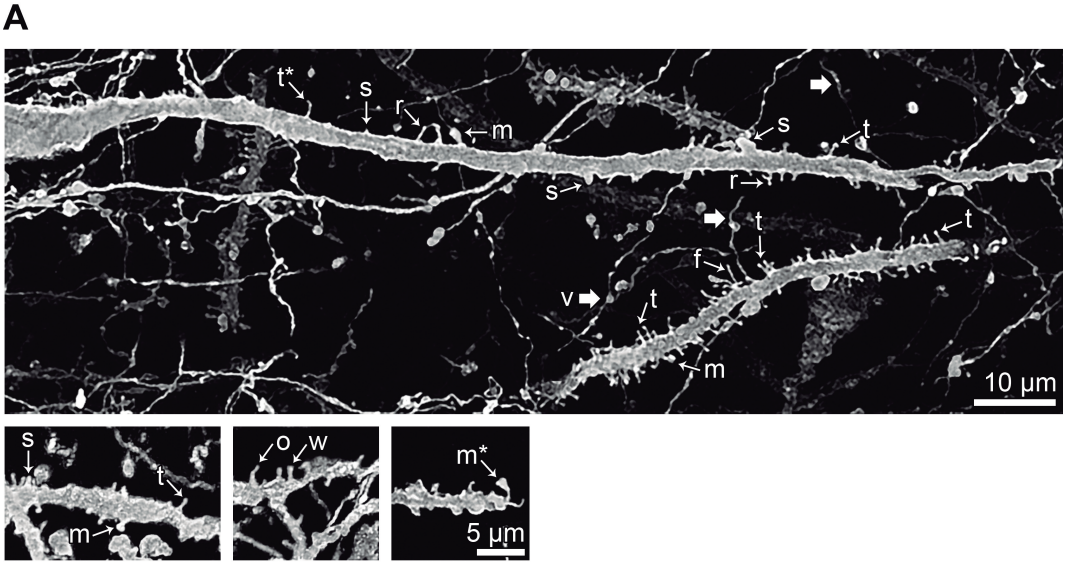


Fig. 2. A. Three-dimensional-reconstructed fluorescent image of Wistar male rat posterodorsal medial amygdala (MePD) neurons under confocal microscopy following application of extracellular sonicated fine powdered DiI in a coronal brain slice. Spines were classified as thin (t), stubby (s), wide (w), "mushroom"-like (m), ramified (r), and "others" (o). At bottom, higher magnification pictures to illustrate the shapes of the dendritic spines. The asterisks indicate a spine with an assumed protruding vesicular spinule. Arrows point to axons with varicosities (v). **B.** Representative drawings of the different shapes of the dendritic spines from the MePD of adult male rats as evidenced by DiI fluorescence and confocal microscopy. **C.** Three-dimensional-reconstructed image under confocal microscopy to show a dendritic shaft and spines from the adult male rat MePD as evidenced by DiI fluorescence (yellow) associated with synaptophysin labeling (red). Note the synaptophysin puncta in close apposition to dendritic shafts and spines of different shapes (indicated by an arrow, for example), although not all of the visible spines appeared contacted by these puncta (asterisk) or others showed a cluster of labeled puncta close to them (arrowhead). In all pictures, background and contrast were slightly adjusted using Adobe Photoshop 7.0 software (USA). Reprinted from Brusco J., Dall'Oglio A., Rocha L.B., Rossi M.A., Moreira J.E. and Rasia-Filho A.A. Descriptive findings on the morphology of dendritic spines in the rat medial amygdala. Neurosci. Lett. 483, 152-156.

connectivity (Nishizuka and Arai, 1983) and the excitatory post-synaptic current frequencies (Cooke and Woolley, 2005), the content of neuropeptides (Micevych et al., 1988; Oro et al., 1988; Malsbury and McKay, 1994; De Vries and Simerly, 2002), and astrocyte density measured by glial fibrillary acidic protein (GFAP) immunoreactivity (Rasia-Filho et al., 2002; Martinez et al., 2006; Johnson et al., 2008).

By linking the behavioral displays elicited by the MePD with gonadal hormonal effects, it became clear that sex steroids can remarkably alter the function of the MePD cells. As reviewed recently (Rasia-Filho et al., 2012), an unilateral implantation of estradiol in this subnucleus restored copulatory behavior in adult castrated male rats (Rasia-Filho et al., 1991) as in adult castrated hamsters after implantation of testosterone or estradiol, but not dihydrotestosterone, in the posterior MeA (Wood, 1996). Male mating behavior increased either after unilateral or bilateral implants of testosterone in the MePD of adult castrated hamsters, indicating redundancy, but not amplification, of the androgenic effects in this brain area (Coolen and Wood, 1999). The MePD role in the central modulation of male copulatory behavior is affected by the individual's sexual experience and involves the evaluation of the sexual receptivity of the female (De Jonge et al., 1992; Stark, 2005). This suggests that the MePD participates in a plastic circuit that changes with emotional/social processing and learning. According to Newman (1999), "These same factors, sex-steroid sensitivity and neuronal connections, are of course dynamically modulated throughout life by sexual maturation, by experience or learning, by reproductive cycles and diurnal cycles, and by disease and aging.… At the very least, these stimuli produce immediate changes in synaptic activity in the nodes of the social behavior network. In some cases the effects are long-lasting changes in the strength of synaptic connections…We will have to demonstrate mini-circuits within this network, each one independently regulating a specific aspect of a particular behavior.".

In effect, most MePD neurons are connected with the medial hypothalamic nuclei related to reproduction (Choi et al., 2005) either directly or indirectly via components of the bed nuclei of the ST (BNST; Dong et al., 2001) or the hippocampus/septum pathway (Petrovich et al., 2001). To the hypothalamus, the MePD sends projections: 1) with sparse terminals or *en passant* buttons to the anterior periventricular nucleus and the arcuate nucleus in the "neuroendocrine motor zone"; to the anterior nucleus and the dorsomedial part of the ventromedial nucleus in the medial 'behavior control column' involved with defensive display, as well to the descending division of the paraventricular nucleus related to ingestive or sympathetic/parasympathetic activities, and to the medial mammillary nucleus or the supramammilary nucleus, this one in the lateral zone; 2) moderate terminals in the ventrolateral part of the ventromedial nucleus in the medial nuclei/"behavior control column" for reproduction; and, 3) dense innervation to the anteroventral periventricular nucleus (AVPV) in the periventricular region; to the lateral part of the medial preoptic nucleus (MPOA) and the ventral premammillary nucleus (PMv) in the medial nuclei/"behavior control column" for reproduction; and, to the posterior nucleus in the lateral zone (Petrovich et al., 2001).

The relevance and functional integration for some of these connections has recently been addressed (Petrovich et al., 2001; Simerly, 2002; Choi et al., 2005; Rasia-Filho et al., 2009, 2012; Quagliotto et al., 2012). It is noteworthy that the rat MePD can affect the occurrence of emotionally-loaded social behaviors, according to Newman's proposition (Newman, 1999). Local neurochemical stimulation and inhibition support the MePD role as a node for the modulation of social behavior neural networks (see further data and comments in Rasia-Filho et al., 2012). Indeed, the MePD deals with the interpretation of the social relevance of both olfactory and vomeronasal stimuli (Meredith and Westberry, 2004; Blake and Meredith, 2011; Dhungel et al., 2011), the central processing of genitosensorial stimulation and modulation of different aspects of the sexual behavior of males (remarkably ejaculation) and females (Rasia-Filho et al., 1991, 2012; Coolen et al., 1997; Pfaus and Heeb, 1997; Coolen and Wood, 1999; Newman, 1999; de Castilhos et al., 2006 linked to Rasia-Filho and Lucion, 1996), maternal behavior (Fleming et al., 1980; Sheehan et al., 2001; Rasia-Filho et al., 2004), aggression (Halász et al., 2002; Nelson and Trainor, 2007; Rasia-Filho et al., 2012), and neuroendocrine responses to stressful stimuli (Dayas et al., 1999; Marcuzzo et al., 2007; Singewald et al., 2008). For example, the MePD projections to the hypothalamic AVPV, MPOA, and PMv are involved with pheromonal stimuli processing, neuroendocrine, behavioral, and sympathetic/parasympathetic responses (Canteras et al., 1995; Petrovich et al., 2001) with some indirect connections via GABAergic efferents from the BNST (Dong et al., 2001; Polston et al., 2004). As noted in a recent review (Rasia-Filho et al., 2009), male rat MePD activation during mating and the synaptic codification of the MePD output activity to hypothalamic areas would disinhibit brain areas involved with sexual activity (Choi et al., 2005) and modulate intromissions and ejaculation (Coolen et al., 1996; Dominguez and Hull, 2001; de Castilhos et al., 2006). Clusters of neurons medially and laterally located within the MePD are respectively involved with the occurrence of these two male activities (Coolen et al., 1996, 1997). Besides, projections to the entorhinal area and to the postpiriform transitional area would serve as other routes for pheromonal stimuli to affect the hippocampal circuitry and memory formation (Petrovich et al., 2001). From these experimental data that demonstrated multiple demands on MePD cells, it was suggested that local neurons receive "different demands from specific pathways, whose inputs are temporally and spatially integrated within neural

networks, triggering the most appropriate action according to the animal's ongoing situation" (Rasia-Filho, 2006; Rasia-Filho et al., 2012 and references therein). At the cellular level, the dendritic spines are candidates for where to look for plastic synaptic properties in these neural circuits. They are notably affected by sex steroids in adult animals.

Dendritic spine plasticity in the MePD

A new frontier in MePD neuroanatomy began when it was possible to look at individual cells within distinct circuits (Choi et al., 2005). Although dendritic spines were recognized as a cellular component for more than a century (see García-López et al., 2010), they have received much attention recently as an active research field for unraveling neuronal plastic properties. MePD neurons are basically spiny and their spines can be found emerging from cell bodies, in the initial axonal cones or, rather, from dendrites (Rasia-Filho et al., 1999; Brusco et al., 2010; Figures 1-3).

