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A QUANTITATIVE COMPARISON OF THE EFFERENT PROJECTIONS OF THE ANTERIOR AND POSTERIOR SUBDIVISIONS OF THE MEDIAL AMYGDALA IN FEMALE MICE

Brett T. DiBenedictis^a, Alexander I. Helfand^a, Michael J. Baum^a, and James A. Cherry^{*,b}

^a Department of Biology, Boston University, Boston, MA 02215, United States

^b Department of Psychology, Boston University, Boston, MA 02215, United States

Abstract

In rodents, many aspects of sociosexual behavior are mediated by chemosignals released by opposite-sex conspecifics. These chemosignals are relayed via the main (MOS) and accessory olfactory systems (AOS) to the medial amygdala (Me). The Me is subdivided into anterior (MeA) and posterior (MeP) subnuclei, and lesions targeting these regions have different effects on proceptive courtship behaviors in female mice. Differential behavioral effects of MeA vs. MeP lesions could reflect a difference in the projections of neurons located in these Me subnuclei. To examine this question, we injected female mice with the anterograde tracer, Fluoro-Ruby into either the MeA or MeP and quantified labeled puncta in 11 forebrain target sites implicated in courtship behaviors using confocal fluorescence microscopy. We found that the MeP more densely innervates the medial and intermediate regions of the posterior bed nucleus of the stria terminalis (pBNST) and the posteromedial cortical amygdala (PMCo), while the MeA more densely innervates the horizontal diagonal band of Broca (HDB) and the medial olfactory tubercle (mOT), a region that may be a component of the circuitry responsible for olfactory-mediated motivated behaviors.

Keywords

Medial Amygdala; axon terminal; sociosexual behavior; olfactory; anterograde tracing

1. Introduction

Female mice prefer to seek out and investigate pheromonal odors released by male conspecifics, even in the absence of prior experience with those odors. These chemosignals are detected and processed by two separate olfactory systems, the main (MOS) and accessory (AOS) olfactory systems, which detect primarily volatile and non-volatile body odor cues, respectively. Both the MOS and AOS contribute to the normal display of sociosexual behaviors in female mice (Keller *et al.*, 2006a,b; Martel & Baum, 2009b). Female urinary stimuli that activate either the MOS or the AOS are sufficient to establish a

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* Corresponding author at: Department of Psychology, Boston University, Boston, MA 02215, United States. Tel.: +1 617 353 3254. jcherry@bu.edu (J.A. Cherry).

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conditioned place preference in male mice, suggesting that either olfactory system suffices to mediate the rewarding effects of opposite-sex odors (Korzan *et al.*, 2013).

The medial amygdala (Me) is a critical node of convergent inputs from the MOS and AOS. Both the main and accessory olfactory bulbs send direct projections to the Me; Me-projecting mitral cells in the ventromedial portion of the main olfactory bulb of female mice are preferentially activated by opposite-sex urinary odors, suggesting that this circuit may relay sexually relevant olfactory information to the Me (Kang *et al.*, 2009). The Me includes anterior, posterodorsal and posteroventral subdivisions, and it has been proposed that these subnuclei play distinct roles in the processing of conspecific body odors. In male Syrian hamsters, the anterior Me (MeA) acts as a “chemosensory filter” that distinguishes between opposite-sex and same-sex odorants (Maras & Petrulis, 2006), while the posterodorsal Me (MePD), which includes a dense population of steroid receptor-expressing neurons (Wood *et al.*, 1993), enhances male hamsters’ attraction toward female hamster body odorants. More recently, we found that in estrous female mice, lesions targeting only the MeP eliminated females’ motivation to preferentially approach urinary odors from testes-intact, as opposed to castrated males, whereas lesions of either the MeA or MeP reduced females’ lordotic responses to male mounts (DiBenedictis *et al.*, 2012).

We hypothesized that the ability of MeP, but not MeA, lesions to disrupt the normal preference of female mice for male chemosignals would be reflected in differences in the downstream projection targets of these two amygdalar subnuclei. Two recent studies examined differences in the efferent projections of Me subdivisions in mice (Pardo-Bellver *et al.*, 2012; Usunoff *et al.*, 2009). One of these studies (Pardo-Bellver *et al.*, 2012) used a small number of subjects (n=2 in one group) with no quantitative index of the strength of efferent Me projections; the other study (Usunoff *et al.*, 2009) examined the MePD but provided no assessment of MeA projections. Here, we largely confirmed and extended the results of Pardo-Bellver and others (2012) in outbred (Swiss Webster) female mice using a method of analysis that allowed for a quantitative assessment of MeA vs. MeP projections to downstream forebrain projection targets. We found that the MeA and MeP differentially target several forebrain sites implicated in motivated behavior, including the posterior BNST (pBNST) and medial olfactory tubercle (mOT).

