GONADAL STEROID RECEPTORS COLOCALIZE WITH CENTRAL NERVOUS SYSTEM NEURONS PROJECTING TO THE RAT PROSTATE GLAND

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Running Head: PRV, Gonadal Steroid Receptors & Prostate

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ABSTRACT

Huddleston, G. G., Song, C. K., Paisley, J. C., Bartness, T. J. and Clancy, A. N. Gonadal steroid receptors colocalize with central nervous system neurons projecting to the rat prostate gland *Am J Physiol Regul Integr Comp Physiol*, 000: 000-000, 2006. — Mating-induced Fos-immunoreactive (-ir) cells are colocalized with androgen receptors (AR), estrogen receptors (ER) or both in limbic and hypothalamic areas known to mediate male rat mating behavior. A steroid-responsive neural network might govern copulatory behavior in male laboratory rats that is analogous to the network described in female rats governing the lordosis response. This hypothesized network in males may synchronize and coordinate sexual behavioral responses with physiological responses of the genitals and the internal organs of reproduction. Therefore, the pseudorabies virus (PRV Bartha), a transneuronal, viral retrograde tract tracer, was microinjected into the prostate gland to label this network. After seven days, brains from infected animals were processed for immunohistochemical labeling of AR, ER and PRV. The majority of PRV-ir cells exhibited either AR-immunoreactivity (ir) or ER-ir in the medial preoptic area, median preoptic nucleus, bed nucleus of stria terminalis, hypothalamic paraventricular nucleus, and zona incerta, areas known to play a role in male rat mating behavior. Other structures such as the central tegmental field/subparafascicular nucleus of the thalamus, central nucleus of the amygdala and medial amygdala, also important in the display of male copulatory behavior, were less reliably labeled. Collectively, a steroid receptor-containing neuronal circuit, largely contained in the diencephalon, was revealed that likely is involved in the autonomic control of the prostate gland and the consummatory aspects of male rat mating behavior.
KEYWORDS: pseudorabies virus, autonomic nervous system, tract tracing, androgen receptor, estrogen receptor, sexual behavior
INTRODUCTION

Gonadal hormones act on hypothalamic and limbic sites in the male rodent brain, promoting sexual behavior. Moreover, mating-induced Fos immunoreactivity (-ir) (a marker of neuronal activity [43]), is expressed in neurons that contain gonadal steroid receptors, suggesting that a brain neural network, composed of interconnected, steroid-sensitive neurons, mediates male mating behaviors. The distribution of forebrain neurons that project to the prostate gland [44, 48, 71] overlaps to a considerable extent with that of the distribution of mating-induced Fos-ir, raising the possibility that the same neurons that mediate mating behavior also control the prostate gland. It is not known at present, however, if the forebrain neurons labeled after inoculation of the prostate gland with a transneuronal viral tract tracer, the pseudorabies virus (PRV, Bartha), contain gonadal steroid receptors. Two areas of overlap are the medial preoptic area (MPO) and bed nucleus of stria terminalis (BST) [31, 42]. Anatomical studies have shown that these areas and the medial amygdala (MEA) that, as for the MPO and BST, also mediates male sexual behaviors [28, 34], are reciprocally interconnected [7, 14, 15, 55]. Neurons in each of these brain regions contain either androgen receptors (AR) alone or estrogen receptors (ER) alone, or both receptor types [23, 54]. Male sexual behavior is altered or attenuated when steroid hormone receptor antagonists to AR or ER are administered into the MPO or the MEA (and likely the BST) [39-41, 63]. Male sexual behavior also is reduced by inactivation of the aromatase enzyme that converts testosterone (T) to estradiol (E2) [45], reinforcing the importance of both androgenic and estrogenic action in the brain [5, 12, 28, 29, 49, 52]. Therefore, because immunohistochemical (IHC) studies indicate that mating-induced Fos-ir is colocalized with either ER-ir or AR-ir or both in several brain areas that also contain neurons that project to the prostate gland [23, 44, 48, 71], we hypothesized that a single steroid-sensitive
brain network mediates both male copulatory behavior and controls the prostate gland. The interconnected brain regions that make up this steroid-sensitive network may synchronize and coordinate sexual behavioral responses with physiological responses of the prostate gland, the penis and other reproductive organs associated with insemination.

To test the hypothesis, we injected PRV that labels entire circuits within the same animal through its infection of only functionally connected neurons (i.e., synaptically connected neurons [8, 9, 56]), into the prostate gland, a peripheral structure activated during mating. We chose to infect the prostate gland with PRV because the expulsion of prostatic fluid temporally is synchronized with the ejaculatory behavioral pattern suggesting that activity in a common supraspinal circuit may trigger both the expulsion of prostatic fluid and the behavioral patterns associated with mating behavior. In addition, to our knowledge, this is the only function of the prostate gland, thus ensuring labeling of a circuit that only functions to secrete prostatic fluid with ejaculation [42]. Therefore, we reasoned that PRV would infect the central neural network underlying mating behavior and we additionally predicted that this network would be composed of steroid-sensitive neurons. This was accomplished by processing the brains for PRV-ir and either ER-ir or AR-ir. The anti-AR antibody PG-21 [51] and the anti-ER antibody 1D5 were chosen for this work because PG-21 is commonly used to located AR-ir neurons in the brain and because the 1D5 antibody was raised against ER-α [32]. Knockout studies in mice suggest that ERα is the major ER subtype that mediates male sexual behavior [47, 53]. The lack of a validated and effective ERβ antibody precluded analysis of this important ER subtype.