As summarized elsewhere (Rasia-Filho et al., 2010), several lines of evidence suggest that spines are complex, multifunctional, integrative units, and form specialized neuronal postsynaptic compartments with neurotransmitter receptor/ionic channels to alter local dendritic (passive and active) biophysical properties (Shepherd, 1996; Benavides-Piccione et al., 2002; Nimchinsky et al., 2002; Kasai et al., 2003; Tsay and Yuste, 2004). Therefore, the pattern of spacing and the shape of dendritic spines can alter single spine membrane potential or couple voltages among neighboring spines and/or dendritic shafts (Harris and Kater, 1994; Hayashi and Majewska, 2005; Bourne and

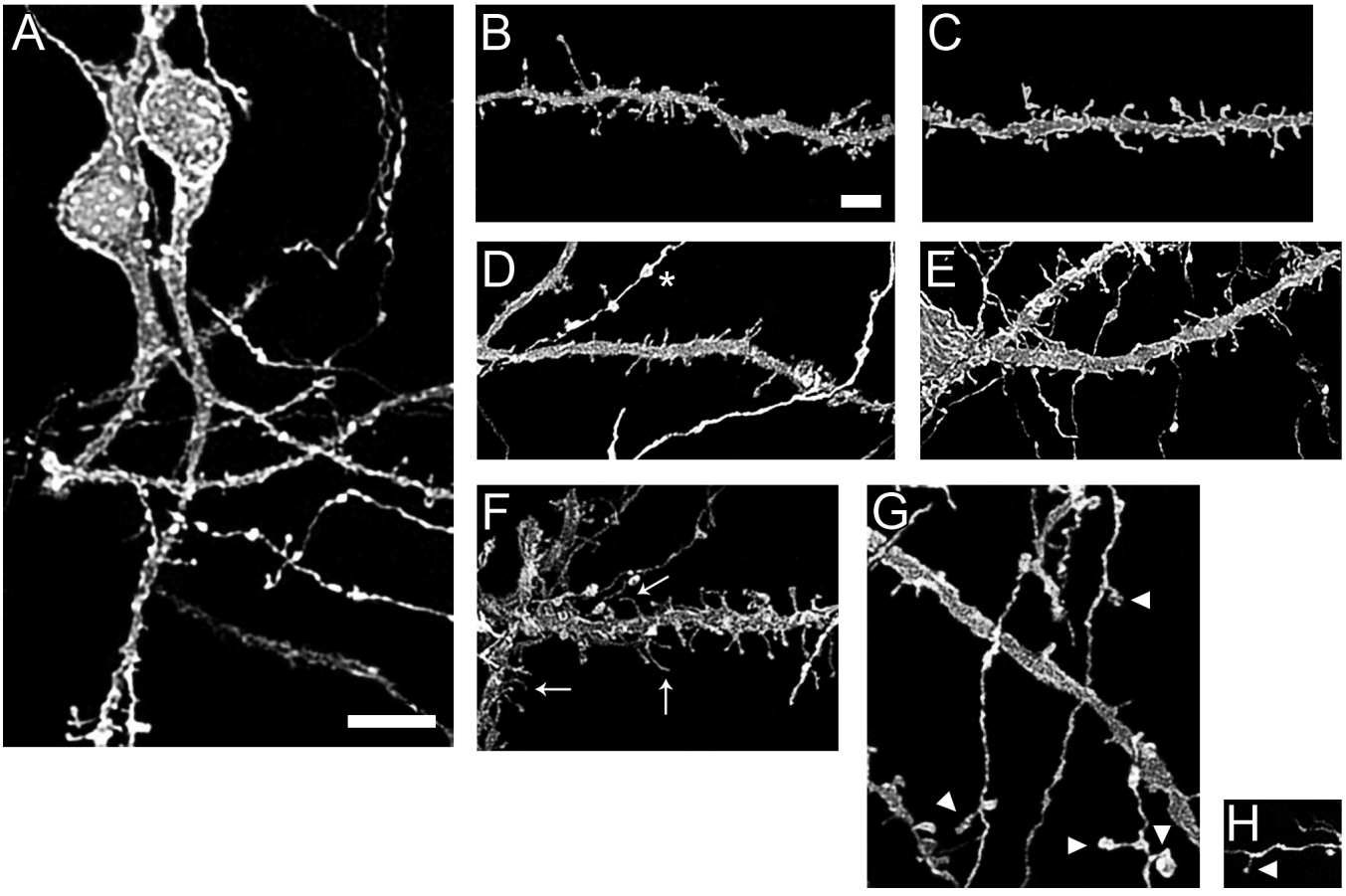


Fig. 3. A. Three-dimensional-reconstructed DiI fluorescent images of the Wistar rat posterodorsal medial amygdala (MePD; coronal brain slices approximately at 3.3 mm posterior to the bregma) neurons under confocal microscopy. **A.** Two multipolar neurons exemplifying the quality of the images obtained with this technique. Note the neuronal components and the presence of dendritic spines. **B-E.** Three-dimensional-reconstructed images at higher magnification to evidence the density and shape of dendritic spines of adult rats. Representative pleomorphic dendritic spines were obtained from the MePD of males **(B)**, females in diestrous **(C),** proestrous **(D)**, and estrous **(E)**. **F.** Note the presence of fillopodium (small arrows) among spines in the dendritic shaft, as well as axons with a regular contour and varicosities (asterisks) or **(G-H)** axonal ramifications with appendages with different shapes (arrow heads). Brightness and background contrast were slightly adjusted (Photoshop CS3 10.0, USA). Bars: A, 10 μm; B-H, 4 μm.

Harris, 2008). This activation can bring about intracellular biochemical cascades with multiple functions, such as to induce and endure long-term electrophysiological changes (Kasai et al., 2003; Segal, 2005). Spines can also serve to establish a biochemical compartmentation to deal with calcium influx, to integrate synaptic function and/or to prevent the increase of ionic concentration to a pathological level during normal synaptic transmission (Segal, 2005). Spines increase membrane surface and the receptive field for the establishment of contacts whose selectivity and functional properties determine the type of synaptically generated electric potential (Harris and Kater, 1994; Nimchinsky et al., 2002; Kasai et al., 2003; but see also Segal, 2010). In effect, few spines are non-synaptic (Arellano et al., 2007), a variable proportion is stable (Zuo et al., 2005), and others can provide new synapses related to changing neuronal inputs (Lendvai et al., 2000; Deng and Dunaevsky, 2005; see also data from Yasumatsu et al., 2008). Spines can show dynamic changes for synaptic plasticity under natural conditions (e.g., along the estrous cycle; Woolley and McEwen, 1992; Brusco et al., 2008) or pathological situations (Campbell et al., 2009). It is likely that changes in spine shape and number may be associated with various cellular processes and have an important functional implication in the synapse-specific implementation of plasticity (Hayashi and Majewska, 2005).

The number and morphology of dendritic spines have been studied using the Golgi method (Ramón y Cajal, 1909; Jones and Powell, 1969; Peters and Kaiserman-Abramof, 1970; Ramón-Moliner, 1970; Valverde, 1970; Fairén et al., 1977; Szentágothai, 1978; Woolley and McEwen, 1992; Dall'Oglio et al., 2010),

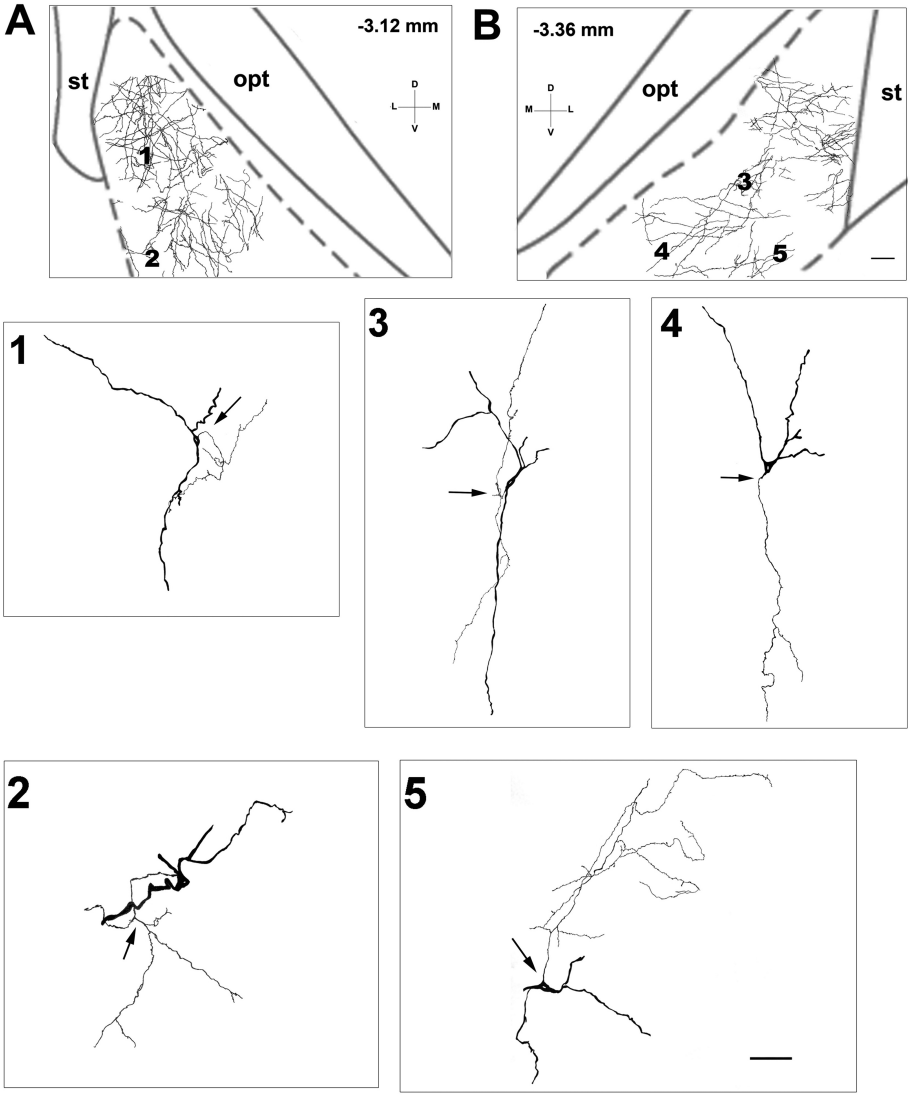


Fig. 4. A,B. Camera lucida drawings of the Golgi-impregnated axonal fibrillar network in the neuropil of the right and left posterodorsal medial amygdala (MePD), close to the optic tract (opt) and stria terminalis (st) in adult male Wistar rats. Images were 3.12 mm and 3.36 mm posterior to the bregma (according to Paxinos and Watson, 1988). Numbers inside the MePD borders represent where the neurons were found. Spatial coordinates are: D, dorsal; L, lateral; M, medial; V, ventral. (1-5) Camera lucida drawings of Golgi-impregnated neurons from the MePD of adult male rats. As a matter of correctness, spine distribution was not drawn for these spiny neurons. Arrows point to axons and their spatial distribution within the MePD. Bars: 50 μm.

