Histol Histopathol (2015) 30: 1303-1311

**http://www.hh.um.es**

**Histology and Histopathology**

*Cellular and Molecular Biology*

# Distribution of glutamate receptors in the posterodorsal medial amygdala of adult male rats

**Francine Dalpian[1], Janaina Brusco[2], Maria Elisa Calcagnotto[3], Jorge E. Moreira[2] and Alberto A. Rasia-Filho[1]**

[1]Department of Basic Sciences/Physiology, Federal University of Health Sciences of Porto Alegre (UFCSPA), RS, [2]Laboratory of Synaptic Structure, Departments of Pathology and Forensic Medicine and Neuroscience and Behavior, Ribeirão Preto School of Medicine, University of São Paulo (FMRP-USP), SP and [3]Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

**Summary.** The rat posterodorsal medial amygdala (MePD) has a remarkable neuronal plasticity and responds to olfactory/pheromonal stimuli to modulate emotional and reproductive behaviors. Glutamate is locally released by incoming sensorial pathways to establish and enforce synaptic inputs. Here, we combined DiI dye and immunolabeling procedure under confocal microscopy to describe the presence and distribution of glutamate receptors on neurons of the MePD of adult male rats. Western blot analysis interrogated binding specificity. Both AMPA (GluA1-4 subunits) and NMDA (GluN1 subunit) receptors were immunolabeled on cell bodies and along proximal and distal dendritic shafts. AMPA receptors were mainly observed on mushroom and stubby/wide spines, whereas NMDA receptors were found on thin spines. Colocalization of AMPA and NMDA receptors occurred in some spines. Filopodium did not show immunolabeled puncta on it. Our results are different from the distribution of glutamate receptors in the amygdaloid lateral nucleus, an upstream area involved with emotional processing, and suggest a region-specific excitatory transmission at proximal and distal dendritic branches. Altogether, these data provide new information for synaptic processing in the MePD likely related to the modulation of social behavior in rats.

*Offprint requests to:* Prof. J.E. Moreira, Laboratory of Synaptic Structure, Deparments of Pathology and Forensic Medicine and Neuroscience and Behavior, Ribeirao Preto School of Medicine, University of Sao Paulo (MRP-USP), SP 14049-900, Brazil. e-mail: cello@fmrp.usp.br

DO: 10.14670/HH-11-626

**Key words:** Extended amygdala, AMPA, NMDA, Neuronal cell body, Dendritic shafts, Spines

## Introduction

The posterodorsal medial amygdala (MePD) is composed of cells with different histogenetic origins (Carney et al., 2010; Bupesh et al., 2011) and is part of the "extended amygdala" in the rat basal forebrain (de Olmos et al., 2004). The MePD is a sexually dimorphic area and serves as an interface for the processing of exteroceptive and interoceptive signals and the action of sex steroids (Newman, 1999; Rasia-Filho et al., 2012a). In effect, the MePD is a nodal point for emotionally-loaded responses and the display of social behaviors in rats (Newman, 1999; Choi et al., 2005; Rasia-Filho et al., 2012b), responds to species-specific socially relevant olfactory/pheromonal (Meredith and Westberry, 2004; Pereno et al., 2011) and genitosensorial stimuli (Coolen et al., 1997), has one of the highest expressions of androgen receptors in the brain (Simerly, 2004), and adjusts the male sexual behavior (mainly for the occurrence of intromission and ejaculation; Coolen et al., 1997; Newman, 1999; Rasia-Filho et al., 2012b).

The study of dendritic shafts and spines in the MePD can help to understand the structural basis of neural circuit function for processing sensorial stimuli and the display of social behaviors in male rats (Dall'Oglio et al., 2008; Brusco et al., 2014). In this sense, the MePD proximal and distal dendritic branches show notable plasticity. For example, compared to females, adult males have dendritic branches that are preferentially

oriented and extend towards the medial and dorsolateral aspects of the MePD (Dall'Oglio et al., 2008). The medially-oriented branches are likely involved with receiving inputs from the accessory olfactory pathway (de Olmos et al., 2004; Pro-Sistiaga et al., 2007). Although most synapses occur on dendritic shafts in the adult male MePD (Brusco et al., 2014), remarkable findings were already described for the density of proximal dendritic spines (de Castilhos et al., 2008; Rasia-Filho et al., 2012a). For example, males have more dendritic spines than females along the different phases of the estrous cycle (Rasia-Filho et al., 2012a). Spines in distal dendritic branches can be affected by androgenic manipulations around puberty (Cunningham et al., 2007). Dendritic spines usually receive excitatory inputs and their shape and number can be associated with biochemical and biophysical signaling for synaptic organization, strength and plasticity (Rochefort and Konnerth, 2012; Yuste, 2013). MePD spines were also implicated in the occurrence of male-typical behaviors since there is a marked reduction in the number of spines in adult castrated males, coincident with the impairment of sexual behavior following testes removal (de Castilhos et al., 2008), or with the dampered development of rough-and-tumble playful attacks in prepubertal castrated rats (Cooke and Woolley, 2009).

Several lines of evidence indicate an important functional role for the main excitatory neurotransmitter glutamate in the MePD of different species (Polston et al., 2001; Simmons and Yahr, 2003; Bian et al., 2008). Incoming pathways use glutamate to code for primary or multimodal sensory information (Bian et al., 2008) and glutamate was found in almost 70% of all ejaculation-activated cells in the MePD of gerbils (Simmons and Yahr, 2003). In male rats, glutamate facilitates the occurrence of ejaculation (Rasia-Filho et al., 2012b) and induces a selective control of the sympathetic output to the cardiovascular system, likely involved with broadening reflex responses for the proper execution of social behaviors (Neckel et al., 2012). Furthermore, the MePD connections to the entorhinal area and postpiriform transitional area likely represent alternative routes for sensorial influences to affect the hippocampal circuitry for the consolidation of episodic memory (Petrovich et al., 2001). Notably, prior sexual experiences and associative learning involving the MePD can lead to permanent modifications in the sexual performance of male rats (Stark, 2005). NMDA-mediated neuronal responses in the medial amygdala (MeA) induced long-term potentiation and memory formation in male rats (Shindou et al., 1993).