2. Results

Injections of the anterograde tracer, Fluoro-Ruby, into the MeA or MeP were largely confined to the target nucleus (Fig. 1A,B), although in the case of larger injections, there was some leakage of tracer (i.e. very few labeled cell bodies) into the rostral MeP in animals given injections intended for the MeA (Fig. 1D). Likewise, there was minimal leakage into the caudal MeA in animals given injections targeting the MeP (Fig. 1E). In some cases, a few labeled cell bodies were observed lateral to the target Me nucleus, minimally affecting adjacent amygdaloid nuclei including the anterior amygdaloid area (subjects 336 and 353), and the anterior cortical amygdaloid nucleus (subjects 337 and 344; see Figure 1). However, labeling due to tracer spread in these adjacent regions was very sparse and in most cases did not occur at all. In no cases were central amygdaloid nuclei labeled.

Both the posteromedial cortical amygdala (PMCo) and posterior bed nucleus of the stria terminals (pBNST) were most densely innervated by the MeP injections (Fig. 2). Many anterogradely labeled fibers originating from neurons in the MeP were observed in the PMCo. Retrogradely labeled cell bodies were also found, suggesting a dense bilateral connectivity between the MeP and PMCo (Fig. 2A') that was not observed between the MeA and PMCo (Fig. 2A). Fluoro-Ruby is predominantly an anterograde tracer, but retrograde labeling can also occur. The pBNST also showed dense labeling in lateral,

intermediate, and medial subregions (STMPL, STMPI, STMPM, respectively) in MeP-injected subjects (Fig. 2B'), while labeling was mostly observed in the STMPL in MeA-injected subjects (Fig. 2B). Several animals (not included in the quantitative analysis) were given an injection of the anterograde tracer, *Phaseolus vulgaris* leucoagglutinin (PHA-L) into the MeA plus an injection of Fluoro-Ruby into the MeP of the same hemisphere in order to obtain a qualitative comparison of pBNST and mOT innervation by the MeA and MeP. Fibers originating from the MeA and MeP were apparent throughout the pBNST but mainly overlapped in the STMPL (Fig. 3A,A'). In contrast to the pBNST, the mOT received relatively dense innervation from the MeA, but was only sparsely innervated by the MeP (Fig. 3B,B').

An important goal of this study was to directly compare the relative magnitude of efferent innervation of MeA vs. MeP projection targets; therefore a quantitative estimation of puncta number in projection targets of the MeA and MeP was performed. Puncta are highly variable in size (Mitchell *et al.*, 2012), and only those that could be reliably identified were counted (Fig. 4). Puncta were quantified from confocal images stacked in the Z-plane of 11 forebrain sites that received considerable innervation by the MeA and/or MeP. These regions included the mOT, the shell and core of the nucleus accumbens (AcbSh, AcbC), the horizontal diagonal band of Broca (HDB), the medial preoptic area (MPA), pBNST subregions (STMPM, STMPI, STMPL), dorsomedial and ventrolateral subdivisions of the ventromedial hypothalamus (VMHdm, VMHvl), and PMCo (Fig. 5). These regions were chosen for two main reasons. First, preliminary qualitative results indicated that these regions received considerable innervation from the MeA and/or MeP. Second, the purpose of this study was to elucidate the degree of innervation of Me forebrain targets that have been previously implicated in odor-driven sociosexual behaviors, since deficits observed in MeA vs. MeP-lesioned animals in our previous study concerned these behaviors (DiBenedictis *et al.*, 2012). Intra-amygdaloid connections, though important, were not included in the present study (with the exception of Me-PMCo projections) because our aim was to focus on specific regions of interest and not to document the full breadth and magnitude of MeA and MeP projections in the female mouse brain (but see Pardo-Bellver *et al.*, 2012). The precise locations of puncta counting domains within each target nucleus analyzed were chosen based on prior studies from our laboratory in which we observed augmented Fos protein expression in response to opposite-sex odors or in response to placement in a chamber where subjects were previously exposed to an anesthetized opposite-sex conspecific (see Pankevich 2006b; Martel & Baum, 2009a; Kang *et al.*, 2009).