intracellular microinjections of Lucifer-Yellow (Duan et al., 2003; Wearne et al., 2005), serial section reconstruction of electron microscopy images (Harris et al., 2006; Arellano et al., 2007), extracellular application of DiI, a diffusible and fluorescent carbocyanine dye, under confocal microscopy (Kim et al., 2007; Rasia-Filho et al., 2009, 2010; Brusco et al., 2010), *in vivo* two-photon imaging (Yang, et al., 2009; Fu and Zuo, 2011) and in cultured central nervous system neurons (Zagrebelsky et al., 2010; Srivastava et al., 2011a,b). Descriptive studies of this highly specialized cellular structure become even more interesting when integrated with the connectional and functional features (Freund and Somogyi, 1983; Izzo et al., 1987; Kisvárday et al., 1990; Benavides-Piccione et al., 2002; Larriva-Sahd, 2008; Lanciego and Wouterlood, 2011; Rasia-Filho et al., 2012) that are needed, for example, to co-ordinate the expression of complex behaviors and timed physiological events. Because of their strategic place to affect somatic voltage, action potential generation, and the electrical/neurochemical output coding for different networks, proximal spines of MePD neurons were counted (Rasia-Filho et al., 2004; de Castilhos et al., 2006, 2008; Marcuzzo et al., 2007; Arpini et al., 2010). Data related to these spines are described below. However, other spine locations along MePD dendrites also provided significant insights about neuronal plasticity (Gomez and Newman, 1991; Cooke and Woolley, 2005; Cunningham et al., 2007). For example, male Sprague Dawley rats castrated before puberty had a reduced dendritic spine number (31-46% lower values than control ones) along the first 70 μm of primary dendrites or along the last 70μm of terminal dendrites, without affecting overall dendritic length or branching of Lucifer Yellow-filled MePD neurons (Cooke and Woolley, 2009). This occurred together with an impaired rate of the typically juvenile rough-and-tumble playful attacks (Cooke and Woolley, 2009). As evidenced using DiI labeling and confocal microscopy, pubertal, gonadally intact Long Evans male rats exposed to testosterone proprionate for 4 weeks had a significant increase (near 67%) in spine number of MePD dendrites at least 20 μm in length and 100 μm from the cell body (Cunningham et al., 2007).

The spine density (i.e., number of spines per unit of dendritic length) in the first 40 μm of dendritic shafts of camera lucida drawing Golgi-impregnated MePD neurons ranged from approximately 2.0 to 2.9 spines/dendritic μm in adult male Wistar rats (Rasia-Filho et al., 2004; de Castilhos et al., 2006, 2008; Marcuzzo et al., 2007; Arpini et al., 2010). Using confocal microscopy and the same rat strain and sex, spine density in proximal branches was (mean ± standard deviation) 1.15±0.67 spines/dendritic μm (Brusco et al., 2010). Methodological differences in the histological preparation, mainly fixation, would account for the discrepant data. As originally depicted, pleomorphic spines were found in the adult Wistar rat MePD, either isolated or in small clusters, notably showing a *continuum* of different shapes and sizes (Rasia-Filho et al., 2004, 2009; Brusco et al., 2010). Spines were classified morphologically as thin, mushroom-like, stubby/wide, ramified, with a gemule appearance or with other transitional aspects (Peters and Kaiserman-Abramof, 1970; Fiala and Harris, 1999; Fig. 2). In the MePD of adult Wistar males, thin spines were the most abundant type (approximately 53%) followed by mushroom-like (22%) and stubby/wide spines (21%); filopodia-like protusions were also found and, together with other less frequent spine shapes, accounted for almost 3% of all sampled spines (Brusco et al., 2010). For comparison between rat brain areas, this proportion of thin spines is basically identical to that found in the stratum radiatum of hippocampal CA1 area (Fiala and Harris, 1999).

As the results in Brusco et al. (2010) indicate, "most of the MePD dendritic spines showed a thin appearance. Two interpretations could be made on spine shape and size modifications by the synaptic signal. One suggests that morphological changes are not required for all forms of synaptic plasticity (see Alvarez and Sabatini, 2007), whereas the other proposes that the diversity of dendritic spines indicates an intrinsic variability for synaptic strength and plasticity (see Arellano et al., 2007). Thin spines are usually more plastic, involve NMDA glutamate receptors, and were named as 'learning spines' (Kasai et al., 2003; Bourne and Harris, 2007). The mushroom-like spines are more stable and make synapses functionally stronger (Kasai et al., 2003; Bourne and Harris, 2007). Stubby spines have a low resistance for the flow of electrical potentials (Korkotian and Segal, 2000)." Ramified spines may enhance the surface available for synaptic contacts but the presence of filopodia is not usual in normal adult brain and might play a role in the establishment of new synapses (Fiala and Harris, 1999; Bhatt et al., 2009). Spinule-like protusions could be involved in structural remodeling and/or intercellular signaling between dendritic spines and its surrounding neuropil (Spacek and Harris, 2004).

Thus, data obtained for the adult male MePD (Brusco et al., 2010) suggest that local "spines can underlie synapses with different plastic capacities, strength, and functional consequences in adult male rats. The dendritic spine geometry would be modeled by synaptic activity to regulate the neuronal function (Hayashi and Majewska, 2005; Schmidt and Eilers, 2009). Dendritic spines can be stable in adulthood (Zuo et al., 2005; Bhatt et al., 2009) but can also show dynamic changes linked with endogenous or environmental influences (Rasia-Filho et al., 2004; Marcuzzo et al., 2007). It is likely that above this basal number, the epigenetic influences of the androgens can increase the dendritic spine density in the MePD. Furthermore, MePD dendrites with stable spines might indicate that neurons can have more steady properties in male-typical neural networks, whereas other modifiable spines would represent an intrinsic plastic capacity for local information processing and behavior modulation.

In this sense, most MePD dendritic spines receive excitatory synapses (Hermel et al., 2006b) and long-term potentiation was described in the MeA (Shindou et al., 1993), which might be related to memory formation in some parallel neural circuits (Petrovich et al., 2001). Prior experiences and associative learning involving the MePD can lead to permanent modifications, at least in the sexual performance of male rats (Stark, 2005)."

However, one must bear in mind what was critically argued by Segal (2010) about dendritic spine plasticity: "… there is an assumption that the size and number of miniature excitatory postsynaptic currents are closely correlated with, respectively, the physical size of synapses and number of spines. However, several recent observations do not conform to these generalizations, necessitating a reassessment of the model: spine dimension and synaptic responses are not always correlated. It is proposed that spines are formed and shaped by ongoing network activity, not necessarily by a 'learning' event, to the extent that, in the absence of such activity, new spines are not formed and existing ones disappear or convert into thin filopodia. In the absence of spines, neurons can still maintain synapses with afferent fibers, which can now terminate on its dendritic shaft. Shaft synapses are likely to produce larger synaptic currents than spine synapses. Following loss of their spines, neurons are less able to cope with the large synaptic inputs impinging on their dendritic shafts, and these inputs may lead to their eventual death. Thus, dendritic spines protect neurons from synaptic activity-induced rises in intracellular calcium concentrations.". These relevant ideas have to be tested in the MePD as well.

To add new data on this issue and to verify the synaptic connectivity of MePD spines, DiI was used concomitantly with the immunolabeling for synaptophysin, a pre-synaptic protein present in contact sites (Fig. 2). This approach proved to be very successful in the rat MePD under confocal microscopy (Rasia-Filho et al., 2010; Brusco et al., 2010). Most spines showed synaptophysin puncta close to its head or neck, whereas other spines had no evident labeled puncta on them or, conversely, multiple puncta appeared on one spine in the adult male rat MePD (Brusco et al., 2010). In adult male Wistar rats, ultrastructural results showed that most contacts (67.5%) were axodendritic, symmetrical and asymmetrical synaptic ones in the MePD neuropil and 92% of them appeared excitatory. Near 23% of all synapses were on dendritic spines, with no inhibitory synapses on them (Hermel et al., 2006b). Although it was suggested that dendritic spines mostly receive an asymmetrical synapse with no dense-cored vesicles (87.5% of the cases; Hermel et al., 2006b), inhibitory and multisynaptic contacts were also found on dendritic spines of the adult male MePD as evidenced in an additional electron microscopy study (Rasia-Filho et al., 2009: Brusco et al., unpublished data). The relevance of this kind of inhibitory contact on spines was hypothesized previously (Marcuzzo et al., 2007). Axospiny inhibitory synapses would account for a small proportion of contacts on MePD neurons. However, some GABAergic terminals and possible inhibitory post-synaptic currents can be dependent on spines (Kisvárday et al., 1990; López-Bendito et al., 2004; Huang et al., 2005). GABA-immunoreactivity occurred in presynaptic terminals on symmetric synapses in the MePD of prepubescent male rats (Cooke and Woolley, 2005). Although the percentage of occurrence of inhibitory spine synapses is small, they actually involve a large amount of contacts per cubic millimeter of brain tissue (Popov and Stewart, 2009). Multisynaptic spines add to the complexity of both the organization and functioning of local synapses as well (Popov et al., 2005). Therefore, the propositions regarding the associations between neurotransmission from the intra-amygdaloid or extra-amygdaloid fibre plexus to MePD dendritic spines have to include the dual possibilities of excitation and inhibition of local neurons in the course of information processing (Rasia-Filho et al., 2004, 2009; Marcuzzo et al., 2007).

Furthermore, the occurrence of "silent synapses" and the proportion of MePD spines that are genetically and functionally developed to be connectionally stable and those spines that are rather labile and plastic remains to be elucidated. One intriguing idea is that MePD dendrites with stable spines may suggest that local neurons can have some steady properties in neural networks, whereas unstable spines would represent an intrinsic plastic capacity for synaptic processing and behavior modulation; in these latter the gonadal hormones would act to modulate spine number and distribution (Rasia-Filho et al., 2009, 2012; Brusco et al., 2010).