The presence and distribution of glutamate receptors on MePD neurons are currently unknown. Here, we describe AMPA and NMDA receptors on the neuronal cell body, on both proximal and distal dendritic shafts, and on different dendritic spines using confocal microscopy. The elucidation of receptor distribution for the main excitatory neurotransmitter in the MePD provides cues to understand the synaptic processing in this area and in the social behavior network of the adult male rat.

## Material and methods

### *Animals*

Adult male Wistar rats (n=4; 3-4 months old) were housed in groups with free access to food and water, and room temperature kept at 22°C in a 12 h light-dark cycle. Experimental procedures were performed in accordance with the international laws for the ethical care (EC Directive 86/609/EEC) and were approved by local Ethics Committees (UFCSPA protocol 033-10 and FMRP/USP protocol 174-11).

### *Immunofluorescence*

Animals were anesthetized with ketamine and xylazine (80 mg/kg and 10 mg/kg, i.p., respectively) and underwent transcardiac perfusion with 200 ml of 0.9% NaCl followed by 300 ml of 1.5% paraformaldehyde in 0.1 M phosphate buffer solution, pH 7.4 (PBS) for over 15 min. The brains were maintained in the same fixative solution for 1 h at room temperature (RT) and coronal brain slices (80 $\mu$m) containing the MePD were obtained using a vibratome (Leica VT 1000S, Leica Microsystems GmbH, Germany). The MePD was localized at 3.0-3.30 mm posterior to the Bregma, lateral to the optic tract and ventral to the stria terminalis (ST; Fig. 1A; according to Paxinos and Watson, 1998; de Olmos et al., 2004; Dall'Oglio et al., 2008; de Castilhos et al., 2008; Brusco et al., 2010, 2014).

Brain sections were blocked with 2% bovine serum albumin (BSA) in Tris Buffered Saline (TBS) for 2 h, and incubated with the primary antibodies. After dilution tests, MePD sections were incubated with the rabbit polyclonal antibody against GluA1-4 subunits to label AMPA receptors (1:50) and the mouse monoclonal antibody against GluN1 subunit to label NMDA receptors (1:100; Synaptic Systems, Germany; catalog # 182403 and 114011, respectively) in 0.5% BSA and 0.1% Tween 20 Tris Buffered Saline (TBST) overnight at 4°C. Then, sections were washed in 0.5% BSA, incubated with anti-rabbit IgG Alexa 594 (1:200) and anti-mouse IgG Alexa 405 (1:200; both from Invitrogen, USA). Secondary antibodies were diluted in 0.5% BSA in TBST, incubated with the brain slices for 2h at 4°C, and washed in TBS. Controls were incubated with the omission of the primary antibodies and, as expected, no labeling was observed (data not shown). Experiments with the omission of one of the two primary antibodies were done to certify negative cross-reaction of antibodies prior to gathering the present experimental results (data not shown).

After the antibody incubation, sonicated fine powdered carbocyanine dye DiI (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate; Molecular Probes, Invitrogen, USA) was placed over the

ST for 24 h at 4°C while sections were immersed in TBS. DiI is a lipophilic dye that diffuses over intact cellular membranes when used extracellularly and allows the identification of the cell body, dendritic shafts and spines (cf. Rasia-Filho et al., 2010 and references therein). Sections were finally washed in distilled water, mounted with Fluoromount (EMS, USA), and visualized with confocal microscopy (SP-5 AOBS, Leica Microsystems GmbH, Germany). Data were obtained from both hemispheres. For the aims of the present work, it is worth noting that previous experimental data did not observe hemispheric lateralization for dendritic spine density (Arpini et al., 2010) or synaptic features (Brusco et al., 2014) in the MePD of adult male rats.

### *Western Blot*

Western blot analysis was also performed to check binding specificity (cf. Alegria-Schaffer et al., 2009; Kurien et al., 2011). Briefly, total protein lysate from the cerebral cortex of adult male rats was run in a SDS-page gel for size separation by electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane and blocked with 5% milk in PBS for 2 h at RT. Primary antibodies (GluA1-4, 1:1500; and, GluN1, 1:1000) in 1% milk were incubated for 1 h at RT and, afterwards, with the secondary antibody for an additional 1 h at RT. Bands were revealed with the addition of the ECL Detection Reagents (ECL Western Blotting kit, GE Healthcare, UK) and captured on film. High and low range molecular weight markers were used ("Thermo Scientific Spectra Multicolor Broad Range Protein Ladder" and "Thermo Scientific Spectra Multicolor Low Range Protein Ladder"; Thermo Fisher Scientific, USA). The final WB labeling for both antibodies are in accordance with the manufacturer's data sheet (Fig. 1B). The non-specific band of around 20kD in the GluA1-4 WB is likely due to protein degradation products.

### *Imaging*

The same protocol used previously was applied (Rasia-Filho et al., 2010). We studied both bitufted and stellate multipolar neurons located in the MePD. Inclusion criteria were: (1) neurons must be located within the MePD, avoiding its ultimate borders; (2) fluorescence for both antibodies and DiI must be stable and on the same focal plane; (3) neurons should be brightly fluorescent and, as much as possible, not "tangled" with neighboring neuronal processes; and, (4) dendrites and spines must be clearly identifiable. Data were obtained from one dendritic branch per neuron from eight different neurons per rat. The fluorescence for the two antibodies was analyzed through the acquired z-planes (approximately 50 z-stack slices of 0.1 $\mu$m step size per reconstructed image) on each dendritic spine. Immunofluorescent puncta distribution for the tested receptors were in accordance with other authors (Sanes and Jessell, 2013). Because the brain sections were 60 $\mu$m thick and antibody penetration was estimated to be of approximately 20 $\mu$m, it was not possible to obtain entire neuronal trees to compare bitufted and stellate neurons as done previously with the Golgi method (Dall'Oglio et al., 2008; de Castilhos et al., 2008).

Z-stack acquisition was obtained with a Leica plan-apochromat 63X, 1.40 NA oil-immersion objective lens on the above mentioned confocal microscope. DiI was imaged using the Helium/Neon laser 543 (emission: 555-590 nm) whereas GluA1-4 and GluN1 receptor subunits labeled with Alexa secondary antibodies were imaged with the 594 (605-658 nm) and 405 (410-490 nm) lasers, respectively. Data were gathered only in those focal planes where a reliable immunolabeling for the studied receptors was coincident with the DiI dye immunofluorescence on the same neuron. The acquisition of multiple labeling was done sequentially with appropriate band pass filters to avoid overlapping detection of fluorochromes. We observed each separate image to check for the receptor immunolabeling and, afterwards, we compared the merged images. We also restricted the number of summed reconstructed images in the "z" confocal plane to those that were most relevant for the demonstration of the results (representative images in Figs. 1-3).

