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A FOREBRAIN-RETROUBRAL PATHWAY INVOLVED IN MALE SEX BEHAVIOR IS GABAERGIC AND ACTIVATED WITH MATING IN GERBILS

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Abstract

The ventral bed nuclei of the stria terminalis (BST) and medial preoptic nucleus (MPN) of gerbils contain cells that regulate male sex behavior via a largely uncrossed pathway to the retrorubral field (RRF). Our goal was to learn more about cells at the pathway source and target. To determine if the pathway uses GABA as its transmitter, we used immunocytochemistry to study glutamic acid decarboxylase₆₇ (GAD₆₇) colocalization with Fluoro-Gold (FG) in the ventral BST and MPN after applying FG to the RRF. To determine if the pathway is activated with mating, we studied FG-Fos colocalization in the ventral BST of recently mated males. The ventral BST expresses Fos with mating and is the major pathway source. To determine to what extent other GABAergic cells in the ventral BST are activated with mating, we studied Fos colocalization with GAD₆₇ mRNA visualized by *in situ* hybridization. We also looked for GAD₆₇ mRNA in RRF cells. Almost all ventral BST and MPNm cells projecting to the RRF (95–97%), and most ventral BST cells activated with mating (89%), were GABAergic. GABAergic cells were also seen in the RRF. RRF-projecting cells represented 37% of ventral BST cells activated with mating. Their activation may reflect arousal and anticipation of sexual reward. Among ventral BST cells that project to the RRF, 14% were activated with mating, consistent with how much of this pathway is needed for mating. The activated GABAergic cells that do not project to the RRF may release GABA locally and inhibit ejaculation.

Keywords

ventral bed nuclei of the stria terminalis; medial preoptic nucleus; sexually dimorphic area; retrorubral field; A8

The ventral bed nuclei of the stria terminalis (BST) and medial preoptic nucleus (MPN) of gerbils and rats contain cells that project to the retrorubral field (RRF) via a largely uncrossed pathway (Finn et al., 1993; Finn and Yahr, 2005). If this pathway is severed by destroying cells at its source and target contralaterally, mating ceases or is severely impaired (Finn and Yahr, 1994, 2005). Ipsilateral pathway lesions have no effect. Since the RRF does not project to the MPN/ventral BST (De Vries et al., 1988; Deutch et al., 1988; Finn et al.,

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1993), the effects of disrupting this pathway can be interpreted in terms of the direction of information flow.

RRF responses to input from this pathway are mediated primarily by non-A8 cells, but the A8 dopamine cells are involved. When the RRF component of the pathway lesion was made with 6-hydroxydopamine instead of a general neurocytotoxin, to destroy only A8 cells, male gerbils were less likely to intromit and ejaculate but continued to mate (Finn and Yahr, 1994).

Our goals here were to identify the MPN/ventral BST-RRF pathway transmitter, to determine if the pathway is activated with mating, and to learn more about cells at its source and target. To determine if the pathway uses γ -aminobutyric acid (GABA) as its transmitter, we used immunocytochemistry (ICC) to study the colocalization of a glutamic acid decarboxylase (GAD) isoform, GAD₆₇, found primarily in cell bodies (Erlander et al., 1991), with Fluoro-Gold (FG), a retrograde axonal tracer, in ventral BST and MPN cells after applying FG to the RRF. GAD is the enzyme that synthesizes GABA. To determine if the pathway is activated with mating, we used ICC to study FG colocalization with Fos in ventral BST cells of recently mated males. The ventral BST contains most of the RRF-projecting cells (Finn et al., 1993; Finn and Yahr, 2005) and expresses Fos with mating (Heeb and Yahr, 1996). To determine if any GABAergic ventral BST cells are activated with mating, we studied Fos colocalization with GAD₆₇ mRNA by *in situ* hybridization (ISH). We also assessed whether any RRF cells contain GAD₆₇ mRNA.

EXPERIMENTAL PROCEDURES

Animals

Gerbils purchased as adults (Sprague-Dawley; Indianapolis, IN) were housed in same-sex pairs under a 14:10-hr light:dark cycle with food and water freely available. The females were used as partners in tests of male sex behavior and were implanted subcutaneously, 5–10 days before use, with 5-mm Silastic capsules with estradiol benzoate to ensure their sexual receptivity. They were not ovariectomized, as this had proved unnecessary. All animals were anesthetized with sodium pentobarbital before surgery (50 mg/kg) or perfusion (100 mg/kg). All procedures were approved by the University of California, Irvine, Institutional Animal Care and Use Committee.

Behavioral testing

Males used for Fos ICC had copulated to ejaculation in at least one screening test. For those tests, they were placed alone in Plexiglas arenas (32–42 cm diameter) 5 min before a female was introduced. The male was given 30 min to intromit. If he did not do so in 15 min, the female was replaced. If he did, he was given 30 min to ejaculate. Males were used for Fos ICC only if they copulated to ejaculation again on the day of perfusion. They were perfused 1–1.5 hr later.