In the MePD of adult Wistar rat brains, no statistically significant difference was found in the density of Golgi-impregnated proximal spines in bitufted or stellate neurons (de Castilhos et al., 2006), neither in the percentage of each different shape of dendritic spines in those neurons (Brusco et al., 2010), nor in the dendritic spine density in both the medial or the lateral aspects of the MePD (de Castilhos et al., 2008), which are differently involved with male sexual behavior (Coolen et al., 1996). There was also no difference in dendritic spine density in dorsally- or in ventrally-located neurons of proestrous females MePD (Arpini et al., 2010), in spite of the local heterogenic expression/distribution of ER-α and ER-ß (Gréco et al., 2001). No evidence was found for a left to right hemisphere difference in the density of MePD proximal dendritic spines when comparing data from adult males and diestrous females (Arpini et al., 2010), which contrasts with prepubertal Sprague Dawley data that showed more dendritic shafts and greater branching (Cooke et al., 2007), as well as more asymmetric excitatory synapses on dendritic spines in the left MePD neurons of males than in females (Cooke and Woolley, 2005). Apart from possible strain differences, other disparities between young and adult animals may be due

to increased levels of gonadal hormones during puberty, which may result in the rearrangement of dendrites, dendritic spines and/or other types of synapses in the MePD of both hemispheres. Indeed, there is a dendritic pruning in MeA neurons during pubertal development in male Syrian hamsters (Zehr et al., 2006) and a MePD synaptic reorganization, indicated by an increase in the number of puncta immunoreactive for vesicular glutamate transporter-2 and post-synaptic density 95, two markers of excitatory synapses, in male Siberian hamsters during puberty (Cooke, 2011).

As found originally by Rasia-Filho et al. (2004), the density of proximal dendritic spines in the MePD of adult Wistar rats is sexually dimorphic (higher in males than in proestrous, estrous or metaestrous females) and is affected by the normal fluctuations in plasma ovarian steroids along the estrous cycle (near 35% of reduction during the transition from diestrous to proestrous) or after the occurrence of motherhood (almost 24% lower in postpartum diestrous females than in age-matched virgin diestrous females). The mean spine density in the initial 40 μm of Golgi-impregnated dendritic branches studied under light microscopy ranged from approximately 2.2 spines/dendritic μm in diestrous to 1.35 spines/dendritic μm in the other phases of the estrous cycle (Rasia-Filho et al., 2004). In another set of experiments using DiI and confocal microscopy under the same methodological conditions described previously (Brusco et al., 2010), adult Wistar males had (mean ± standard deviation for proximal dendrites) 1.3±0.3 spines/dendritic μm (n=6 rats, 2.5±1.4 neurons per rat), whereas females had 0.9±0.1 spines/dendritic μm in diestrous (n=4 rats, 4.2±2.6 neurons per rat), 0.6±0.2 spines/dendritic μm in proestrous (n=4 rats, 4.2±1.5 neurons per rat), and 0.6±0.1 spines/dendritic μm in estrous (n=5 rats, 3.8±1.6 neurons per rat; Fig. 3). These data agree with the interpretation of previous Golgi results, i.e., that the number of proximal dendritic spines is different between sexes in rats (Rasia-Filho et al., 2004) and are in accordance with previous confocal data obtained in males (Brusco et al., 2010). In these newly sampled MePD neurons, diestrous females had approximately 51% of thin spines, 31% of stubby/wide, 12% of mushroom-like, and 6% belonging to the other spine shapes. Proestrous females showed near 53% of thin spines, 28% of stubby/wide, 10% of mushroom-like, and 9% belonging to the other spine shapes. Estrous females showed around 47% of thin spines, 34% of stubby/wide, 14% of mushroom-like, and 5% belonging to the other spine shapes. These novel descriptive data indicate that males have more than threefold mushroom-like spines than females along the estrous cycle. Taking into account the putative stability of this kind of spine and the relative percentages of the other shapes along the estrous phases, the present data expand the observations on cyclic synaptic plasticity in the female rat MePD (Rasia-Filho et al., 2004, 2009).

Gonadal steroid withdrawal and replacement hormonal therapies affected MePD dendritic spine density as well. Adult male castration notably reduced dendritic spine density 90 days after testes removal, leaving a lower basal value of stable spines in the MePD at this time (de Castilhos et al., 2008). This finding reinforces the proposition that androgens can affect the MePD neuropil and local synaptic organization, as previously indicated by the reduction in substance P immunoreactivity and MePD volume (Malsbury and McKay, 1994) or dendritic atrophy after castration (Gomez and Newman, 1991). Considering morphology and function, it is interesting to note that the decrease in the MePD spine density 90 days following testes removal coincides temporally with the marked postcastration reduction in ejaculatory and intromission behaviors in rats (Rasia-Filho et al., 1991; Rasia-Filho and Lucion, 1996; de Castilhos et al., 2008). It remains to be determined whether MePD spine reduction following gonadectomy is the cause or the consequence of male sexual behavior impairment.

In adult Wistar female rats, estradiol and progesterone replacement beginning 1 week after castration increased spine number to supra-physiological levels when compared to the normal number of spines during the proestrous phase (Rasia-Filho et al., 2004; de Castilhos et al., 2008). In this experiment, ovariectomized (OVX) females received one of the following treatments: (1) rats that received 2 injections of sesame oil as vehicle (0.1 mL, s.c.), 24 h apart each one, and a third injection of oil 48 h later; (2) rats that received 2 injections of estradiol benzoate (EB) (10 μg/0.1 mL, s.c.), 24 h apart each one, and a third injection of sesame oil 48 h later; and, (3) rats that received 2 injections of estradiol benzoate (10 μg/0.1 mL, s.c.), 24 h apart each one, but the third injection was of progesterone (500 μg/0.1 mL, s.c.) 48 h later (de Castilhos et al., 2008). The proximal dendritic spine density of females treated with EB and progesterone reached statistically significant values when compared to both oil-treated (near 68% more spines) or only EB-treated rats (around 42%; de Castilhos et al., 2008). The higher MePD spine density under the effects of EB and progesterone indicates a complex modulation of spine number by ovarian steroids and their receptors under different experimental or physiological conditions (compare data in Rasia-Filho et al., 2004; de Castilhos et al., 2008; see a parallel discussion in Martinez et al., 2006).

Based on dendritic morphology and spine data acquired until now, it was suggested that adult Wistar rat males and females might be receiving inputs from different spatially-oriented neural pathways (Dall'Oglio et al., 2008a). This might not occur during the pre-pubertal period in Sprague Dawley male rats submitted to gonadectomy (Cooke and Woolley, 2009). In the latter case, spine density did not show distinct changes in one particular set of inputs to the MePD, as evaluated by the general reduction in spines whatever the dendritic projection was (Cooke and Woolley, 2009). Apart from the possible differences due to the rat strain, sample size,

or methodological approach, in adult Wistar rats, MePD dendritic shafts and spines might be receiving inputs from different circuits. That is, there were more dendritic branches oriented dorsolaterally and medially in males, whereas diestrous females had predominant dorsal and ventromedial orientated dendrites (Dall'Oglio et al., 2008a). The medial dendritic orientation suggests that males are rather gathering synaptic inputs from the superficial "molecular layer", where vomeronasal information passes through (Scalia and Winans, 1975; de Olmos et al., 2004; Pro-Sistiaga et al., 2007). Males also have a higher number of dendritic shaft synapses in the medial part of the ventromedially surrounding 'molecular layer' and more spine synapses in its ventral aspect (Nishizuka and Arai, 1983). "On the other hand, morphological (dendritic spine density changes) and biochemical switches (as found for the expression of different neuropeptides) suggest that the female MePD play a relevant role in altering the course of information through interconnected circuitries during the estrous cycle (Oro et al., 1988; Fergunson et al., 2001; Polston et al., 2001; Rasia-Filho et al., 2004; Simerly, 2004). For instance, fewer dendritic spines in the MePD occur during proestrous and estrous females, coincident with a decrease in the number of synapsin reactivity, which could be associated with synaptic pruning (Rasia-Filho et al., 2004; Oberlander and Erskine, 2008). Thus, it was also hypothesized that labile dendritic spines in the MePD, which change their number along the estrous cycle, would serve to modulate phasic synaptic inputs, whereas dendritic shafts would serve to receive more stable afferences (Rasia-Filho et al., 2009). It would be interesting to know whether the synaptic inputs to dendritic spines and shafts are neurochemically different and/or whether they come from different subpopulations of input neurons…" (Rasia-Filho et al., 2012; additional comments in Rasia-Filho et al., 2009).

Another clue for elucidating the plasticity of the MePD neurons is provided by the aspect of the axonal network in the neuropil of this subnucleus. Because dendritic spines usually establish contact with one axon (Hermel et al., 2006b; but see Rasia-Filho et al., 2009; Brusco et al., 2010), the study of the features of the axonal organization in the MePD deserves further consideration. Axonal morphology of the MePD of adult male Wistar rats was studied by the "single-section" Golgi method and its variant (Gabbott and Somogyi, 1984; Izzo et al., 1987; Bolam, 1992) with light microscopy and DiI labeling on confocal microscopy. Different Golgi procedures provided separate possibilities for the evaluation of axonal features and extensions. In our hands, the Golgi procedure developed by Izzo et al. (1987) provided the best results. The spatial distribution of local axons partially resembled the one previously observed in the MePD of mice (Valverde, 1962; Fig. 4). Isolated axons in the MePD neuropil were often observed with both Golgi and DiI procedures. In these cases there were no obvious conditions to classify them as afferences or efferences because the axonal fibrillar pattern of the MePD resulted from these mixed circuits with branchings in various directions (Fig. 4). In this regard, axons might be coming from the ipsilateral MeA subnuclei or other intra-amygdaloid connections (including fibers from the intercalated nuclei or from the cortical ones) or from various extra-amygaloid connections (Nishizuka and Arai, 1983; Canteras et al., 1995; McDonald, 1998; Pitkänen, 2000; Meredith and Westberry, 2004).