Receptor localization on each dendritic spine was indicated when the pixels of the receptor punctum and the DiI labeled spine were on the same focal plane, or if there was an overlap between both in at least one focal plane (Deng and Dunaevsky, 2005; Brusco et al., 2010). Whenever necessary, the images were evaluated in the orthogonal plane to decide whether a receptor was present on the dendritic spine (Fig. 2). However, sometimes the association of the puncta with different membrane segments was not obvious. It is an inherent limitation to the present study the distance between the receptor and the fluorophore in indirect immunofluorescence location to the spine and dendritic shafts. We did not test the colocalization of receptors by the Pearson's linear correlation coefficient to avoid poor measures and misleading conclusions (Dunn et al., 2011).

All images were obtained with a resolution of 2048x2048 pixels per frame avoiding over and undersaturated pixels, which generated a voxel size of 55x55x300 nm. The images were three-dimensionally (3D) reconstructed using the LAS AF software (Leica Microsystems, Germany). Dendritic spines were observed in 3D to evaluate their morphology. The presence, shape, diameter and length of a visible head and neck on each spine were taken into consideration for the classification as stubby/wide, thin, mushroom or "others", which included ramified spines (according to Fiala and Harris, 1999; Brusco et al., 2010, 2014). Intermediate spine shapes were included in one of the above-mentioned categories according to the most evident aspects of their head and neck. A filopodium was recognized as a long and thin protrusion without an apparent head (Fiala and Harris, 1999), but it was not

included as a "spine type" since filopodium does not show a postsynaptic density in ultrastructural reconstructions and there is a current debate about its role in spinogenesis, synaptogenesis or spine retraction (Brusco et al., 2014 and references therein).

The distribution of imunolabeling for the studied receptors was analyzed on the cell body and on dendritic shafts and spines of proximal (along the first 50 $\mu$m of primary dendrites; Brusco et al., 2010; Rasia-Filho et al., 2012a) and distal branches (at least 25 $\mu$m in length, 100 $\mu$m away from the cell body; Cunningham et al., 2007). The presence of receptors was evaluated in 147 immunolabeled spines along proximal and distal dendrites (based on Murakoshi et al., 2011).

Figures were manipulated at minimum to provide representative results according to our empirical observations. Image contrast was slightly adjusted using Adobe Photoshop 8.0 software (USA).

## Results

MePD neurons showed immunolabeling for AMPA and NMDA receptors on the cell body (Fig. 1C), on proximal and distal dendritic shafts (Fig. 2A-C at lower magnification and D-G at higher magnification) as well as on dendritic spines (Figs. 2D-G, 3A-C) of both bitufted and stellate neurons from the right and left MePD (Fig. 2A,B). Immunolabeled puncta occurred either isolated or in clusters, and both receptors colocalized in adjacent dendritic segments (Fig. 2D-G).

Further observations indicated that AMPA and NMDA receptors can be found on differentially shaped spines along proximal and distal dendrites in the MePD (Figs. 2D-G, 3A-C, Table 1), mostly on the spine heads and, less frequently, on the spine neck or base (Figs. 2D-G, 3A-C). AMPA receptors were usually observed on stubby/wide and mushroom spines, whereas NMDA