Nomenclature

The gerbil MPNm and ventral BST were initially identified as the two major cell groups of the sexually dimorphic area (SDA) of the gerbil hypothalamus (Commings and Yahr, 1984), but based on homologies discussed in Finn et al. (1993), the medial SDA, the area lateral to it and the SDA *pars compacta* correspond, respectively, to the MPNm, lateral MPN (MPNI) and central MPN of rats (Swanson, 1992). The lateral SDA corresponds to (Finn and Yahr, 2005) the magnocellular subnucleus of the BST (BSTmg) plus the parts of the ventral subnucleus lying ventral and lateral to the BSTmg in rats (Ju and Swanson, 1989), including the dorsal parts of the preoptic subnucleus as described by Moga et al. (1989).

FG injections

Using slightly different protocols, two sets of males were given FG (Fluorochrome, Englewood, CO), 6–20 days before perfusion, via a glass pipette (tip diameter 20- to 80- μ m) stereotactically (Kopf; Tujunga, CA) directed toward the RRF [2.4 mm caudal to Bregma, 1.2 mm lateral and 6.4 mm ventral to the midline sinus (GAD₆₇ ICC); 3.0 mm caudal, 1.4 mm lateral and 7.05 mm (Kopf Ultraprecise) ventral to Bregma (Fos ICC)]. Starting a few minutes later, an alternating [9 sec on/9 sec off (Fos); 10/10 (GAD₆₇)], +3 μ A direct current was applied for 15–20 min to the solution in the pipette [2% FG in 0.1M acetate buffer (pH 3.3; Fos); 3% FG in deionized, purified water (GAD₆₇)], which was withdrawn 10–20 min after the injection.

Tissue fixation

Males were pericardially perfused with saline and then freshly prepared 4% paraformaldehyde (pH 7.3–7.4) in 0.1 M Na phosphate buffer (PB). Brains were removed, postfixed in this fixative at 4 °C for 1.5 hr, and stored in it overnight at 4 °C with 20% sucrose added for cryoprotection. The next day, brains were frozen and cut coronally at 30 μ m.

Immunocytochemistry

Unless noted in a specific protocol, the following applied. Procedures were done at room temperature, rinses involved three changes of solution over 15–30 min, and both incubations and rinses involved gentle agitation. Vectastain ABC kits (Vector Labs; Burlingame, CA) were used per instructions. The chromogen was 3,3'-diaminobenzidine (DAB; Sigma, St. Louis, MO) used at 0.05% in Tris-buffered saline (TBS; pH 7.6) and developed with increasing concentrations of H₂O₂ (0.006–0.01%). Sections were rinsed and stored in PB containing 0.9% NaCl (PBS) until mounted onto slides coated with gelatin/chrom-alum. They were then air dried, dehydrated with graded alcohols, delipidized with xylene (Fos) or Histoclear (GAD₆₇; National Diagnostics; Atlanta, GA) and coverslipped using DePeX (BDH Laboratory Supplies; Poole, UK). To determine if either secondary antibody mimicked or interfered with binding of the other, some sections were processed for both antigens with one primary antibody omitted. This produced staining for the antibody used that was like that seen when tissue was processed for it alone.

FG-GAD₆₇ colocalization—For GAD₆₇ ICC, which preceded FG ICC, sections were incubated for 1 hr in PBS + 10% normal goat serum (NGS), then for 20 hr at 4 °C in PBS + 1% NGS + rabbit anti-GAD₆₇ immunoglobulin G (IgG) K2 (AB108; Chemicon; Temecula, CA) at 1:2,000, and then for 1 hr in PBS + 1.5% NGS + biotinylated goat anti-rabbit IgG (Vector) at 1:200. For FG ICC, sections were incubated in PBS + 10% NGS for 20 min, then overnight at 4 °C in PBS containing 0.3% Triton-X 100 (TXB) + 1% NGS + rabbit polyclonal anti-FG IgG (Chemicon) at 1:1,000 or 1:4,000, and then for 1 hr in PBS + 1.5% NGS + biotinylated goat anti-rabbit IgG (Vector) at 1:200. Vector VIP (purple reaction product) and SG (blue-gray reaction product) peroxidase substrate kits were used to visualize FG in alternate sections.

FG-Fos colocalization—Tissue used for FG-Fos colocalization was from five males with FG injections centered in the RRF used in a previous study (Simmons and Yahr, 2002). Procedures are described there in detail. Fos ICC, which preceded FG ICC, used a polyclonal rabbit anti-c-Fos IgG (Ab-5; Calbiochem; San Diego, CA) at 1:30,000–50,000, goat anti-rabbit IgG at 1:400, and DAB as the chromogen. FG ICC was done as described above except that the primary antibody was used at 1:5,000–7,500 and the secondary antibody at 1:400, and FG was visualized with a 1:4 dilution of a Vector SG substrate kit.