In the adult male Wistar rat MePD a single Golgi-impregnated axon per neuron was found emerging from the cell body or, sometimes, from a primary dendrite. Axons had regular contours and they likely represent only the subpopulation of unmyelinated nerve fibers (Lanciego and Wouterlood, 2011). The presence of only an initial axonal cone in well-impregnated MePD neurons (Rasia-Filho et al., 1999; Dall'Oglio et al., 2008a) indicates that the Golgi reaction was impaired by the myelin sheath. The axons usually presented varicosities but the occurrence of non-classical *en passant* synapses related with these varicosities was not studied by electron microscopy until now. Axonal length was variable due to technical reasons. Local axons in coronal sections showed a tortuous course with a fibrillar aspect composed by parallel and oblique fibers in relation to the OT. Some axons were found going dorsally to the ST (Fig. 4). Supposedly, those neurons would be classified as projecting ones, but the end-target of these axons could not be determined with the present approach. Other neurons had axons directed to the medial "molecular layer" or were projected ventrally or provided an apparent innervation to its close surrounding space (Fig. 4). Some neurons appeared to have recurrent axons but their actual existence has to be confirmed. The pattern of ramification of the sampled axons was not very extensive and the number of collaterals was notably fewer than some interneurons in the rat cerebral cortex (Fig. 4). Local axonal morphology, branching and lengths did not allow the reliable classification of MePD cells as interneurons or projecting ones. This issue needs additional effort using electrophysiological recordings. As noted here, there is an interesting research field opened to new and exciting discoveries.

In addition, one morphological aspect of the MePD axons focused our attention: the presence of axonal ramifications along the axon length appearing as pleomorphic protusions from the axon membrane. They appeared as a kind of growth cone restricted to the end of the axon (Fig. 3). The appearance of each protusium under confocal microscopy was usually as a small, thin appendage extending from the parent axon. Varying shapes were observed, including a single fine prolongment that resemble a filopodium, those appearing like "spines" with a neck and a bulbous head or ramified (Fig. 3). They were not as complex as the large end bulbs of Held in the anteroventral cochlear nucleus (Lorente de Nó, 1981; Ryugo and Fekete, 1982). However, these axonal protusions appear similar to terminals found in the accessory olfactory bulb (Larriva-

Sahd, 2008) or the auditory cortex (Szentágothai, 1978), two highly plastic structures in the rat brain. It is unclear whether these axonal appendages may be in the process of forming new local connections or if they had already made mature, stable connections, adding further clues for synaptic processing in the MePD of adult animals. Interestingly, among local spines, filopodia were also observed arising from dendritic shafts of adult rats (Brusco et al., 2010). Based on these data, a working hypothesis regarding the formation and stability of axospiny synapses is as follows: 1) dendritic filiopodia grow to find an axon aiming to form a new contact, 2) an axon sends out a protusium for a new synaptic site, or 3) both phenomena may occur concomitantly (Nimchinsky et al., 2002). The spinogenesis and/or plasticity of dendritic spines may now be linked with the axonal properties in the neuropil of the adult rat MePD. Likewise, astrocytic morphology and function may be integrated in this dynamic scheme as the "third synaptic element" and for which sexually dimorphic or gonadal hormones effects were already depicted in the MePD of rats (Rasia-Filho et al., 2002, 2012; Martinez et al., 2006; Johnson et al., 2008; Morris et al., 2008).

Subcellular effects of sex steroids in the MeA

Because of its profound effects on cellular and synaptic growth, estrogen may be thought of as a trophic factor (Pfaff and Cohen, 1987). These effects may be direct or indirect via second messenger pathways, ultimately resulting in the generation of gene products (Woolley and Cohen, 2002, Scharfman and MacLusky, 2005; Srivastava et al., 2011a,b), which may elicit growth or regulatory effects, such as those for dendritic spines described above. Notably, different responses of the dendritic spines in the MePD to estrogen occur during physiological cyclic fluctuations of sex steroids or after ovariectomy and hormonal replacement, which also contrast with the region-specific effects reported in the VMH or the CA1 hippocampal field in female rats (compare data in Woolley et al. 1990; Calizo and Flanagan-Cato, 2000; Rasia-Filho et al., 2004; Brusco et al., 2008; de Castilhos et al., 2008).

Among the candidates that are likely to mediate estrogen effects on synaptic and cellular growth and neuroprotection, in general, are the cyclic AMP response element-binding protein (CREB)-related gene products, brain-derived neurotrophic factor (BDNF), the anti-apoptotic protein B-cell lymphoma-2 (Bcl-2) and the activity-regulated cytoskeleton-related protein (Arc). Furthermore, an emerging and exciting literature describes some of the rapid effects of estrogen on signal transduction cascades that lead to local protein synthesis (cf. Srivastava et al., 2011a,b for review) and/or rearrangement of the actin-based cytoskeleton (Kramár et al., 2009a,b; see also data in Sekino et al., 2007) and subsequent numerical and morphological alterations in dendritic spines.

CREB may be activated by phosphorylation at Serine-133 by various kinases, including: protein kinase A (PKA), calcium/calmodulin-dependent protein kinase IV (CaMK IV) and ribosome S6 Kinase (RSK) (cf. reviews in Lonze and Ginty, 2002; McClung and Nestler, 2008). Phosphorylated CREB (pCREB) is capable of forming homo- or heterodimers with cyclic AMP responsive modulator protein (CREM) or activating transacting factor (ATF). It can then bind to a cAMP response element (CRE), which consists of the palindromic consensus sequence TGACGTCA of promotor regions of genes (cf. reviews in Lonze and Ginty, 2002; McClung and Nestler, 2008). Along with the relevant co-activators the complex can activate transcription of CREB-related genes, such as those described above.

Previous studies (Rachman et al., 1998) indicated an ameliorative effect of estrogen in the forced swim test, a rodent model for depressive-like behavior. This finding prompted a search for molecular correlates of this action in brain areas implicated in emotional processing, including the amygdaloid nuclei and hippocampal fields. Since CREB is a target of antidepressant action (Duman et al., 1997; cf. also Carlezon et al., 2005), attention was focused on this gene transcription factor in elucidating some of the molecular mechanisms that may be involved in estrogen effects in the forced swim paradigm. Specifically, the effects of estrogen on the CREB signaling pathway, including BDNF and Bcl-2 in the whole MeA were examined. In addition to the aforementioned effects of estrogen on dendritic spines in the MeA, this brain structure was selected for study for several reasons: its relevance to reproduction (Newman, 1999; McDonald, 2003; Rasia-Filho et al., 2012); its abundance of ERs (Simerly et al., 1990; Li et al., 1997; Shughrue et al., 1997, 1998; Laflamme et al., 1998; Österlund et al., 1998; Gréco et al., 2001; Isgor et al., 2002) and its documented involvement in neuroendocrine responses to emotional stress (Dayas et al., 1999; Ebner et al., 2004; Marcuzzo et al., 2007), in which pCREB also appears to play a role (Kuipers et al., 2006). The MeA or its subdivisions have also been shown to be sensitive to steroid hormone manipulations (Nishizuka and Arai, 1981; Wood and Newman, 1995; Gréco et al., 2001; Rasia-Filho et al., 2004; de Castilhos et al., 2008), as described above, or defective androgen receptors (Morris et al., 2005). Our initial biochemical studies employed the whole amygdala because of the difficulty in dissecting out fresh subregions of this brain area. Nevertheless, this approach can provide cues for the subcellular effects of gonadal hormones in the MeA subnuclei as well.

The following section describes some of the effects of estrogen on the CREB signaling pathway, including its actions on CaMK IV, CREB, pCREB and BDNF and Bcl-2 in the MeA of OVX, estrogen-treated female rats. The central amygdala (CeA) served as a control throughout. In addition, the importance of dose and time effects of hormone treatment and a description of some preliminary findings regarding strain-dependent

differences in plasma steroid hormone levels and in pCREB levels in limbic brain areas of intact and OVX female rats are given. Some of the molecular mechanisms of dendritic spine growth in relation to signal transduction cascades and studies relating some of these cascades to sexual dimorphisms are considered. Comparisons to other brain areas, where experimental data is currently available, are needed to provide insights into MeA features and, more specifically, for further experiments aimed at the rat MePD.

Effects of long-term estrogen treatment on CRE-DNA binding, CREB, pCREB and BDNF in the amygdala and integrated areas

First, an effect of long-term estrogen treatment (10μg estradiol benzoate [EB] for 14 days) on CRE-DNA binding activity in neuroanatomical areas related to emotional processing in OVX rats using the gel-mobility shift assay on nuclear protein extracts from the amygdala, hippocampus, frontal cortex and, as a control, the cerebellum was determined (Carlstrom et al., 2001). Hormone treatment of OVX rats over the two week period resulted in an increase in CRE-DNA binding activity in the nuclear extract of amygdala of these rats compared to OVX controls, but not in extracts of hippocampus, frontal cortex, or cerebellum. Acute estrogen treatment (100 μg for one hour), on the other hand, resulted in an increase in CRE-DNA binding activity in the frontal cortex of OVX rats treated with estrogen compared to OVX controls. However, no differences were seen in the whole amygdala, hippocampus or cerebellum using this regimen. Higher CRE-DNA binding was associated with increases in levels of total and pCREB in amygdala during long-term estrogen treatment as seen by quantitative analysis of Western blots (Carlstrom et al., 2001). No differences in these parameters were seen with the acute estrogen treatment. The lack of effect of the long-term estrogen treatment in the hippocampus may be due to masking of the positive signal in the CA1 and CA3 regions, as whole hippocampus was used in these experiments.The importance of this consideration is underscored by studies showing hetereogeneity of dose and time effects of estrogen on neuron-specific neuronal protein (NeuN) and pCREB in the hippocampus of OVX rats, where EB responsiveness to different regimens varies depending on regional specificity of the hippocampus among its subregions and throughout its extent (Bakkum et al., 2011).