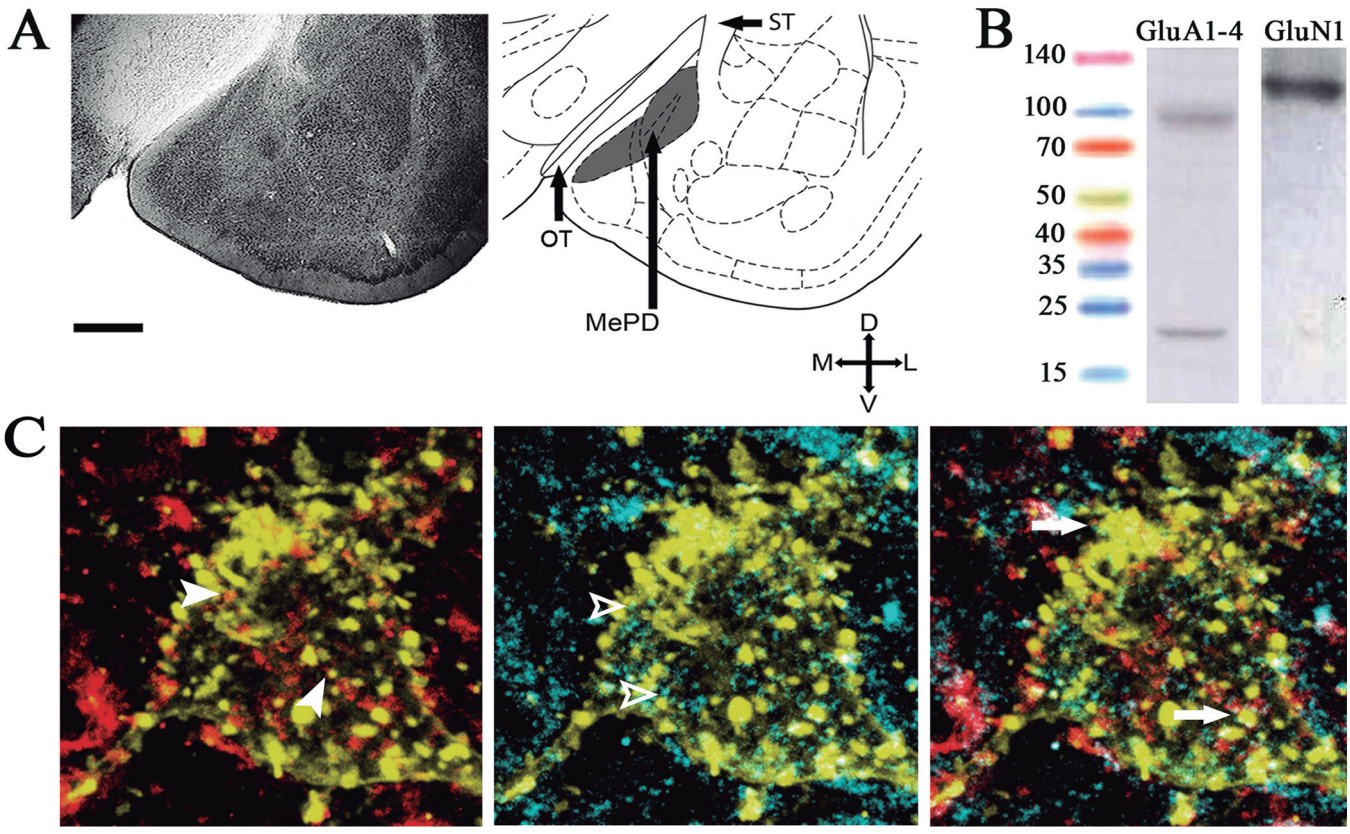


**Fig. 1. A.** (Left) Cresyl violet staining of a coronal brain section shows the ventral location of the posterodorsal medial amygdala (MePD) in the adult male rat brain (3.30 mm posterior to the bregma). (Right) Schematic diagram of a matched coronal brain section adapted from the atlas of Paxinos and Watson (1998). OT: optic tract, ST: stria terminalis. Spatial coordinates: D, dorsal; V, ventral; M, medial; L, lateral. Reprinted from Brusco J, Dall'Oglio A, Rocha LB, Rossi MA, Moreira JE, and Rasia-Filho A.A. "Descriptive findings on the morphology of dendritic spines in the rat medial amygdala". Neuroscience Letters 483, 152-156, 2010. Copyright with permission from Elsevier (no. 3097760021389). **B.** Western blot of the cerebral cortex of adult male rats showing AMPA (GluA1-4, 100KDa) and NMDA (GluN1, 120 KDa) receptors antibody reactivity. **C.** Neuronal soma of a male rat MePD labeled with DiI (yellow) and antibodies for AMPA (red) and NMDA (cyan) receptors. Arrowheads point to spines labeled exclusively with AMPA GluA1-4 receptors antibody (red). Empty arrow-heads point to spines labeled exclusively with NMDAR (cyan). Arrows point to spines labelled with both AMPA and NMDA receptors. Image contrast was slightly adjusted with Adobe Photoshop 8.0 software (USA). Scale bar: A, 800 µm; C, 5 $\mu$m.

receptors were more frequent on thin spines (Table 1).

Multiple puncta for the same receptor (Fig. 3B) and double-labeling for AMPA and NMDA receptors (Fig. 3C) were observed colocalized on spines as well (Table 1). Filopodia were the least frequent dendritic protrusion and did not show immmunolabeling for any of the tested receptors (Fig. 3D).

## Discussion

This work provides information for the presence and distribution of synaptic receptors in the rat MePD. Here, we demonstrate that AMPA and NMDA receptors are localized on neuronal cell bodies, on both proximal and distal dendritic shafts of MePD neurons, and that dendritic spines of different shapes show these excitatory receptors.

Axo-somatic synapses were reported in the male rat MePD (see Fig. 3B in Hermel et al., 2006). Here, excitatory receptors were found in the neuronal cell body and it is interesting to consider that synaptic currents in the soma can promptly and strategically affect the neuronal firing pattern (Sanes and Jessell, 2013). Furthermore, synapses on dendritic shafts are more