Visualization of FG injection sites—Every other section through the RRF of males given FG in the RRF was processed for FG ICC with the forebrain sections. For males used to study FG-Fos colocalization, the intervening sections were processed for tyrosine hydroxylase (TH) ICC to visualize A8 cells. TH ICC, which preceded FG ICC, was the same as the Fos ICC described above except that normal horse serum replaced NGS, monoclonal mouse anti-TH IgG (Incstar; Stillwater, MN) was used at 1:10,000, and biotinylated horse anti-mouse IgG (Vector) was used at 1:200. See Simmons and Yahr (2002) for details.

GAD₆₇ mRNA colocalization with Fos

Tissue used to colocalize Fos with GAD₆₇ mRNA was from four males used in a previous study (Simmons and Yahr, 2003). The ISH procedure, which used free-floating sections, is described in detail there. ISH was used instead of ICC because it produced less nuclear staining, which made it easier to see Fos there. Briefly, ISH preceded Fos ICC and used GAD₆₇ antisense riboprobes transcribed from 3.2-kb cDNA clones (gift of Dr. A. J. Tobin; UCLA) and labeled with digoxigenin (Boehringer Mannheim, Indianapolis, IN). Sections were rinsed once in PB + 0.1% Triton X-100, twice in PB + 0.75% glycine, twice in PB and incubated in water + 0.1M triethanolamine + 2.5N hydrochloric acid + 2.5% acetic anhydride. After two rinses in 2X standard saline sodium citrate (SSC, pH 7.2), they were dehydrated in alcohols, chloroform, and ethanol again (100%, 95%) before being incubated overnight (55 °C) in a humid chamber in hybridization buffer containing riboprobe at 1:1,500 (about 0.25 µg/µl). Sections were rinsed in 50% formamide in 2X SSC and digested with RNase A (20 µg/ml). After rinses in 2X SSC, PBS, and PBS with 0.1% Triton X-100, sections were incubated in blocking solution (5% sheep serum, 5% bovine serum albumin, 0.1% Triton X-100 in PBS) and then in it + sheep anti-digoxigenin IgG, conjugated to alkaline phosphatase (Boehringer Mannheim), at 1:1,000. After rinses in buffer, sections were incubated in a BCIP/NBT Alkaline Phosphatase kit IV solution (Vector) to which 0.1% Triton X-100 + 1 drop/5 ml of levamisole (Vector) was added until reaction product (blue-purple) was seen microscopically (usually 2–4 hr). Forebrain sections were processed for Fos ICC, mounted, dehydrated, delipidized in xylene, and coverslipped using Permount (Fisher; Pittsburgh, PA). Midbrain sections were mounted with the forebrain sections.

Controls—Other sections were processed with a sense GAD₆₇ riboprobe (gift of Dr. Stuart Tobin, UCLA); it produced no staining. Some of them were also processed for Fos ICC, which produced staining indistinguishable from that seen with antisense probe or Fos ICC alone.

Histological analysis

Identification of FG injection sites—Midbrain sections were examined at 100–250X under brightfield illumination to identify the necrotic center of the injection site. If necrosis was not seen, the center of the injection site was identified as the area of densest FG staining in the neuropil. The RRF was identified by cells immunoreactive (IR) for TH (A8 cells) or by tissue landmarks using rat brain maps and atlases (Swanson, 1992; Paxinos and Watson, 1997). Males were used only if the FG injection was centered in the RRF.

Quantification of FG colocalization with GAD₆₇—Two observers independently counted cells IR for FG, GAD₆₇ or both in alternate sections (4–5/side for the MPNm; 3–4/side for the ventral BST) from three males while viewing the tissue at 500X with brightfield illumination. A grid was superimposed on the tissue with a drawing tube. Recording blood vessels and other landmarks as well as FG-IR cells allowed observers to progress systematically through the areas. Each FG-IR cell was examined for GAD₆₇. Observers' counts for each male were averaged across sides for each cell group. The percentage of

RRF-projecting cells that were GABAergic was computed for each male by dividing the number of double-labeled cells by the total that were FG-IR. Sections from a fourth male were used to chart locations of FG-IR cells with and without GAD₆₇ in detail using three sections separated from each other by 90 μ m.

Quantification of FG colocalization with Fos—Cells IR for Fos, FG or both were counted in the ventral BST on the side of the FG injection in 2–3 non-adjacent sections while viewing the tissue at 312X. A square (0.2 mm/side) superimposed on the tissue with a drawing tube was positioned parallel to the midline and covered most or all of the ventral BST while minimally including other cells. FG-IR cells (*i.e.*, soma that were distinct due to speckled, blue reaction product in the cytoplasm) in or near the box were traced. Then, Fos-IR nuclei (*i.e.*, round profiles of a size appropriate for neuronal nuclei that were distinct due to brown reaction product in them) were traced. Cells meeting both criteria were marked as double-labeled.