In support of qualitative observations, results of puncta analysis showed that the STMPI ($t(df = 8) = 5.350, p < 0.001$), STMPM ($t(df = 8) = 7.445, p < 0.001$), and PMCo ($t(df = 9) = -8.632, p = 0.001$) possessed significantly more labeled puncta on efferent fibers originating from the MeP, as compared to the MeA. In contrast, the HDB ($t(df = 10) = 3.567, p = 0.005$) and mOT ($t(df = 10) = 2.408, p = 0.037$) showed denser innervation by the MeA. Other ventral striatal and hypothalamic structures displayed relatively equal innervation from the MeA and MeP, although there was a trend toward denser innervation by the MeP in the STMPL ($t(df = 8) = 2.263, p = 0.053$) and VMHdm ($t(df = 8) = -2.208, p = 0.058$) (Fig. 6).

3. Discussion

The present results demonstrate that the MeA and MeP differentially target multiple ventral telencephalic sites that have been previously implicated in the control of sociosexual behaviors. The pBNST is the major recipient of efferent projections from the Me as a whole, but our analysis revealed that the STMPI and STMPM subdivisions receive significantly more input from the MeP, while the STMPL receives an equal, but lesser degree of input

from both the MeA and MeP. Aspects of this pattern of innervation of the pBNST by the Me differs from those observed by Pardo-Bellver *et al.*, (2012). Specifically, those workers reported that the MeA most densely innervates the STMPI, while the STMPM is equally innervated by all Me subnuclei. While it is possible that this discrepancy could reflect strain differences (C57BL/J6 & CD1 vs. Swiss Webster in the present study), an alternative explanation is that puncta analysis, averaged across tracer injections given in multiple animals, is more sensitive to discrete differences in the pattern of innervation of these subnuclei. For example, the MeA and MeP may send a similar number of axonal projections to the STMPI (as has been previously reported using qualitative measurements of fiber innervation). However, projections from the MeP may make more en passant synaptic connections within the region, as indexed by labeled puncta, while the majority of fibers from the MeA may represent fibers of passage, producing fewer labeled puncta. Puncta evaluation is therefore a fundamentally different method of analysis, which makes direct comparisons with past studies using qualitative measures of fiber density potentially confounding. A major strength of the current method is that puncta values are averaged across multiple animals, which accounts for individual variability and may provide a more complete view (i.e. rostral to caudal) of the outputs of a given brain region. Our approach contrasts with the alternative approach (Pardo-Bellver *et al.*, 2012; Usunoff *et al.*, 2009) in which as few as one or two discrete tracer injections, which may or may not cover a substantial volume of the targeted nucleus, are used to assess efferent projections of amygdalar subnuclei.

Puncta are clearly defined boutons sprouting from labeled fibers and have been shown to contain synaptic proteins (Bozdagi *et al.*, 2000). They are therefore thought to denote the presence of synapses. For example, long-term potentiation in hippocampal neurons, which is associated with an increase synapse numbers (Toni *et al.*, 1999), is accompanied by long lasting increases in puncta number (Antonova *et al.*, 2009). In the present study, we did not probe for the presence of pre and/or post-synaptic proteins and cannot therefore define a punctum as representing a synapse, but rather as a method for quantifying the amount of labeling observed in Me projection targets.

The BNST has been directly implicated in the control of sociosexual behaviors. In female hamsters, an intact BNST is necessary for the display of precopulatory behaviors including ultrasonic vocalizations and vaginal scent marking (Kirn & Floody, 1985; Martinez & Petrulis, 2011), while in male hamsters excitotoxic lesions of the BNST eliminate opposite-sex odor preference (Been & Petrulis, 2010). The BNST as a whole, but particularly the anterior BNST, densely innervates the ventral tegmental area (VTA) predominantly via GABAergic inputs. The BNST is thus a potent regulator VTA activity, principally acting by disinhibiting dopaminergic projection neurons (Georges & Aston-Jones, 2001; Kudo *et al.*, 2012). This polysynaptic pathway linking the MeP with the VTA (via the BNST) may underlie the preference of female mice for opposite-sex odors (DiBenedictis *et al.*, 2012). The activation of this pathway may also contribute to the increase in Fos expression previously observed in the VTA in response to opposite-sex odors (Moncho-Bogani *et al.*, 2005) in lieu of any major direct Me-VTA projections. Moreover, amygdaloid fibers terminating in the AcbC and AcbSh also traverse the BNST (Novejarque *et al.*, 2011).