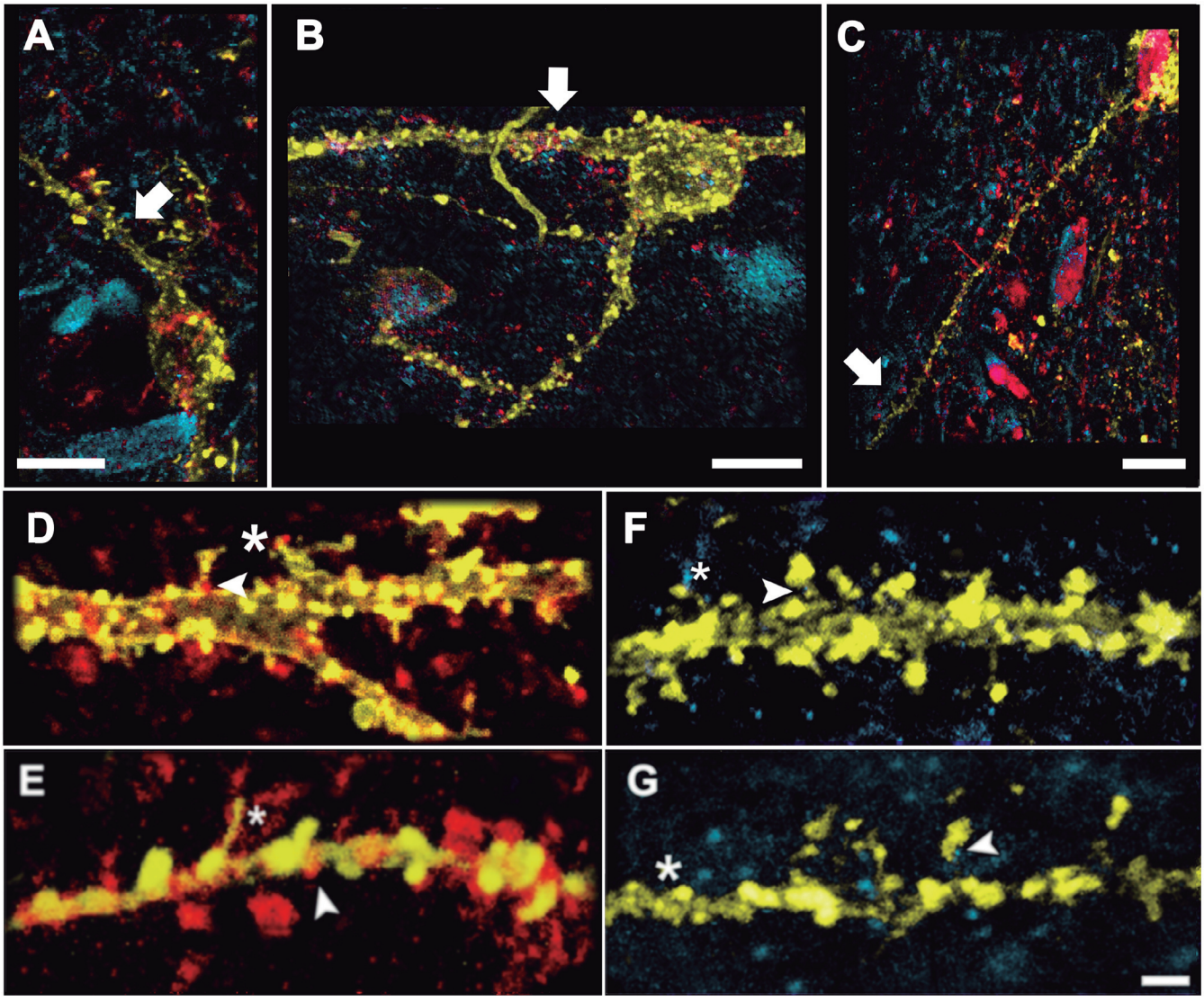


**Fig. 2.** Immunofluorescence for AMPAR and NMDAR in DiI labeled bitufted **(A)** and stellate **(B)** spiny neurons of the rat posterodorsal medial amygdala (MePD). Arrows point to proximal **(A, B)** and distal **(C)** dendritic branches from where data were obtained after 3D reconstruction of z-stack images. At higher magnification, **(D, E)** AMPA GluA1-4 (red) and **(F, G)** NMDA GluN1 (cyan) receptors on proximal **(D, F)** and distal **(E, G)** dendritic shafts and on differently shaped dendritic spines. Puncta are present on the head (asterisk), neck or base (arrowhead) of the spines. Image contrast was slightly adjusted with Adobe Photoshop 8.0 software (USA). Scale bar: A-C, 10 $\mu$m; D-G, 2 $\mu$m.

frequent than synapses on dendritic spines in the MePD (Brusco et al., 2014). Synapses on dendritic shafts can be more efficient at producing higher postsynaptic potentials than synapses on dendritic spines (Ivenshitz and Segal, 2010). On the other hand, the density of dendritic spines in the MePD shows sex differences (approximately 30% higher in males than in proestrus females) and is modified by castration and substitutive hormonal therapy (Rasia-Filho et al., 2012a), which indicates an evident site for synaptic plastic properties. Spines are complex, multifunctional, integrative units that form specialized neuronal postsynaptic compartments with neurotransmitter receptor/ionic channels to alter local dendritic (passive and active) biophysical properties (Fiala and Harris, 1999; Arellano et al., 2007; Rochefort and Konnerth, 2012; Yuste, 2013). In the adult male rat MePD there is a prevalence of thin spines (near 53%; Brusco et al., 2010), which would be a potentially more labile type of spine compared to other morphological types. It is possible that modifiable spines in the MePD represent an intrinsic plastic capacity for local information processing and behavior modulation whereas stable spines relate to steady properties in male-typical neural networks (Rasia-Filho et al., 2012a). The glutamate involvement in these functional and heterogeneous electrophysiological properties can now be tested in the adult male rat, as done previously in mice (Bian et al., 2008; Niimi et al., 2012).

Excitatory synapses on the spine head or neck were

**Table 1.** Dendritic branches.

| Proximal dentritic branches<br>Spine shape | GluA1-4 | GluN1 | Colocalization |
|---|---|---|---|
| Thin | 5 | 13 | 3 |
| Stubby/Wide | 10 | 1 | 2 |
| Mushroom | 18 | 0 | 2 |
| Others | 0 | 0 | 1 |
| Total | 33 | 14 | 8 |
| **Distal dentritic branches**<br>**Spine shape** | **GluA1-4** | **GluN1** | **Colocalization** |
| Thin | 12 | 23 | 8 |
| Stubby/Wide | 23 | 4 | 0 |
| Mushroom | 13 | 0 | 3 |
| Others | 2 | 2 | 2 |
| Total | 50 | 29 | 13 |

Number of dendritic spines with AMPA GluA1-4 and NMDA GluN1 receptors, or colocalization of both counted in diferentially shaped spines along proximal and distal dendrites in the MePD.