From the drawings, FG-IR cells within the box or overlapping its top or right edges were counted. Those overlapping the left or bottom edges were counted if at least half of the soma was in the box. Fos-IR cells were counted if the nucleus met the same criteria or if the cell was double labeled and included in the FG-IR cell count. Counts for each male were divided by the number of sections used. The percent of RRF-projecting cells activated with mating was computed by dividing the number of double-labeled cells by the total number of FG-IR cells. The percent of Fos-IR cells labeled from the RRF was computed by dividing the number of double-labeled cells by the total number of Fos-IR cells.

Quantification of Fos colocalization with GAD₆₇ mRNA—Cells IR for Fos, positive for GAD₆₇ mRNA or both were counted unilaterally (either side) in one section as described for Fos-FG colocalization. Cells positive for GAD₆₇ mRNA were recognized by diffuse blue-purple reaction product in the cytoplasm. The percentage of Fos-IR cells that were GABAergic and the percentage of GABAergic cells activated with mating were computed as above.

Images

Digitized images were acquired with a Zeiss AxioImager M2 light microscope using a 2.5X, 10X or 40X oil Plan Apo objective and AxioVision software v4.7. To better reproduce the appearance of the tissue at microscopy, tonal and/or color qualities of images were adjusted in Adobe Photoshop CS3 (levels, curves and color balance tools). Figures were assembled in Adobe Illustrator CS3.

RESULTS

In males given FG in the RRF, almost every FG-IR cell in the ventral BST ($95 \pm 1\%$) and MPNm ($97 \pm 1\%$) was also GAD₆₇-IR. These data are summarized in Table 1 and illustrated in Figure 1. Figure 2A shows an example of an FG injection site. The resulting retrograde labeling and the double-labeling by GAD₆₇ are shown in Figure 2B-C.

RRF injections of FG labeled six times as many ventral BST as MPNm cells (Table 1). Most of the RRF-projecting cells in the MPNm were in its rostral aspect (Fig. 1, top). In the ventral BST, 85% of the retrogradely labeled cells were ipsilateral to the RRF injection; in the MPNm, 66% were ipsilateral (Table 1). FG-IR cells were also seen in the MPNl and lateral and dorsal to the ventral BST, primarily below the top of the anterior commissure, but were not as dense in any of those sites as in the rostral MPNm or ventral BST.

In mated males, most Fos-IR cells in the ventral BST contained GAD₆₇ mRNA ($89 \pm 7\%$) and many projected to the RRF ($37 \pm 2\%$). Among ventral BST cells labeled from the RRF, 14% were Fos-IR after mating. Among ventral BST cells containing GAD₆₇ mRNA, 15% were Fos-IR after mating. These data are summarized in Table 2 and illustrated in Figure 2D-H. Cells containing GAD₆₇ mRNA were also seen in the RRF (Fig. 2I).

DISCUSSION

By combining retrograde tracing from the RRF with visualization of GAD₆₇, we showed that the pathway from the ventral BST/MPN to the RRF, which regulates male sex behavior in gerbils and rats, is GABAergic in gerbils. Almost all MPNm and ventral BST cells labeled from the RRF contained GAD₆₇. Knowing the transmitter of a pathway can suggest selective ways to manipulate it. Knowing that a GABAergic pathway originates or terminates in an area can affect the interpretation of GABA manipulations there. Knowing that some GAD cells in an area are projection rather than local neurons can affect the interpretation of GAD colocalization with regulatory molecules, such as hormone receptors. The present study verified earlier work (Finn et al., 1993; Finn and Yahr, 2005) indicating that the source cells for this pathway are densest in the rostral MPNm and ventral BST and are most abundant in the ventral BST.

In the ventral BST, 14% of the RRF-projecting cells were activated with mating based on Fos expression. The only context we have in which to consider how much of the pathway is activated with mating is how much of it is needed for mating. That is roughly 13%. When we made pathway lesions in gerbils and rats (Finn and Yahr, 1994, 2005), the target-side lesions were quite complete, but the source-side lesions varied. Yet whether the source cells left intact on the side of the source lesions were in the rostral MPNm, caudal ventral BST or dorsolateral to the ventral BST, the effect on male sex behavior was the same. Mating was largely unaffected until less than 25% of them, or roughly 13% of the bilateral pathway, remained intact. When more of the pathway was lost, mating declined. Retained function, as assessed by copulatory rate, correlated with the integrity of the pathway. Thus, while the similarity between how little of the pathway is needed for mating - its functional redundancy - and how little of it is activated with mating could be coincidental, it could also indicate that the pathway becomes activated during mating only to the extent necessary and sufficient to support the behavior.