To further ascertain the effect of estrogen in the MeA and CeA, immunolabeling for pCREB using immunoperoxidase techniques was performed. Long-term EB treatment resulted in a significant increase in relative total immunolabeled nuclei in the MeAV; no differences were seen in the MePD or MeAD or MePV subdivisions. However, this finding does not preclude an influence of the CREB pathway in these areas. Intraamygdaloid connections or connections from other brain areas into the MePD, for example, can influence local synaptic growth and development within the MePD. Alternatively, the MePD may be sensitive to other estrogen regimens or other complex ER interactions and responses. Moreover, estrogen effects on the CREB signaling pathway in this brain structure may be subject to strain differences and/or sex differences, as discussed below. Estrogen effects on pCREB protein levels and CRE-DNA activity in the amygdala may presage CREB-binding protein (CPB) recruitment and the initiation of transcriptional activity (cf. discussion in Carlstrom et al., 2001).

The aforementioned estrogen regimen, however, did not elicit any changes in basal or cAMP-stimulated activity of protein kinase A (PKA) or in immunolabeling of the α-isoform of the catalytic subunit of PKA (PKAα-C) in the amygdala of OVX rats following EB treatment compared to OVX controls (Carlstrom et al., 2001). Therefore, an alternative path to CREB phosphorylation, i. e., CaMK IV was explored. Upon activation by calcium/calmodulin kinase kinase (CaMKK) CaMK IV can phosphorylate CREB at serine 133. The long-term estrogen regimen increased protein levels of CaMK IV in the nuclear fraction of whole amygdala and in the MeA and basomedial amygdala, but not CeA or basolateral amygdala, by quantitative Western blot analysis and immunogold labeling, respectively (Zhou et al., 2001). CaMK IV may be involved in dendritic spine growth and synaptic connectivity (Soderling, 2000) and may, therefore, mediate estrogen action on dendritic spines in the MeA.

Indeed, some of the studies on immunolabeling in the MeA of OVX rats given long-term estrogen treatment were confirmed using gold immunolabeling (Zhou et al., 2005). Estrogen treatment increased immunolabeling of CREB and pCREB in the MeA and basomedial amygdala, but not CeA or basolateral amygdala (Fig. 5). Also, long-term estrogen treatment increased gold immunolabeling and mRNA levels as seen with *in situ* polymerase chain reaction (PCR) of BDNF in the MeA and basomedial amygdala and CA1 and CA3 regions of the hippocampus, but not in any other amygdaloid or hippocampal regions examined (Zhou et al., 2005)

Dose and time effects of estrogen on the CREB signaling pathway, pCREB and Bcl-2 in the MeA and on behavior

The aforementioned data suggested a neuroprotective effect of estrogen in the MeA. However, estrogen treatment to OVX females led to opposite results on the density of MePD dendritic spines than seen with normal ovarian steroids fluctuations (Rasia-Filho et al., 2004; de Castilhos et al., 2008). That is, a reduced number of spines is found in the MePD concomitant with the physiological elevation of estradiol and progesterone during the proestrous phase, whereas supraphysiological steroid substitutive therapy to OVX females promoted an abnormal increase of spine counts

(compare Rasia-Filho et al., 2004; de Castilhos et al., 2008).Because hormonal regimens and time courses for hormone replacement therapies vary significantly, it is important to address the manner in which different regimens affect the CREB pathway. This issue is underscored by the emergence of low-dose hormone therapies, which have been developed with the assumption that the associated risks are reduced for women (cf. van de Weijer et al., 2007). Also, some animal studies report contradictory findings regarding the effectiveness of different estrogen regimens. Factors that may influence the efficacy of hormone treatments on the brain and/or behaviors include: regimen (Wise et al., 2001; Sohrabji and Lewis, 2006; Walf and Frye, 2006); dose, manner of delivery [continuous or repeated injections at specific intervals, length of treatment; acute versus chronic treatment; cf. Gibbs, 2000)]; length of time of ovariectomy (Singh et al.,1995; Cavus and Duman, 2003; Tanapat et al., 2005; Caruso et al., 2010; Lagunas et al., 2010); age of animals (Diz-Chaves et al., 2012) and/or hormone deprivation (Daniel et al., 2006)

We traditionally used the 10 μg for 14 days estrogen regimen based on previously published data (Rachman et al., 1998; Carlstrom et al., 2001; Zhou et al., 2005) so as to optimize the detection of hormonal action on cellular responses and relate our findings to previously published biochemical, molecular, and immunocytochemical data on estrogen effects on the CREB pathway, including pCREB (Gu et al., 1996; Zhou et al., 1996; Ábrahám et al., 2003; Szegö et al., 2006; cf. review in Rønnekleiv et al., 2007). However, to address some of the aforementioned issues, the effects of EB on neuronal numbers (using neuron-specific protein [NeuN] immunolabeling) and brain region volume in the MeA and CeA using stereology were determined (Fan et al., 2008a). Ovariectomized rats were injected with vehicle for 14 days; 2.5 μg EB for 4 or 14 days; or 10 μg EB for 14 days. NeuN-labeled neuronal number may be related to neuronal survival and upregulation of CREB signaling, therefore the effect of these regimens on levels of pCREB labeling in the MeA and CeA was also tested. The 2.5 μg EB for 14 days regimen increased the mean number of NeuN-labeled neurons and pCREB-labeled cells in the MeA compared to vehicle or 2.5 μg EB for 4 days, indicating that the effect was time-dependent (Fan et al., 2008a). There was also an increase in volume of the MeA with 2.5 μg EB for 14 days compared to vehicle or 2.5 μg EB for 4 days. No differences in these parameters were seen in CeA. These data indicate a neuroanatomical heterogeneity of a time effect of EB on cells expressing NeuN and pCREB in the MeA versus CeA. The time-related increase in pCREB immunolabeling may be due to an increase in the number of cells expressing pCREB or an upregulation of pCREB cells already expressing pCREB. Also, Zhou et al. (2001) showed an estrogen-induced increase in levels of the upstream regulator of CREB, CaMK IV and a decrease in levels of calcineurin (Zhou et al., 2004), a negative regulator of pCREB, in the MeA, but not CeA, of OVX rats with the 10 μg EB for 14 days regimen. Funabashi et al. (1995) have demonstrated a negative effect of proestrous or estrogen on calcineurin mRNA levels in the hypothalamic ventromedial nucleus (VMN), and Sharrow et al. (2002) have also shown a negative effect of proestrous or estrogen on protein levels and activity of calcineurin in the hippocampus.

The EB-induced increase in the mean numbers of NeuN-immunolabled neurons may be due to an increase in neurogenesis, an increase in neurotrophic and/or survival factors, such as BDNF and Bcl-2, respectively, and/or a decrease in neuronal death. Relevant to the MeA, Fowler et al. (2005) noted estrogen effects on neurogenesis in the adult posterior MeA of meadow voles. Carillo et al. (2007) have demonstrated an increase in neuron number in the MeAV in estrous female rats compared to diestrous rats. There was no

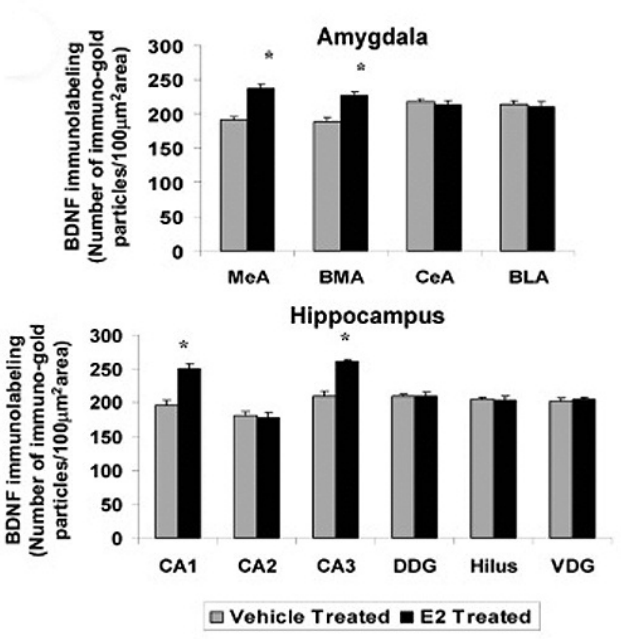


Fig. 5. Quantitation of BDNF immunogold labeling (number of immunogold particles per 100 μm^2 area) shows that estrogen treatment significantly increases levels of BDNF immunolabeling in specific subdivisions of the amygdala (top) and, for comparison, in the hippocampus (bottom). A significant increase in immunogold labeling is seen in the medial (MeA, $p<0.01$) and basomedial (BMA, $p<0.01$), but not central (CeA) or basolateral (BLA) amygdala of the estrogen-treated group (n=6) compared to the ovariectomized (OVX) control animals (n=6). A significant increase in levels of BDNF immunolabeling is seen in the CA1 ($p<0.001$) and CA3 ($p<0.001$) regions of the hippocampus, but not in the CA2, dorsal dentate gyrus (DDG), hilus, or ventral dentate gyrus (VDG) of the estrogen-treated group (n=6) compared to the OVX control (n=6) animals. Values are the mean ± S.E.M. and are represented as the mean OD/pixels of area. * Significantly different from vehicle-treated rats. Reprinted with kind permission from Neuroendocrinology; Zhou J., Zhang H., Cohen R. S. and Pandey S.C. (2005). Effects of estrogen treatment on expression of brain-derived neurotrophic factor and cAMP response element-binding protein expression and phosphorylation in rat amygdaloid and hippocampal structures. Neuroendocrinology 5, 294-310. Copyright Karger.

increase in bromodeoxyuridine or GFAP immunoreactivity, suggesting that an increase in the number of glial cells did not contribute to the increase in cell number. Also, because of the variability in cell number between estrous and diestrous rats, there was a transient sex difference between males and diestrous females.

An EB-induced increase in volume in the MeA, but not CeA, was seen with both of the 14 day EB treatments. Other studies demonstrated sex differences in the volume of unilateral MeA, with the nucleus of the adult male being larger than that seen in females; the difference was evident at postnatal (PN) day 21 (Mizukami et al., 1983). Estrogen increased the volume from PN days 1 to 30 compared to the non-treated female, whereas similar administration of estrogen did not affect the lateral nucleus in a comparable way (Mizukami et al., 1983). After day PN 30, the volume in estrogen-treated females was similar to that seen in the males. Other studies document the maintenance of MePD volume by estradiol following castration of males (Cooke et al., 2003). In the present study, differences in volume among EB regimens may be due to estrogen effects on cell survival and/or an effect of ovariectomy on apoptosis or other forms of degeneration. Several lines of evidence indicate that estrogen maintains soma size (Cooke et al., 2003; Cooke and Woolley, 2005) and synaptic structures in the MeA (Ebner et al., 2004; Cooke and Woolley, 2005; Cooke et al., 2007) and the role of estrogen in neuroprotection is well-documented (cf. reviews in Garcia-Segura et al., 2001; Scott et al., 2012). In this regard, the EB-induced volume changes may be due to a region-specific effect of estrogen on BDNF (Zhou et al., 2005) or Bcl-2 (Fan et al., 2008b) in the MeA, but not CeA.