The VMH and MPA play important roles in controlling sexual behavior in females of many rodent species (Blaustein & Erskine, 2002; Clark *et al.*, 1981; Malsbury *et al.*, 1977; Meerts & Clark, 2009), and the MePD projection to the VMH conveys reproductively significant olfactory information in mice and ferrets (Choi *et al.*, 2005; Wersinger & Baum, 1997). In the present study we found that these regions receive relatively equal input from the MeA and MeP. This result corroborates our previous work showing that both MeA and MeP-

lesioned female mice exhibited deficits in their display of lordosis in response to male mounts (DiBenedictis *et al.*, 2012).

The posteromedial cortical amygdala receives substantial input (as measured by labeled puncta) from both the MeA and MeP, with the most robust innervation originating from the MeP. These projections are reciprocal, as has been previously described in rats and hamsters (Coolen & Wood, 1998; Pitkänen *et al.*, 2000), suggesting that the pattern of Me output may be modified by intra-amygdaloid interactions. Insofar as the PMCo directly targets regions of the ventral striatum, including the islands of Calleja and adjoining olfactory tubercle complex (Novejarque *et al.*, 2012; Ubeda-Banon *et al.*, 2008), this circuit may also be involved in motivating appetitive behavioral responses to salient opposite-sex chemosignals.

In contrast to the BNST and PMCo, we observed significantly more labeled puncta on fibers originating from the MeA in the horizontal limb of the diagonal band of Broca (HDB). The HDB is a major cholinergic brain center whose activity has been linked to attention. Pharmacological disruption of cholinergic signaling has been shown to impair performance in olfactory-based behavioral tasks (Roman *et al.*, 1993), while enhancing acetylcholine levels improved olfactory discrimination (Doty *et al.*, 1999). Furthermore, the HDB provides major centrifugal inputs to the MOB (Ma & Luo, 2012), which presumably modulates odor perception. It is possible that salient pheromonal information reaching the MeA is passed to the HDB, which in turn heightens the animal's level of attention toward the pheromonal stimulus, thereby enhancing olfactory acuity and promoting chemoinvestigation.

We also observed a pathway directly linking the MeA (and to a lesser extent, the MeP) with the ventral striatum, including the mOT, AcbSh, and AcbC. While Meaccumbal projections were relatively sparse, there was robust innervation of the mOT by the MeA, but not by the MeP. Because this projection is monosynaptic, pheromonal information transmitted by this pathway may be subject to limited signal modification and could be involved in mediating the reinforcing effects of opposite-sex odors. Indeed, the mOT has been implicated in the reinforcing effects of cocaine, MDMA, and amphetamines (Ikemoto, 2003; Shin *et al.*, 2008; Shin *et al.*, 2010) and in male rat sexual behavior (Hitt *et al.*, 1973). The mOT has also been hypothesized to play an important role in odor hedonics (Wesson & Wilson, 2011). We propose that male pheromonal signals reach the Me via the main and accessory olfactory bulbs and that this information is relayed to the ventral striatum by way of both indirect polysynaptic (MeP-BNST-VTA-mOT and MeP-PMCo-mOT) and direct monosynaptic (MeA-mOT) pathways which may act synergistically to motivate female mice to seek out and investigate these odors, a critical first step leading to successful procreation. Based on our previous work (DiBenedictis *et al.*, 2012), it is likely that indirect inputs to the mOT, originating from the MeP, are alone sufficient to drive proceptive chemoinvestigatory behaviors, while the direct pathway from the MeA is not. Future work will assess the functional role of the mOT in hardwired olfactory-driven motivated behaviors in female mice.

4. Experimental Procedure

Thirty-five ovary-intact female Swiss Webster mice (Charles River Laboratories, Wilmington, MA, USA) were purchased at 5-6 weeks of age and maintained on a reversed 12:12 h light:dark cycle with food and water available *ad libitum*. All procedures were approved by the Boston University Charles River Campus Institutional Animal Care and Use Committee. Female subjects used for quantitative analysis were assigned to one of two groups: MeA Fluoro-Ruby-injected or MeP Fluoro-Ruby-injected. Subjects were injected with tracer in both hemispheres to maximize the probability of obtaining an accurate

injection. However, projections were quantified only within a single hemisphere for each animal, and only mice with accurate injections in either the MeA (n=6) or MeP (n=6) were included in this study. Mice with inaccurate injection placement and/or appreciable spread of the anterograde tracer into adjacent amygdaloid nuclei were not used (n=21).