For parsimony, we assume that functional redundancy is independent of the specific cell types involved. Thus, if RRF-projecting cells consist of subsets that contact different RRF cell types or release GABA with different peptides, we assume that the ratio of those subsets within any set of activated cells stays the same as different sets are activated at different times that the behavior is displayed. We have no way to know what determines which set of cells is activated with any display of mating. However, data on male rats allowed to copulate to ejaculation twice suggest that repeating the behavior, even minutes later, involves activation of a second set of cells that is about the same size as the set activated with the first mating bout (Baum and Everitt, 1992; Coolen et al., 1996). The increase in Fos-IR cells in the medial preoptic area (MPOA), medial amygdala and parts of the BST in male rats allowed to copulate to ejaculation twice, which involved only a few additional intromissions, was as large as the increase seen after the first ejaculation, relative to what was seen in males allowed to intromit only 5–8 times. If more and more cells are activated with each copulatory bout, it is even possible that exhaustion of the supply of cells available for activation underlies the onset of sexual satiety.

In the ventral BST, 15% of GABAergic cells were activated with mating. Unlike the situation for ventral BST cells that project to the RRF, we have no data that suggest what

percent of its GABAergic cells are needed for mating. We do know, though, that higher percentages (27–41%) of GABAergic cells were activated with mating in the MPNm (assessed caudally), posterodorsal preoptic nucleus (PdPN) and lateral part of the posterodorsal medial amygdala (MeApd) in the same males (Simmons and Yahr, 2003). However, the pattern of Fos expression in the ventral BST with mating, which is described below, is quite different from the patterns seen in these other areas (Heeb and Yahr, 1996). The PdPN and lateral MeApd express Fos only at ejaculation. They are also much smaller than the ventral BST. The MPNm expresses Fos when males are exposed to an environment associated with mating and at ejaculation.

Among ventral BST cells activated with mating, 37% project to the RRF. Since 38% of ventral BST cells activated with mating are activated by arousal (general + sexual), it is possible that the arousal-activated cells are the RRF-projecting cells. During mating, Fos expression in the ventral BST increases incrementally (Heeb and Yahr, 1996). If we express its cumulative Fos-cell densities relative to the total seen after one ejaculation (100%), 12% reflects handling (being placed in a familiar arena where no social contact occurs). Spending 5 min in the arena in which the males gained their sexual experience brings that up to 38%. After a receptive female is introduced, Fos expression continues to rise as the male investigates her anogenital region (51%), mounts (71%), intromits (84%) and ejaculates (100%). The fact that the percent of activated cells activated by general plus sexual arousal (38%) is almost identical to the percent that project to the RRF (37%) could be coincidence. Or, arousing stimuli may activate ventral BST cells that project to the RRF.

This possibility is consistent with data implicating the RRF in arousal and anticipation of reward. In gerbils, the RRF is activated by handling (Heeb and Yahr, 1996). In rats trained to press a lever for electrical stimulation of the lateral hypothalamus or ventral tegmental area (VTA), more stimulation is needed to maintain lever pressing after the RRF has been inactivated by infusions of lidocaine (Waraczynski and Parsons, 2000). The A8 cells of the RRF project to the VTA (Swanson, 1982; Hasue and Shammah-Lagnado, 2002), which is active in rats during copulatory movements (Hernandez-Gonzalez et al., 1997), and to the nucleus accumbens (NA). Both the NA and VTA are implicated in sexual arousal and reward (Liu et al., 1998; Balfour et al., 2004; Kippin et al., 2004; Guevara et al., 2008; Wallace et al., 2008; Pitchers et al., 2010a,b).

Among ventral BST cells activated with mating, 89% are GABAergic, which includes the GABAergic cells that project to the RRF. Since activation of ventral BST cells did not reach a similar level (84%) until after many intromissions, the activated GABAergic cells that do not project to the RRF may respond to genital stimulation or other stimuli available only through direct contact with a receptive female. The similar percentages could be coincidental. However, in rats, GABA action in the MPOA inhibits ejaculation and the resumption of mating afterward (Fernández-Guasti et al., 1986a,b). Thus, if GABAergic ventral BST cells activated with mating that do not project to the RRF release GABA locally, they may participate in such processes to affect the pace of mating. In gerbils, the ventral BST projects to the MPNm (Finn et al., 1993) and both affect ejaculation via connections with the amygdala (Sayag et al., 1994). GABA action in the ventral BST may also exert effects similar to GABA action in the adjacent MPOA.

Which RRF cells are contacted by MPN/ventral BST efferents is unknown, as are which RRF efferents affect male sex behavior, but the net effect of both projections is excitatory for mating. Otherwise, mating would not decrease when they are destroyed (Finn and Yahr, 1994, 2005). The simplest model that could account for this is a monosynaptic, excitatory connection between the projections, but that would require GABA to excite RRF cells. While GABA excites some neurons in adult mammals (Choi et al., 2008), it is usually

inhibitory. Thus, a more likely minimal model is for the ventral BST/MPN input to inhibit RRF interneurons that inhibit RRF projection neurons that promote mating.