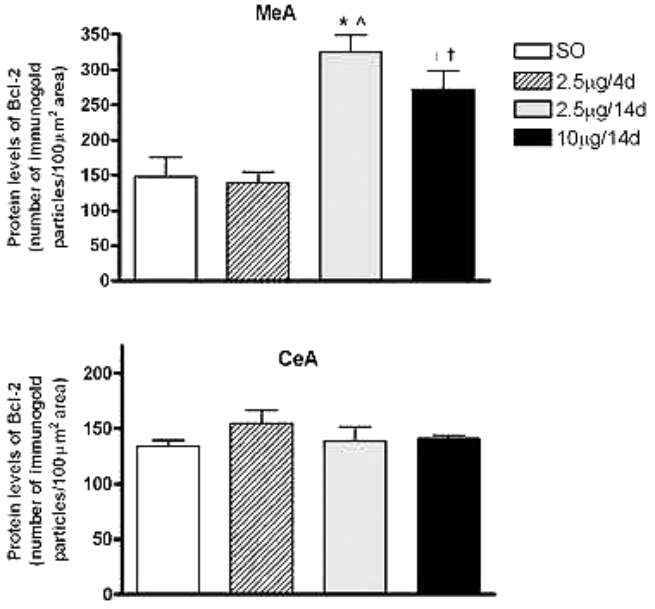


Fig. 6. Quantitation of Bcl-2 immunogold labeling (number of immunogold particles per 100 μm^2 area) in the rat medial amygdala (MeA, top) and central amygdala (CeA, bottom). There was a significant difference among treatment groups in MeA ($F_{3,22}$=15.4, p<0.001). Significant increases in immunolabeling were seen in the 2.5μg estradiol benzoate (EB) for 14 days (2.5 μg/14 days) vs. sesame oil (SO, p<0.001) and 2.5μg EB for 4 days (2.5 μg/4 days, p<0.001) groups and 10 μg estradiol benzoate (EB) for 14 days (10 μg/14 days) vs. SO (p<0.01) and 2.5 μg/4 days (p<0.01) groups. None of the groups displayed differences in gold immunolabeling in the CeA. Values are the mean ± SEM. *p<0.001; !p<0.01 compared with SO; ^p<0.001; †p<0.01 compared with 2.5 μg/4 days. Adapted from Fan L., Pandey S.C. and Cohen R.S. (2008). Estrogen affects levels of Bcl-2 protein and mRNA in medial amygdala of ovariectomized rats. J. Neurosci. Res. 86(16), 3655-3664. Copyright John Wiley and Sons.

The survival factor Bcl-2 is a CREB-related gene product and is implicated in mediating some of estrogen's action on neuroprotection. Therefore, the effects of estrogen on levels of Bcl-2 gold immunolabeling in the MeA and CeA of OVX rats treated with the abovementioned estrogen regimens were determined (Fan et al., 2008b). The 2.5 μg and 10 μg EB for 14 days regimens increased levels of Bcl-2 gold immunolabeling compared with vehicle and 2.5 μg EB for 4 days in MeA, but not CeA (Fig. 6). In addition, Bcl-2 mRNA levels in vehicle and 2.5 μg EB for 14 day groups were determined. There was a significant increase in Bcl-2 mRNA levels in MeA, but not CeA, of EB-treated OVX rats compared with vehicle controls.

Disparate effects of estrogen within the MeA may be due to estrogen effects on classical ERs or membrane ERs, which appear to mediate intracellular signaling pathways and are, also, implicated in cell proliferation, neuroprotection, and growth and survival (Gingerich et al., 2010; Srivastava et al., 2011a). Membrane ERs include: mER-Gαq, the orphan G-protein-coupled receptor, GPR30/GPER1, and the plasma membrane-associated, putative ER-X (cf. Mermelstein and Micevych, 2008 for review). In terms of sex differences, Mermelstein and colleagues have shown that activation of ER-α leads to mGluR1a signaling and phosphorylation of CREB via phospholipase C regulation of MAPK, whereas stimulation of ERα or ERß resulted in mGluR2/3 signaling, with a concomitant decrease in L-type channel-mediated phosphorylation of CREB in cultured female hippocampal pyramidal neurons (Boulware et al., 2005). These bi-directional effects of estrogen were sex-specific (Boulware et al., 2005); that is, age-matched cultures from males did not display an estradiol-induced increase in MAPK-dependent CREB phosphorylation or the estradiol-mediated decrease in CREB phosphorylation following L-type channel activation. The two pathways were dependent on calveolin proteins. Also, cultures from male rats did not display alterations in CRE-dependent transcription following estradiol treatment (Boulware et al., 2007; Mermelstein and Micevych, 2008). These effects appear to be mediated via membrane-localized receptors that stimulated group I and group II metabotropic mGluR signaling (Boulware et al., 2005). The importance of ER subtypes in mediating rapid estrogen effects on signal transduction pathways and subsequent growth and alterations to dendritic spine

morphology has been recently reviewed by Srivastava et al. (2011a,b; see also discussion below).

Implications for the estrogen effects on signaling pathways in the synaptic growth, development, and generation of sexual dimorphisms in the brain

Estrogen's effects on the CREB pathway and CREB-related gene products are likely to effect the survival of neurons and specific circuits and, consequently, estrogen-related behaviors. Growth effects of estrogen on cells and synapses are well-documented (Woolley and Cohen, 2002; Scharfmn and Maclusky, 2005; Srivastava et al., 2011a,b). Some of these effects may be mediated by increased BDNF (cf. Zhou et al., 2005) or survival factors, such as Bcl-2. The trophic effects of estrogen acting via genomic or non-genomic pathways may increase the threshold for stress-related damage, for example (see discussion below).

As mentioned, estrogen has profound effects on dendritic spines in the MeA subnuclei (e.g., Rasia-Filho et al., 2004; de Castilhos et al., 2008), as well as in other brain areas, where some of these actions appear to be mediated by pCREB (Murphy and Segal, 1997; Zhou et al., 2005). For example, estrogen treatment of cultured hippocampal neurons increased dendritic spine density, and this effect was blocked by prior treatment with antisense oligonucleotides against CREB mRNA (Murphy and Segal, 1997).

Another CREB-related gene product relevant to dendritic spine development is Arc. Flanagan-Cato et al. (2006) demonstrated the induction of Arc expression in the ventrolateral subdivision of the VMN by mating, and that the induction was consistent regardless of previous sexual experience. In these studies, there was a reduction in dendritic spines in previously mated animals. Spine density was measured five days following mating, so that the effect of mating is long lasting. However, the paradoxical result of an increased Arc induction with decreased dendritic spine density may be explained by temporal changes in spine density missed after the five day period. Also, the observed changes in spine density may have occurred in neurons other than those displaying Arc induction and/or that Arc induction is not causally related to the later action on spine density (Flangan-Cato et al., 2006). Chamniansawat and Chongthammakun (2010) demonstrated that estrogen rapidly increases the expression of Arc through non-genomic phosphoinositide-3 kinase (PI-3K)-, mitogen-activated protein kinase (MAPK)-, and ER-dependent pathways in SH-SY5Y cells.

In addition to its effect on dendritic spines, estrogen also appears to have an effect on the structure of the postsynaptic density (PSD), the dense area behind the postsynaptic membrane. Early morphological studies showed changes in the length and curvature of PSDs with the long-term regimen of estrogen (10 μg EB for 14 days) in the midbrain central gray of OVX rats (Chung et al., 1998). Also, there was an increase in the number of perforated PSDs, a putative sign of increased synaptic plasticity. Estrogen appears to regulate the expression of the PSD scaffolding protein PSD-95 in the hippocampus. Akama and McEwen (2003) demonstrated that estrogen induced a rapid increase in PSD-95 new protein synthesis in NG108-15 neurons and that this new protein synthesis depends upon Akt (protein kinase B), an intermediate in signal transduction involved in the initiation of protein translation. Waters et al. (2009) showed that ERα- and ERß-specific agonists regulate the expression of (PSD-95) in the stratum radiatum of the hippocampus. Srivastava et al. (2010) also showed that ERß activity increases dendritic spine density and PSD-95 accumulation in membrane regions of synapses.

Recently, studies have implicated the coupling of membrane-associated ERs, such as those associated with dendritic spines, with intracellular signaling pathways in mediating the rapid effects of the neurosteroid estrogen on synaptic proteins, connectivity, and synaptic function in pyramidal neurons (cf. Srivastava et al., 2011a,b for review). The studies indicate that this neurosteroid employs particular signal transduction cascades in disparate brain areas. These characteristics of synapses may form the basis for fine-tuning of neural circuitry and may contribute to differences in circuitry that, in turn, are translated to differences in learned behaviors in males and females, for example. In the model proposed by Srivastava et al. (2011a,b) estrogen affects local protein synthesis in dendritic spines by reducing translational repression with the consequent upregulation of synaptic proteins, such as PSD-95 and GluA1 which in turn may alter dendritic spine structure and the facilitation of long-term potentiation (Srivastava et al., 2011a,b). Rapid effects of estrogen may also involve the subsynaptic cytoskeleton, of which actin is a major constituent. Kramár et al. (2009a,b) have presented a scheme detailing the putative substrates for the rapid effects of estrogen on synaptic function in the adult hippocampus, involving the signaling cascade RhoA, ROCK and LIM-K, which can inactivate cofilin, a blocker of actin filament assembly. In this way estrogen can mediate synaptic structural plasticity and function (Kramár et al., 2009a). Other cascades appear to be triggered by the binding of 17-estradiol to ER-α, which induces phosphorylation of the moesin and WAVE-1 cascade, in turn leading to actin remodeling and actin branching, respectively (Sanchez and Simoncini, 2010). These types of signaling pathways may be involved in the generation of sexual dimorphism of dendritic spines and/or other dimorphic brain structures and is a subject for further investigation in the MePD.