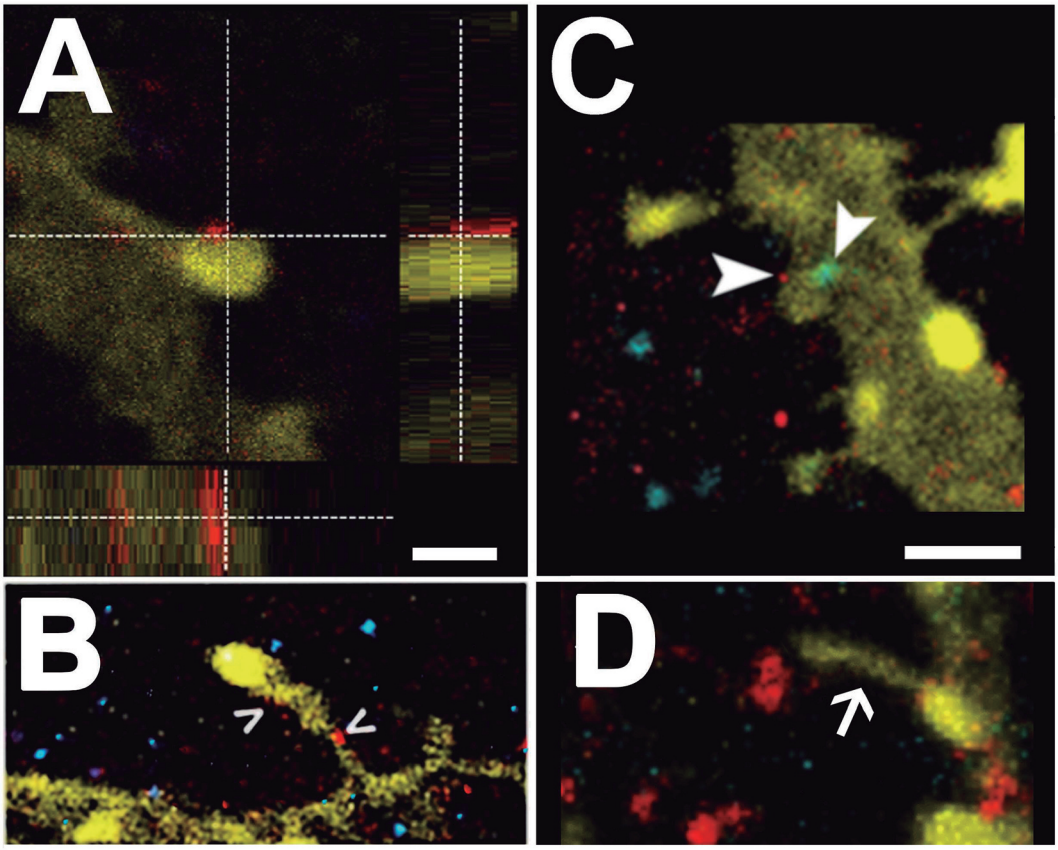


**Fig. 3.** AMPA and NMDAR in dendritic spines of posterodorsal medial amygdala (MePD) neurons. **A.** Orthogonal sections confirm the presence of AMPA receptors (red) on the head of a thin spine (yellow). **B.** Two AMPA receptors (red) puncta (arrows) on the same dendritic spine. **C.** Colocalization of AMPA (red) and NMDA (cyan) receptors (arrows) on a stubby spine. **D.** Filopodium with no immunolabeled puncta. Scale bars: 2 µm.

described with electron microscopy and 3D reconstructions of serial-sections in neurons of the adult rat MePD (Brusco et al., 2014). Here, in agreement with that ultrastructural data, we also observed spines of different shapes with receptors for glutamate in the MePD. Glutamate receptors can move along the membranes modifying the number and composition of receptors that are available at the synaptic active zone (Collingridge et al., 2004; Lin et al., 2004; Frick and Johnston, 2005). These changes are determined by a local intracellular pool of fast recycling receptors available at synapses regulated by proteins and signaling mechanisms that associate or interact with receptor subunits, contributing to synaptic plasticity (Collingridge et al., 2004). The use of immunogold with electron microscopy or the combination of immunolabeling for receptors and scaffold proteins such as PSD95 under confocal microscopy can link the present results and reveal the location of glutamate receptors in synaptic sites or in endomembranes.

We observed AMPA receptors on mushroom and stubby/wide spines, whereas NMDA receptors occurred on thin spines in the MePD. We also found immunolabeled puncta for AMPA and NMDA receptors in different parts of the dendritic spine. The existence of multisynaptic spines in the MePD was previously shown by the presence of various puncta of synaptophysin on the same spine with confocal microscopy (Brusco et al., 2010) and with 3D reconstruction of electron microscopy sections (Brusco et al., 2014). Accordingly, these basic data encourage electrophysiological studies employing the specific release of glutamate on differently shaped spines to reveal the impact of the morphologically distinct spines, alone and in clusters, in the activity of MePD neurons. Conversely, filopodium did not show AMPA or NMDA labeling, demonstrating that this kind of protrusion involved in spinogenesis or spine retraction (García-López et al., 2010) does not share major excitatory receptors in the adult rat MePD.

Furthermore, synaptic activity in proximal and distal dendritic spines would also serve as a detector for temporal-spatial synchronization of afferent neuronal activity. Synapses on different portions of the dendritic shaft would modulate local excitability for the compartmentalization of information processing or for signal propagation along dendrites that, ultimately, would alter connectional input, strength, integration, and synaptic plasticity (Dall'Oglio et al., 2008; Frick and Johnston, 2005). They can also maintain or rearrange the number and shape of dendritic spines (Segal, 2010; Lin et al., 2004), and determine the firing rate and dynamic pattern of activity across the rat social behavior network (Newman, 1999; Choi et al., 2005; Rasia-Filho et al., 2012b). In this regard, socially relevant information modulated by the MePD is distinctly coded by both glutamatergic and GABAergic inputs. AMPA and NMDA-mediated excitatory postsynaptic currents in the MePD evoked by stimulation of the accessory olfactory bulb (Bian et al., 2008; Niimi et al., 2012) can initiate a rapid and specific response to pheromonal stimuli (Park et al., 2014). To establish a counterpart, pheromonal information is also sent to the rat MePD via GABAergic fibers from the intercallated amygdaloid nuclei (Meredith and Westberry, 2004). Strong colocalization of Fos and GAD-67 mRNA in MePD neurons after odor exposure (Donato Jr et al., 2010) or Fos and GABA, calretinin, and calbindin after detection of conspecific pheromones were observed in the extended amygdala, notably in the MePD (Pereno et al., 2011). Local GABAergic neurons can form microcircuitries in the MePD or have bidirectional connections with neighboring amygdaloid nuclei in mice (Bian et al., 2008; Bian, 2013). Altogether, these data indicate the important role of both the excitatory and inhibitory balance and control of incoming pheromonal and olfactory sensorial inputs to the MePD. After this intrinsic processing, the MePD projections reach hypothalamic nuclei and circuitries whose functional consequence are the timely display of neuroendocrine secretion and reproductive behavior in rats (see additional comments in Rasia-Filho et al., 2012a,b).

Compared to the present results, it is now evident that not all amygdaloid nuclei display the same distribution of glutamate receptors on local neurons. That is, in the lateral nucleus of the amygdala, a MePD upstream area involved in emotional and memory processing (Farb et al., 1995), most glutamate-immunoreactive synapses are present on spines or small dendrites, but are rarely seen on soma or proximal dendrites (Farb et al., 1992). This is not the case of the MePD where the presence of AMPA and NMDA receptors on the soma and along either proximal or distal dendritic branches suggests that these different amygdaloid parts have different and region-specific synaptic mechanisms to code for their role in emotional information processing and behavioral modulation (Farb et al., 1992).

### *Conclusion*

The present work adds data to the amygdaloid region-specific features for the main excitatory synaptic receptors on the neuronal body, dendritic shafts, and dendritic spines in the male MePD, a relevant component of the rat social behavior network (Newman, 1999; Choi et al., 2005; Rasia-Filho et al., 2012b). From our findings, we can also assume that "the morphological heterogeneity of spines, even for a local small portion of the dendrites, is consistent with the idea that synaptic strength is regulated locally, at the level of a single spine" (Arellano et al., 2007; see also Frick and Johnston, 2005; Segal, 2010; Rochefort and Konnerth, 2012). Our data can direct future studies to understand the synaptic organization and sexual dimorphism of the MePD (Rasia-Filho et al., 2012a; Brusco et al., 2014), as well as the local selection of neuroglial assemblies, postsynaptic temporal dynamics, and neural circuit oscillations (Klausberger and Somogyi, 2008).