The effects of pathway lesions made using 6-hydroxydopamine, a catecholamine-specific neurocytotoxin, instead of a general neurocytotoxin in the RRF suggest that both A8 and non-A8 RRF efferents affect male sex behavior but that non-A8 efferents play a larger role (Finn and Yahr, 1994). When A8 cells were destroyed, male gerbils were less likely to intromit or ejaculate. When all RRF cells were destroyed, few males even mounted (Finn and Yahr, 1994).

Overall, much less is known about the non-A8 than the A8 efferents of the RRF, but the projection to the infralimbic cortex, another area implicated in reward, arises from non-A8 cells in rats (Swanson, 1982). The infralimbic cortex expresses Fos with mating, sends efferents to the VTA that contact mating-activated cells (Balfour et al., 2006) and, perhaps in conjunction with the prelimbic cortex, allows male rats to curtail mating after it has acquired aversive consequences (Davis et al., 2010). Thus, non-A8 efferents to the infralimbic cortex might affect mating by affecting its rewarding properties.

But in addition or instead, the RRF may affect male sex behavior by affecting movement. In cats, the RRF projects to pontine reticular nuclei that affect muscle tone and locomotion (Lai et al., 1993), and many of those efferents appear to be glutamatergic, though glutamatergic cells were not seen in the rat RRF (Nair-Roberts et al., 2008). The RRF also responds to testosterone, and most RRF cells with androgen or estrogen receptors are non-A8 cells (Kritzer, 1997). Thus RRF cells of all types merit further study in regard to male sex behavior, which should also improve our general understanding of the mammalian midbrain.

Acknowledgments

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References

- Balfour ME, Brown JL, Yu L, Coolen LM. Potential contributions of efferents from medial prefrontal cortex to neural activation following sexual behavior in the male rat. *Neuroscience*. 2006; 137:1259–1276. [PubMed: 16388908]
- Balfour ME, Yu L, Coolen LM. Sexual behavior and sex-associated environmental cues activate the mesolimbic system in male rats. *Neuropsychopharmacol*. 2004; 29:718–730.
- Baum MJ, Everitt BJ. Increased expression of c-fos in the medial preoptic area after mating in male rats: Role of afferent inputs from the medial amygdala and midbrain central tegmental field. *Neuroscience*. 1992; 50:627–646. [PubMed: 1436507]
- Choi HJ, Lee CJ, Schroeder A, Kim YS, Jung SH, Kim JS, Kim DY, Son EJ, Han HC, Hong SK, Colwell CS, Kim YI. Excitatory actions of GABA in the suprachiasmatic nucleus. *J Neurosci*. 2008; 28:540–549.
- Commings D, Yahr P. Adult testosterone levels influence the morphology of a sexually dimorphic area in the Mongolian gerbil brain. *J Comp Neurol*. 1984; 224:132–140. [PubMed: 6715577]
- Coolen LM, Peters HJPW, Veening JG. Fos-immunoreactivity in the rat brain following consummatory elements of sexual behavior: A sex comparison. *Brain Res*. 1996; 738:67–82. [PubMed: 8949929]
- Davis JF, Loos M, Di Sebastiano AR, Brown JL, Lehman MN, Coolen LM. Lesions of the medial prefrontal cortex cause maladaptive sexual behavior in male rats. *Biol Psychiatry*. 2010; 67:1199–1204. [PubMed: 20346444]
- De Vries GJ, Gonzales CL, Yahr P. Afferent connections of the sexually dimorphic area of the hypothalamus in male and female gerbils. *J Comp Neurol*. 1988; 271:91–105. [PubMed: 2454962]