Sexually differentiated intracellular signaling pathways have been implicated in mediation of sex-specific responses to estrogen in the brain (Gillies and McArthur, 2010; cf. also McCarthy et al, 2002; Simerly, 2002; Auger, 2003). Sex differences in CREB phosphorylation were observed in neonatal rat brain (Auger et al., 2001; McCarthy et al., 2002; Auger, 2003). Specifically, male rat pups displayed more pCREB-

immunoreactive positive cells than females in the sexually dimorphic areas of the MPOA, the VMN, the arcuate nucleus, and also in the CA1 region of the hippocampus; similar differences were not seen in two thalamic nuclei, which display little to no gonadal steroid hormone receptors (Auger et al., 2001). No sex differences were seen in the total number of CREB immunoreactive cells. Furthermore, males and testosterone-treated females displayed more pCREB immunolabeling in the VMN compared to female controls. No differences were seen in pCREB immunolabeling in any other of the areas examined. Auger et al. (2001) suggest that some of the effects of testosterone may be mediated by pathways associated with CREB phosphorylation.

Furthermore, upon phosphorylation of CREB on serine 133, coregulatory proteins may be recruited that assist in the transcription of CREB-related gene products. One of these proteins is CBP, which can also function as a nuclear receptor coactivator by interacting with steroid receptor co-activator-1 (Molenda et al., 2002), thereby enhancing steroid receptor action (Shibata et al., 1997) (cf. also Auger, 2003). CBP is also found in the amygdala (Stromberg et al., 1999). Estradiol treatment of neuronal hippocampal cultures increases the expression of CBP within 24 hours (Murphy and Segal, 1997), a time course that is consistent with the time course for estradiol-induced increases in dendritic spines. Auger et al. (2002) showed sex differences in CBP expression in neonatal rat brain, with males expressing higher levels of CBP within the VMN, MPOA, and arcuate nucleus. Infusion of antisense oligodeoxynuleotides to CBP into the hypothalamus of neonatal rats interfered with the defeminizing, but not masculinizing, effects of testosterone, suggesting that CBP expression in the developing rat brain is sexually dimorphic (Auger et al., 2002).

Auger et al. (2001; cf. McCarthy et al., 2002 and Auger, 2003, for reviews) also reported that changes from excitatory versus inhibitory GABA represent a pivotal point in steroid-mediated sexual differentiation of the brain. There appears to be an heterogeneity in responses to GABA with regard to excitatory or inhibitory on pathways that impact CREB phosphorylation depending on sex and region of the developing brain (Auger, 2003). That is, depending upon sex and brain area GABA can be excitatory or inhibitory on signal cascades associated with CREB phosphorylation. Whereas GABA is primarily inhibitory in the adult brain, GABA has excitatory effects during development (Cherubini et al., 1991). The timing of the shift from depolarizing to hyperpolarizing is brain-region specific; for example, it is relatively early in the sexually dimorphic hypothalamus (McCarthy et al., 2002). Elevated testosterone levels are aromatized to estradiol, which is responsible for many of the features of the masculine brain. Relative to the present studies, GABA-mediated sex differences in CREB phosphorylation may lead to differences in the transcription of CREB-related gene products, such as BDNF (Berninger et al., 1995; cf. also Auger, 2003, for references). Neurotrophic factors may then impact other processes, including synaptogenesis, which differentiates male and female brains (cf. McCarthy et al., 2002; Auger, 2003). However, the disparate effect of estrogen on CREB signaling cascades in male and female brains is not restricted to the neonatal period. For example, ÁAhám and Herbison (2005) noted major sex differences in rapid, non-genomic effects of estrogen on pCREB immunoreactivity in adult, gonadectomized mouse brain.

In addition, Garcia-Segura and colleagues have demonstated the importance of astrocytes in mediating estrogen effects on synaptic plasticity in the brain (Garcia-Segura et al., 1999; cf. also Chowen et al., 2000; Garcia-Segura and McCarthy, 2004). McCarthy and colleagues have presented studies underscoring the importance of immature astrocytes, which are responsive to estradiol and play a role in the establishment of sex differences in synaptic patterns in the arcuate nucleus. Arcuate astrocytes appear more complex, with an increased number of primary, secondary and tertiary processes (Mong et al., 2002), a phenomenon mediated by estrogen upregulation of glutamic decarboxylase (GAD), thereby increasing the synthesis of GABA (Davis et al., 1996). The increased structural changes in astrocytes are inversely correlated with dendritic spine development, which also exhibit increased density on neurons of female rat pups compared to those seen on neuons of males (Mong et al., 2001). The MePD shows differences in glial number and complexity in male and female rats (Rasia-Filho et al., 2002; Martinez et al., 2006; Johnson et al., 2008; Morris et al., 2008). There is an increase in GFAP immunoreactivity in proestrous females concomitant with a decrease in dendritic spines in the MePD (Rasia-Filho et al., 2004; Martinez et al., 2006). It is therefore of interest to investigate the involvement of local glial cells and GABA in cellular and synaptic organization of the MePD, a line of research that remains open to further contributions (see parallel comments in Perea et al., 2009; Faissner et al., 2010; Halassa and Haydon, 2010).

Other studies of sexual differences in behavior as they relate to levels of CREB, pCREB and BDNF, come from the laboratory of Lin et al. (2009), who examined levels of these molecules in stress-related areas of the brain (for CREB and pCREB levels: CA1, CA2 and CA3 regions of the hippocampus, paraventricular nucleus of the thalamus, amygdala, anterior cingulate area, dorsal part and infralimbic area of the prefrontal cortex; for BDNF levels: the dentate gyrus and prelimbic area of the prefrontal cortex) of male and female rats following stress recovery. Stress resulted in decreased levels of pCREB in male CA1, CA2 and CA3 regions, paraventricular nucleus, amygdala and dorsal part of the prefrontal cortex and CREB levels in CA2, but these molecular alterations were not seen in females.

The aforementioned data on estrogen-induced

changes in levels of components of the CREB signaling pathway suggest that these may be translated into behavior alterations. The percent of estrogen-induced increases in protein levels of CREB, pCREB, BDNF and mRNA levels of BDNF in the MeA displays a range of approximately 24 to 30%. Changes in pCREB levels of this magnitude either in alcohol-withdrawn rats following chronic alcohol administration or pharmacological manipulations of CREB phosphorylation in the CeA resulted in alterations in their behavior in the elevated plus maze, a test for anxiety-like behavior (Pandey et al., 2003). That is, the rats displayed a decrease in pCREB and a concommittant reduction in open arm activity in the elevated plus maze. Similarly, a reduction in protein levels of BDNF in the amygdala and hippocampal structures of CREB haplodeficient mice resulted in displays of depressive- and anxiety-like behaviors (Pandey et al., 2004). These data open new experimental possibilities to link subcellular, morphological and functional effects on the MeA subnuclei modulation of social behaviors. Experiments should involve females in addition to males, as usually used (see Rasia-Filho et al., 2012).

In effect, studies focusing on sex differences in signal transduction pathways provide fruitful avenues for unraveling the molecular and cellular bases for sexual dimorphisms in brain structure, such as those seen in dendritic spines and neuronal densities described above. These investigations may provide insight into the mechanisms that underlie differences in behavior between males and females in animal studies and in the clinical arena. Because of its well-documented role in synaptogenesis, dendritic spine formation and neuroprotection, the CREB signaling pathway appears be one of the likely candidates that generate some of these differences.

Strain differences in estrogen effects on CREB and pCREB

Finally, data from other laboratories indicate strain-dependent differences in response to some behavioral paradigms in rats (cf. Einat, 2007 for review; cf. also O'Mahony et al., 2011). To determine if plasma steroid hormone and limbic brain pCREB levels are different between strains in intact and OVX rats, estrogen, testosterone, progesterone, adreno-corticotrophic hormone (ACTH), and corticosterone levels were measured using radioimmuinoassays (RIAs) in Sprague Dawley and Wistar rats. Phosphorylated CREB levels were determined using immunogold labeling and densitometric image analysis in intact Sprague Dawley and Wistar rats. In intact rats, there was a significant increase in plasma estrogen, ACTH, and corticosterone levels in Sprague Dawley compared to Wistar rats; no differences were seen in plasma progesterone or testosterone levels between the strains (Hanbury, Pandey and Cohen, unpublished observations). In OVX rats, there were no significant differences in plasma estrogen, progesterone, testosterone or corticosterone between the strains (Hanbury, Fan, Pandey and Cohen, unpublished observations). There were significant increases in pCREB levels in intact Sprague Dawley compared to Wistar rats in the CA1, CA3 and dentate gyrus of the hippocampus, the MeA and CeA, or shell and core of the nucleus accumbens (Hanbury, Fan, Pandey and Cohen, unpublished observations). These data suggest that plasma steroid hormone and limbic pCREB levels may predispose different rat strains to disparate behaviors (cf. Einat, 2007 for review; cf. also O'Mahony et al., 2011). Moreover, these data reinforce observations that apparently discrepant results can be obtained in different rat strains even when using similar methodologies. This appeared to occur and was commented for some MePD morphological findings in the last years (e.g., Hermel et al., 2006b).

Conclusions

The adult rat MePD is a relevant area for investigating profound effects of sex steroids in the rat brain. Various morphological findings support its sexual dimorphisms and effects of sex steroids in adulthood. Alterations in dendritic spine number and morphology are examples of hormonal influences on synaptic structure and differ in males and females, the phases of the estrous cycle, or the effects supra-physiological hormonal replacement therapies. The shape of dendritic spines, the presence and aspect of dendritic fillopodium and axonal protusium in the MePD neuropil of adult animals are relevant for synaptic strength and reinforce the evidence for local cellular plasticity. Subcellular effects of estrogen in the MeA include the transcription of CREB-related gene products, such as, BDNF, Bcl-2, and Arc, which in turn affect synaptic and cellular growth and neuroprotection. Hormonal actions on various signal transduction cascades, and local protein synthesis may affect the neuronal and dendritic spine cytoskeleton and function. Various working hypotheses are raised from these experimental data. Taken together, they provide additional and exciting insights about the modulatory actions of gonadal hormones in a rat forebrain area and in integrated brain circuits relevant for reproduction and other social behaviors.

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