*Acknowledgements.* Grants from CNPq (no. 141534/2008-7 and 201560/2010-0) and FAPESP (no. 2009/01571-6, 2011/10753-0, 2012/19011-0, and CinAPCe 05/56447-7 to JEM). The authors are thankful to Dr. Lenaldo Branco Rocha (UFTM, Brazil), Mrs. Maria Teresa P. Maglia, Mrs. Vani M. Alves, Mrs. Elizabete R. Milani, Mrs. Carol K. da Fonseca (FMRP/USP, Brazil) and Dr. Aline Dall'Oglio for their assistance. AARF is a CNPq researcher.

*Conflict of Interest.* Authors disclose any actual or potential conflict of interest.

## References

Alegria-Schaffer A., Lodge A. and Vattem K. (2009). Performing and optimizing Western blots with an emphasis on chemiluminescent detection. Methods Enzymol. 463, 573-599.

Arellano J.I., Benavides-Piccione R., DeFelipe J. and Yuste R. (2007). Ultrastructure of dendritic spines: correlation between synaptic and spine morphologies. Front. Neurosci. 1, 131-143.

Arpini M., Menezes I.C., Dall'Oglio A. and Rasia-Filho A.A. (2010). 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.

Bian X. (2013). Physiological and morphological characterization of GABAergic neurons in the medial amygdala. Brain Res. 1509, 8-19.

Bian X., Yanagawa Y., Chen W.R. and Luo M. (2008). Cortical-like functional organization of the pheromone-processing circuits in the medial amygdala. J. Neurophysiol. 99, 77-86.

Brusco J., Dall'Oglio A., Rocha L.B., Rossi M.A., Moreira J.E. and Rasia-Filho A.A. (2010). Descriptive findings on the morphology of dendritic spines in the rat medial amygdala. Neurosci. Lett. 483, 152-156.

Brusco J., Merlo S., Ikda E.T., Petralia R.S., Kachar B., Rasia-Filho A.A. and Moreira J.E. (2014). Inhibitory and multisynaptic spines, and hemispheric synaptic specialization in the posterodorsal medial amygdala of male and female rats. J. Comp. Neurol. 522, 2075-2088.

Bupesh M., Legaz I., Abellán A. and Medina L. (2011). Multiple telencephalic and extratelencephalic embryonic domains contribute neurons to the medial extended amygdala. J. Comp. Neurol. 519, 1505-1525.

Carney R.S., Mangin J.M., Hayes L., Mansfield K., Sousa V.H., Fishell G., Machold R.P., Ahn S., Gallo V. and Corbin J.G. (2010). Sonic hedgehog expressing and responding cells generate neuronal diversity in the medial amygdala. Neural Dev. 27, 5-14.

Choi G.B., Dong H-W, Murphy A.J., Valenzuela D.M., Yancopoulos G.D., Swanson L.W. and Anderson D.J. (2005). Lhx6 delineates a pathway mediating innate reproductive behaviors from the amygdala to the hypothalamus. Neuron 46, 647-660.

Collingridge G.L., Issac J.T.R. and Wang Y.T. (2004). Receptor trafficking and synaptic plasticity. Nature Rev. Neurosci. 5, 952-962.

Cooke B.M. and Woolley C.S. (2009). Effects of prepubertal gonadectomy on a male-typical behavior and excitatory synaptic transmission in the amygdala. Dev. Neurobiol. 69, 141-152.

Coolen L.M., Peters H.J.P.W. and Veening J.G. (1997). Distribution of Fos immunoreactivity following mating versus anogenital investigation in the male rat brain. Neuroscience 77, 1151-1161.

Cunningham R.L., Clairborne B.J. and McGinnis M.Y. (2007). Pubertal exposure to anabolic androgenic steroids increases spine densities on neurons in the limbic system of male rats. Neuroscience 150, 609-615.

Dall'Oglio A., Gehlen G., Achaval M. and Rasia-Filho A.A. (2008). Dendritic branching features of posterodorsal medial amygdala neurons of adult male and female rats: further data based on the Golgi method. Neurosci. Lett. 430, 151-156.

de Castilhos J., Forti C.D., Achaval M. and Rasia-Filho A.A. (2008). Dendritic spine density of posterodorsal medial amygdala neurons can be affected by gonadectomy and sex steroid manipulations in adult rats: a Golgi study. Brain Res. 1240, 73-81.

de Olmos J.S., Beltramino C.A. and Alheid G. (2004). Amygdala and extended amygdala of the rat: a cytoarchitectonical, fibroarchitectonical, and chemoarchitectonical survey. In: The rat nervous system. Paxinos G. (ed). Elsevier Academic Press. San Diego. pp 509-603.

Deng J. and Dunaevsky A. (2005). Dynamics of dendritic spines and their afferent terminals: spines are more motile than presynaptic boutons. Dev. Biol. 277, 366-377.

Donato Jr J., Cavalcante J.C., Silva R.J., Teixeira A.S., Bittencourt J.C. and Elias C.F. (2010). Male and female odors induce Fos expression in chemically defined neuronal population. Physiol. Behav. 99, 67-77.

Dunn K.W., Kamocka M.M. and McDonald J.H. (2011). A practical guide to evaluating colocalization in biological microscopy. Am. J. Physiol. Cell Physiol. 300, C723-C742.

Farb C.R., Aoki C., Milner T., Kaneklo T. and LeDoux J. (1992). Glutamate immunoreactive terminals in the lateral amygdaloid nucleus: a possible substrate for emotional memory. Brain Res. 593, 145-158.

Farb C.R., Aoki C. and LeDoux J.E. (1995). Differential localization of NMDA and AMPA receptor subunits in the lateral and basal nuclei of the amygdala: a light and electron microscopic study. J. Comp. Neurol. 362, 86-108.

Fiala J.C. and Harris K.M. (1999). Dendrite structure. In: Dendrites. Stuart G., Spruston N. and Häusser M. (eds). Oxford University Press. New York. pp 1-34.

Frick A. and Johnston D. (2005). Plasticity of dendritic excitability. J. Neurobiol. 64, 100-115.