- Deutch AY, Goldstein M, Baldino F Jr, Roth RH. Telencephalic projections of the A8 dopamine cell group. *Ann NY Acad Sci.* 1988; 537:27–50. [PubMed: 2462395]
- Erlander MG, Tillakaratne NJK, Feldblum S, Patel N, Tobin AJ. Two genes encode distinct glutamate decarboxylases. *Neuron.* 1991; 7:91–100. [PubMed: 2069816]
- Fernández-Guasti A, Larsson K, Beyer C. GABAergic control of masculine sexual behavior. *Pharm Biochem Behav.* 1986a; 24:1065–1070.
- Fernández-Guasti A, Larsson K, Beyer C. Effect of bicuculline on sexual activity in castrated male rats. *Physiol Behav.* 1986b; 36:235–237.
- Finn PD, De Vries GJ, Yahr P. Efferent projections of the sexually dimorphic area of the gerbil hypothalamus: anterograde identification and retrograde verification in males and females. *J Comp Neurol.* 1993; 338:491–520. [PubMed: 7510729]
- Finn PD, Yahr P. Projection of the sexually dimorphic area of the gerbil hypothalamus to the retrorubral field is essential for male sexual behavior: Role of A8 and other cells. *Behav Neurosci.* 1994; 108:362–378. [PubMed: 8037881]
- Finn PD, Yahr P. Projection from the ventral bed nucleus of the stria terminalis to the retrorubral field in rats and the effects of cells in these areas on mating in male rats versus gerbils. *Horm Behav.* 2005; 47:123–138. [PubMed: 15664016]
- Guevara MA, Martinez-Pelayo M, Arteaga SM, Bonilla-Jaime H, Hernández-González M. Electrophysiological correlates of the mesoaccumbens system during male rat sexual behaviour. *Physiol Behav.* 2008; 95:545–552. [PubMed: 18722395]
- Hasue RH, Shammah-Lagnado SJ. Origin of the dopaminergic innervations of the central extended amygdala and accumbens shell: A combined retrograde tracing and Immunohistochemical study in the rat. *J Comp Neurol.* 2002; 454:15–33. [PubMed: 12410615]
- Heeb MM, Yahr P. *C-fos* immunoreactivity in the sexually dimorphic area of the hypothalamus and related brain regions of male gerbils after exposure to sex-related stimuli or performance of specific sexual behaviors. *Neuroscience.* 1996; 72:1049–1071. [PubMed: 8735229]
- Hernández-González M, Guevara M, Morali G, Cervantes M. Subcortical multiunit activity changes during rat male sexual behavior. *Physiol Behav.* 1997; 61:285–291. [PubMed: 9035260]
- Hull, EM.; Meisel, RL.; Sachs, BD. Male sexual behavior. In: Pfaff, DW.; Arnold, AP.; Etgen, AM.; Fahrbach, SE.; Rubin, RT., editors. *Hormones, Brain and Behavior.* San Diego: Academic Press; 2002. p. 3-137.
- Ju G, Swanson LW. Studies on the cellular architecture of the bed nucleus of the stria terminalis in the rat: I. Cytoarchitecture. *J Comp Neurol.* 1989; 280:587–602. [PubMed: 2708568]
- Kippin TE, Sotiropoulos V, Badii J, Pfaus JG. Opposing roles of the nucleus accumbens and anterior lateral hypothalamic area in the control of sexual behavior in the male rat. *Eur J Neurosci.* 2004; 19:698–704. [PubMed: 14984420]
- Kritzer MF. Selective colocalization of immunoreactivity for intracellular gonadal hormone receptors and tyrosine hydroxylase in the ventral tegmental area, substantia nigra, and retrorubral fields in the rat. *J Comp Neurol.* 1997; 379:247–260. [PubMed: 9050788]
- Lai YY, Clements JR, Siegel JM. Glutamatergic and cholinergic projections to the pontine inhibitory area identified with horseradish peroxidase retrograde transport and immunohistochemistry. *J Comp Neurol.* 1993; 336:321–330. [PubMed: 7505295]
- Liu YC, Sachs BD, Salamone JD. Sexual behavior in male rats after radiofrequency or dopamine-depleting lesions in nucleus accumbens. *Pharmacol Biochem Behav.* 1998; 60:585–592. [PubMed: 9632244]
- Mitchell JB, Stewart J. Effects of castration, steroid replacement, and sexual experience on mesolimbic dopamine and sexual behavior in the male rat. *Brain Res.* 1989; 491:116–127. [PubMed: 2765876]
- Moga MM, Saper CB, Gray TS. Bed nucleus of the stria terminalis: cytoarchitecture, immunocytochemistry, and projection to the parabrachial nucleus in the rat. *J Comp Neurol.* 1989; 283:315–332. [PubMed: 2568370]
- Nair-Roberts RG, Chatelain-Badie SD, Benson E, White-Cooper H, Bolam JP, Ungless MA. Stereological estimates of dopaminergic, GABAergic and glutamatergic neurons in the ventral tegmental area, substantia nigra and retrorubral field in the rat. *Neuroscience.* 2008; 152:1024–1031. [PubMed: 18355970]

- Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. 3. San Diego: Academic Press; 1997.
- Pitchers KK, Balfour ME, Lehmann MN, Richtand NM, Yu L, Coolen LM. Neuroplasticity in the mesolimbic system induced by natural reward and subsequent reward abstinence. *Biol Psychiatry*. 2010a; 67:872–879. [PubMed: 20015481]
- Pitchers KK, Frohmader KS, Vialou V, Mouzon E, Nestler EJ, Lehman MN, Coolen LM. Δ FosB in the nucleus accumbens is critical for reinforcing effects of sexual reward. *Gene Brain Behav*. 2010b; 9:831–840.
- Sayag N, Hoffman NW, Yahr P. Telencephalic connections of the sexually dimorphic area of the gerbil hypothalamus that influence male sexual behavior. *Behav Neurosci*. 1994; 108:743–757. [PubMed: 7986368]
- Simmons DA, Yahr P. Projections of the posterodorsal preoptic nucleus and the lateral part of the posterodorsal medial amygdala in male gerbils, with emphasis on cells activated with ejaculation. *J Comp Neurol*. 2002; 444:74–94.
- Simmons DA, Yahr P. GABA and glutamate in mating-activated cells in the preoptic area and medial amygdala of male gerbils. *J Comp Neurol*. 2003; 459:290–300. [PubMed: 12655511]
- Swanson LW. The projections of the ventral tegmental area and adjacent regions : A combined fluorescent retrograde tracer and immunofluorescence study in the rat. *Brain Res Bull*. 1982; 9:321–353. [PubMed: 6816390]
- Swanson, LW. Brain maps: structure of the rat brain. Amsterdam: Elsevier; 1992.
- Wallace DL, Vialou V, Rios L, Carle-Florence TL, Chakravarty S, Kumar A, Graham DL, Green TA, Kirk A, Iñiguez SD, Perrotti LI, Barrot M, DiLeone RJ, Nestler EJ, Bolaños- Guzmán CA. The influence of deltaFosB in the nucleus accumbens on natural reward-related behavior. *J Neurosci*. 2008; 28:10272–10277. [PubMed: 18842886]
- Waraczynski M, Parsons M. Temporary inactivation of the retrorubral fields decreases the rewarding effect of medial forebrain bundle stimulation. *Brain Res*. 2000; 885:154–165. [PubMed: 11102569]