Subjects were administered carprofen analgesic (5 mg/kg, s.c.) preoperatively and for 2 days after surgery. Mice were anesthetized under continuous 2% isoflurane vapor, and the head was secured in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). A midline incision was made to expose the skull, and a small craniotomy was then drilled bilaterally over each injection site. For both MeA- and MeP-injected animals, lateral coordinates were 2.0 mm and depth coordinates (from dura) were 4.7 mm. Anterior-Posterior (from interaural line) coordinates were 4.0 mm (MeA) and 2.7 mm (MeP). A pulled glass micropipette (20 μ m tip diameter) filled with the fluorescent anterograde tracer, Fluoro-Ruby (5% in 0.01 M PB, pH 8.0, Molecular Probes), was lowered into either the MeA or MeP. Next, a (+5 μ A) pulsatile cathodal current (7s on, 7s off) was passed for 2 minutes to deliver the tracer iontophoretically. The electrode was then retracted while passing a constant (−5 μ A) anodal current. A separate group of animals received dual anterograde tracer injections in the MeA and MeP (n=3): the anterograde tracer, PHA-L; 2.5% in 0.01 M PB, pH 8.0) was injected into the MeA, and Fluoro-Ruby was injected into the MeP within the same hemisphere. Data were not collected from these mice; they were used only to depict qualitatively the overlap in MeA vs MeP projections shown in Figure 3. Craniotomies were covered with bone wax, and the incision was closed by suture and treated with 2% topical lidocaine. Five days after surgery, subjects were perfused and their brains were subsequently removed, fixed, cryoprotected and sectioned coronally at 30 μ m using a cryostat (DiBenedictis *et al.*, 2012).

Fluorescent immunocytochemistry was used to visualize PHA-L labeling in dual anterograde tracer-injected animals. Tissues were blocked for 1 h in 5% donkey serum and subsequently incubated with primary (Vector Laboratories, goat anti-PHA-L, 1:4000, overnight at 4°C) and secondary antibodies (Life Technologies, Alexa Fluor 488 donkey anti-goat, 1:600, 1 h at room temperature) followed by a 10 min incubation in 4',6-diamidino-2-phenylindole (DAPI; Sigma, 1:200, RT) to label cell nuclei.

Fluorescently labeled (Fluoro-Ruby) puncta (terminal and en passant boutons) were counted to quantify the relative magnitude of efferent projections from the MeA vs. MeP. Eleven forebrain regions previously implicated in courtship behaviors were chosen for analysis. Confocal images (60X) of projection targets were obtained using 30 laser scans (every 1.0 μ m) in 30 μ m depth samples using an Olympus FluoView 10i confocal microscope. These images were then stacked in the depth ('Z') plane, and a 100- μ m² area was selected for each region of interest. Puncta were counted in each region using the 'cell counter' plugin in ImageJ by an investigator blind to location of the tracer injection. Only puncta that were clearly visible and sufficiently large (between 0.8 μ m and 1.6 μ m in diameter, similar in range to that used by Pro-Sistiaga *et al.*, 2009) were included in our analysis. Limitations of this analysis, such as the amount of tracer delivered, the percentage of neurons that took up the tracer, and the counting of spurious puncta were assumed to be the same between groups, rendering this type of analysis suitable for a comparative (targets of MeA vs. MeP input) study (Pro-Sistiaga *et al.*, 2007). The number of labeled puncta for each animal was averaged for each group and compared in each forebrain region between the MeA and MeP-injected subjects using a 2-tailed Student's t-test.

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Highlights

- The anterior and posterior Me differentially innervate downstream targets
- The MeP, but not the MeA, densely innervates the BNST and PMCo
- The MeA, but not the MeP, densely innervates the HDB and mOT
- Circuits from the Me to the mOT may underlie innate approach behaviors

Figure 1.