García-López P., García-Marín V. and Freire M. (2010). Dendritic spines and development: towards a unifying model of spinogenesis- a present day review of Cajal's histological slides and drawings. Neural Plast. 2010, 769207.

Hermel E.E.S., Faccioni-Heuser M.C., Marcuzzo S., Rasia-Filho A.A. and Achaval M. (2006). Ultrastructural features of neurons and synaptic contacts in the posterodorsal medial amygdala of adult male rats. J. Anat. 208, 565-575.

Ivenshitz M. and Segal M. (2010). Neuronal density determines network connectivity and spontaneous activity in cultured hippocampus. J. Neurophysiol. 104, 1052-1060.

Klausberger T. and Somogyi P. (2008). Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. Science 321, 53-57.

Kurien B.T., Dorri Y., Dillon S., Dsouza A. and Scofield R.H. (2011). An overview of Western blotting for determining antibody specificities for immunohistochemistry. Methods Mol. Biol. 717, 55-67.

Lin H., Huganir R. and Liao D. (2004). Temporal dynamics of NMDA receptor-induced changes in spine morphology and AMPA receptor

recruitment to spines. Biochem. Biophys. Res. Commun. 316, 501-511.

Meredith M. and Westberry J.M. (2004). Distinctive responses in the medial amygdala to same-species and different-species pheromones. J. Neurosci. 24, 5719-5725.

Murakoshi H., Wang H. and Yasuda R. (2011). Local, persistent activation of Rho GTPases during plasticity of single dendritic spines. Nature 472, 100-104.

Neckel H., Quagliotto E., Casali K.R., Montano N., Dal Lago P. and Rasia-Filho A.A. (2012). Glutamate and GABA in the medial amygdala induce selective central sympathetic/parasympathetic cardiovascular responses. Can. J. Physiol. Pharmacol. 90, 25-36.

Newman S.W. (1999). The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network. Ann. NY Acad. Sci. 877, 242-257.

Niimi K., Horie S., Yokosuka M., Kawakami-Mori F., Tanaka K., Fukayama H. and Sahara Y. (2012). Heterogeneous electrophysiological and morphological properties of neurons in the mouse medial amygdala in vitro. Brain Res. 1480, 41-52.

Park S.K., Kim J.H., Yang E.S., Ahn D.K., Moon C. and Bae Y.C. (2014). Ultrastructure and synaptic connectivity of main and accessory olfactory bulb efferent projections terminating in the rat anterior piriform cortex and medial amygdala. Brain Struct. Funct. 219, 1603-1613.

Paxinos G. and Watson C. (1998). The rat brain in stereotaxic coordinates. Academic Press. San Diego.

Pereno G.L., Balaszczuk V. and Beltramino C.A. (2011). Detection of conspecific pheromones elicits Fos expression in GABA and calcium-binding cells of the rat vomeronasal system-medial extended amygdala. J. Physiol. Biochem. 67, 71-85.

Petrovich G.D., Canteras N.S. and Swanson L.W. (2001). Combinatorial amygdalar inputs to hippocampal domains and hypothalamic behavior systems. Brain Res. Rev. 38, 247-289.

Polston E.K., Heitz M., Barnes W., Cardamone K. and Erskine, M.S. (2001). NMDA-mediated activation of the medial amygdale initiates a downstream neuroendocrine memory responsible for pseudopregnancy in the female rat. J. Neurosci. 21, 4104-4110.

Pro-Sistiaga P., Mohedano-Moriano A., Ubeda-Bañon I., Arroio-Jimenez M.D.M., Marcos P., Artacho-Pérula E., Crespo C., Insausti R. and Martinez-Marcos A. (2007). Convergence of olfactory and vomeronasal projections in the rat basal telencephalon. J. Comp. Neurol. 504, 346-362.

Rasia-Filho A.A., Brusco J., Rocha L.B. and Moreira J.E. (2010). Dendritic spines observed by extracellular DiI dye and immunolabeling under confocal microscopy. Protocol Exchange/Nature Protocols. doi: 10.1038/nprot.2010.153

Rasia-Filho A.A., Dalpian F., Menezes I.C., Brusco J., Moreira J.E. and Cohen R.S. (2012a). Dendritic spines of the medial amygdala: plasticity, density, shape, and subcellular modulation by sex steroids. Histol. Histopathol. 27, 985-1011.

Rasia-Filho A.A., Haas D., de Oliveira A.P., de Castilhos J., Frey R., Stein D., Lazzari V.M., Back F., Pires G.N., Pavesi E., Winkelmann-Duarte E.C. and Giovenardi M. (2012b). Morphological and functional features of the sex steroid-responsive posterodorsal medial amygdala of adult rats. Mini Rev. Med. Chem. 12, 1090-1106.

Rochefort N.L. and Konnerth A. (2012). Dendritic spines: from structure to in vivo function. EMBO Rep. 13, 699-708.

Sanes J.R. and Jessell T.M. (2013). The formation and regeneration of synapses. In: Principles of neural science. 5rd edition. Kandel E.R., Schwartz J.H., Jessell T.M., Siegelbaum S.A. and Hudspeth A.J. (eds). McGraw Hill. New York. pp 1233-1258.

Segal M. (2010). Dendritic spines, synaptic plasticity and neuronal survival: activity shapes dendritic spines to enhance neuronal viability. Eur. J. Neurosci. 31, 2178-2184.

Shindou T., Watanabe S., Yamamoto K. and Nakanishi H. (1993). NMDA receptor dependent formation of long-term potentiation in the rat medial amygdala neuron in an in vitro slice preparation. Brain Res. Bull. 31, 667-672.

Simerly R.B. (2004). Anatomical substrates of hypothalamic integration. In: The rat nervous system. 3rd edition. Paxinos G. (ed). Academic Press. San Diego. pp 335-368.

Simmons D.A. and Yahr P. (2003). GABA and glutamate in mating-activated cells in the preoptic area and medial amygdala of male gerbils. J. Comp. Neurol. 459, 290-300.

Stark C.H. (2005). Behavioral effects of stimulation of the medial amygdala in the male rat are modified by prior sexual experience. J. Gen. Psychol. 132, 207-224.

Yuste R. (2013). Electrical compartmentalization in dendritic spines. Annu. Rev. Neurosci. 36, 429-449.

Accepted April 30, 2015