Figure 1.

Camera lucida drawings of the gerbil MPNm and ventral BST showing locations of cells that were retrogradely labeled by an FG injection in the right RRF. Filled circles indicate FG-IR cells that were also GAD₆₇-IR. Open circles indicate FG-IR cells that either were not GAD₆₇-IR or for which the GAD₆₇-IR status could not be clearly determined. The sections drawn were at 90-μm intervals. LSv = ventral part of the lateral septal nucleus; ac = anterior commissure; 3v = third ventricle; oc = optic chiasm; fx = fornix; SCN = suprachiasmatic nucleus of the hypothalamus.

Figure 2.

Brightfield photomicrographs of coronal sections through the ventral BST and RRF of male gerbils showing colocalization of GAD₆₇ with FG, Fos with FG, and GAD₆₇ with Fos. A) FG injection site in the RRF of a gerbil used to study colocalization of GAD₆₇ and FG. Brown reaction product = GAD₆₇; blue-grey = FG. Note the necrosis at the center of the injection site. Scale bar = 500 μ m and also applies to panel D. B) Distribution of GAD₆₇-IR cells in the ventral BST and surrounding MPOA relative to cells retrogradely labeled from the RRF by the FG injection shown in panel A. Scale bar = 100 μ m and also applies to panels E and G. C) Higher magnification of the ventral BST in panel B showing cells that were retrogradely labeled from the RRF, contained GAD₆₇, or both (arrows). Scale bar = 40 μ m and also applies to panels F, H and I. D) FG injection site in the RRF of a gerbil used to study colocalization of Fos and FG. Brown reaction product = TH; dark blue = FG. Note the A8 cells, visualized by TH ICC, at the injection site and contralaterally, and the necrosis at the center of the injection site. E) Distribution of Fos-IR cells in the ventral BST and surrounding MPOA relative to cells that were retrogradely labeled from the RRF by the FG injection shown in panel D. Brown reaction product = Fos; blue = FG. F) Higher magnification of the ventral BST in panel E showing cells that were retrogradely labeled from the RRF, contained Fos, or both (arrows). G) Distribution of cells in the ventral BST and surrounding MPOA that were Fos-IR after mating (brown), expressed GAD₆₇ mRNA (dark blue) or both. H) Higher magnification of the ventral BST in panel G showing cells that expressed Fos, GAD₆₇ mRNA or both (arrows). I) Cells in the RRF of a male gerbil (same as in panels G and H) that expressed Fos, GAD₆₇ mRNA or both (arrows). Note: Arrows in C, F, H and I point to some of the double-labeled cells.

Table 1

Number of Ventral BST and MPNm Cells^a that were FG-IR after FG Injections in the RRF and Percentage of those Cells that were also GAD₆₇-IR

	FG-IR Cells	% GAD ₆₇ -IR
Ventral BST, ipsi ^b	498 ± 4	95% ± 1%
Ventral BST, contra	85 ± 2	95% ± 2%
MPNm, ipsi	84 ± 12	97% ± 1%
MPNm, contra	44 ± 1	97% ± 1%

^aMean (± SEM) per 0.0025 mm² in 30- m sections. N = 4.

^bIpsi- or contralateral to the FG injection in the RRF.

Table 2

Number of Ventral BST Cells^a that were FG-IR after FG Injections in the RRF or that Expressed GAD₆₇-mRNA (GAD+) Relative to the Number and Percentage that were also Fos-IR after Mating

Marker	N	Cells with Marker	Cells Fos-IR	Cells with both labels	% FG-IR or GAD+ Cells that were Fos-IR	% Fos-IR Cells that were FG-IR or GAD+
FG-IR	5	108 ± 7	41 ± 5	15 ± 2	14% ± 1%	37% ± 2%
GAD+	3	286 ± 20	49 ± 3	43 ± 3	15% ± 1%	89% ± 7%

^aMeans (± SEM) per 0.040 mm² in 30- μ sections, ipsilateral to FG injection.