A,B, Confocal image showing Fluoro-Ruby injection sites in the anterior (A) and posterior (B) medial amygdala. C,D, Schematic reconstructions of coronal sections showing the extent of individual Fluoro-Ruby injections in female mice targeting the anterior (n=6) medial amygdala. Top row: subjects 336, 340, 342; Bottom row: subjects 353, 354, 358. E,F, Schematic reconstructions of coronal sections showing the extent of individual Fluoro-Ruby injections in female mice targeting the posterior (n=6) medial amygdala. Top row: subjects 337, 341, 344; Bottom row: subjects 343, 345, 346. Sections are ordered sequentially from anterior (left) to posterior (right), with the numbers shown on the bottom right of each plate representing the distance in mm posterior to bregma. Colored regions within each plate represent individual injections, identified by the animal code. Adapted from Franklin and Paxinos (Franklin & Paxinos, 2008). MeA, anterior medial amygdala; MeP, posterior medial amygdala; opt, optic tract.

Figure 2.

The posterior medial amygdala (MeP) densely innervates the posteromedial cortical amygdala (PMCo) and the posterior bed nucleus of the stria terminalis (pBNST). Epifluorescent images show dense anterograde fiber labeling in the PMCo and pBNST (A', B') in MeP Fluoro-Ruby-injected animals, but not MeA Fluoro-Ruby-injected animals (A, B). Retrogradely labeled cell bodies were also observed in the PMCo of subjects given Fluoro-Ruby injections into the MeP (A'), indicated by filled white arrows.

Figure 3.

Overlapping anterograde fiber/puncta labeling in the posterior bed nucleus of the stria terminalis (A; pBNST) and medial olfactory tubercle (B; mOT) of a female mouse given injections of PHA-L (green) and Fluoro-Ruby (red) into the MeA and MeP, respectively. A, confocal image showing that the intermediate and medial portions of the pBNST receive input mostly from the MeP (red), while the lateral portion receives relatively equal input from both the anterior and posterior Me. A', 60× (oil) Z plane-stacked confocal image of the boxed area shown in A. Labeled fibers with puncta originating from the MeA (green) and MeP (red) were seen throughout the posterolateral stria terminalis (STMPL), shown with DAPI nuclear counterstain (blue cell bodies). B, confocal image showing synaptic input to the mOT almost exclusively from the anterior Me (green) with very sparse labeling from the MeP (red). B', 60× (oil) Z plane-stacked confocal image of the boxed area shown in B showing innervation of the mOT predominantly by the MeA (green fibers).

Figure 4.

Z-plane stacked confocal images (60× magnification) showing anterograde fiber/puncta labeling in coronal sections of the medial portion of the posterior stria terminalis (STMPM) following Fluoro-Ruby injections into either the anterior (A, MeA) or posterior (B, MeP) medial amygdala. C, Filled white arrows point to labeled puncta, magnified from the boxed region that were included for analysis (0.8µm-1.6µm diameter); open arrows point to possible puncta that were very small (<0.8µm diameter) and therefore excluded from analysis.

Figure 5.

Modified schematic from the mouse brain atlas of Franklin and Paxinos (Franklin & Paxinos, 2008) showing the location of forebrain regions in which labeled puncta (dark gray boxes; $100^2 \mu\text{m}$) were counted. Counting regions included the medial olfactory tubercle (A), the shell (left) and core (right) of the nucleus accumbens (B), the horizontal diagonal band of Broca (C), the medial preoptic area (D), medial (left), intermediate (middle), and lateral (right) posterior bed nucleus of the stria terminalis (E), dorsomedial (left) and ventrolateral (right) subdivisions of the ventromedial hypothalamus (F), and posteromedial cortical amygdala (G). Numerical values represent the distance in mm from bregma for each section.

Figure 6.

Quantitative estimation of labeled puncta in animals given Fluoro-Ruby injections into either the MeA or MeP. Brain regions in which puncta were counted: STMPPL, STMPI, STMPM, posterolateral, intermediate, medial divisions of the bed nucleus of the stria terminalis; PMCo, posteromedial cortical amygdala; HDB, horizontal diagonal band of Broca; MPA, medial preoptic area; VMHdm, VMHvl, dorsomedial, ventrolateral portions of the ventromedial hypothalamus; mOT, medial olfactory tubercle; AcbC, AcbSh, nucleus accumbens core, shell. Data are expressed as the mean ($\pm$ SEM) calculated from confocal images merged in the Z-plane. Sample sizes for each group are shown in parentheses.

* $P < 0.040$, ** $P < 0.001$ (Two-tailed Student's t-test).