MATERIALS AND METHODS

Animals
Male (371-605g) Sprague-Dawley rats, at least 70 days old, were obtained from Charles River Laboratories, Raleigh, North Carolina. Animals were housed singly in polycarbonate cages, 22 x 44 x 18 cm, and given free access to food and water in the university vivarium on a 14:10 reversed light:dark cycle (lights off at 0930 EST). All housing and experimental procedures were approved by the Georgia State University Institutional Animal Care and Use Committee and were in accordance with Public Health Service guidelines.

**PRV injection and dissection**

Males were anesthetized with pentobarbital sodium (40 mg/kg i.p., Veterinary Laboratories, Inc., Lenexa, Kansas) and the prostate gland exposed via a small ventral incision directly over the bladder. PRV (14 µl; 3 x 10^8 plaque forming units per µl) was injected into the rat prostate glands using a microsyringe. Specifically, to each animal (N = 20), 14 µl PRV was delivered into the two prostatic lobes as follows: 7 µl PRV was distributed into the first lobe by injecting 1 µl followed by holding the syringe in place for at least one minute, then slightly moving the syringe to inject the next 1 µl at a different site in the lobe. Thereafter, a second prostatic lobe was identically injected PRV as in the first lobe. This injection technique insures that it is highly likely that PRV was confined only to the prostate gland and did not encroach into the abdominal cavity or adjacent sites. Animals were monitored daily for signs of infection or urinary discomfort. After 7 days, animals were given a lethal dose of pentobarbital sodium (100-150 mg/kg) and perfused transcardially with physiological saline followed by a minimum of 300 ml of 4% paraformaldehyde. The craniums were partially opened and immersed in the same perfusion fixative. The next day, brains were removed from skulls and transferred to 30% sucrose in 0.1 M sodium phosphate buffer (PB, pH 7.4) where they remained for three days. Brains were immersed in 0.1 M PB overnight, cut on a coronal plane into 40 µm sections on a
freezing stage sliding microtome and stored in 0.1 M PB. Standard IHC was used (see below) to visualize infected neurons.

IHC

**Dual Labeling for ER-ir and PRV-ir.** Sections were rinsed in 0.1 M PB followed by a 24 h incubation in a blocking solution of 0.1 M PB + 0.4% Triton X (TX) + 5% normal goat serum (NGS) (Jackson Laboratories, Bar Harbor, ME) at room temperature with agitation. Sections were then sequentially incubated with two different primary antibodies: first, with the rabbit polyclonal anti-PRV antibody (RB132, a generous gift of Dr. Lynn Enquist, Princeton University) diluted 1:10,000 in a solvent of 2% NGS and 0.1% sodium azide in 0.1 M PB + 0.4% TX for 24 h at room temperature with agitation, and second with the 1D5 mouse monoclonal anti-ER antibody (Zymed Laboratories, South San Francisco, catalogue # 18-7149: 1 µg/ml) in a mixture of 2% NGS in 0.1 M PB + 0.4% TX for 48 h at room temperature with agitation. After 0.1 M PB washes (3 x 5 min), sections were incubated in a mixture of two secondary antibodies: (i) biotinylated goat anti-rabbit secondary antibody (1:350 dilution, Vector Laboratories, Burlingame, CA catalogue # BA-1000, Lot No. A0708) and (ii) gold-conjugated goat anti-mouse secondary antibody (1:200 dilution, Jackson Laboratories, catalogue # 115-185-100, Lot No. 44811) in a solution of 0.1 M PB + 0.4% TX + 3% NGS for 2 h at room temperature with agitation. For the peroxidase reaction, sections were washed 3 x 5 min in 0.1 M PB, incubated in one-tenth-strength avidin-biotinylated peroxidase complex (Vector Laboratories, Vectastain Elite standard ABC peroxidase kit) in 0.1 M PB for 60 min at room temperature with agitation, washed in 0.1 M PB (3 x 5 min), and then incubated in a mixture of diaminobenzidine (DAB, Sigma, St. Louis, MO: 0.2 mg/ml) and H₂O₂ (Sigma: 0.1 µl/ml) in 0.1 M PB for 5 min at room temperature. For the silver-enhancement reaction, sections were
washed in deionized water (3 x 5 min), incubated in silver-enhancement reagents (BBI Silver Enhancement kit, Ted Pella, Redding, CA prepared according to the manufacturer’s directions) for 20 min at 4°C and then washed again in deionized water (3 x 5 min). Finally, after additional washes in 0.1 M PB (3 x 5 min), sections were mounted onto gel coated-slides, dehydrated in alcohol, cleared with xylene and cover-slipped with DPX mounting medium. Positive control sections from previously verified PRV infected animals were included in each assay.

**Dual Labeling for AR-ir and PRV-ir.** Sections adjacent to those used in the ER + PRV-ir IHC assay were dually processed for AR-ir and PRV-ir similarly, with a few exceptions. At the appropriate steps in the procedure, sections were sequentially incubated with two different primary antibodies: first, with a porcine polyclonal anti-PRV antibody (1:30,000, generous gift of Dr. Lynn Enquist) in a solvent of 5% NGS in 0.1 M PB + 0.4% TX, for 24 h at 4°C, and second with the PG-21 rabbit polyclonal anti-AR antibody (3 µg/ml: Upstate USA, Charlottesville, VA, Cat. No. 06-680, Lot No. 24732) in the same solvent for 48 h at 4°C. They were then washed in 0.1 M PB (3 x 5 min), incubated in a mixture of two secondary antibodies: (i) biotinylated goat anti-swine secondary antibody (1:200: Vector BA-9020, Lot No. K0223) and (ii) gold-conjugated goat anti-rabbit secondary antibody (1:200: BBI LM.GAR5, Batch No. 2697) in a solution of 0.1 M PB + 0.4% TX + 5% NGS for 2 h at room temperature with agitation. Positive control sections from previously verified PRV infected animals also were included here as well.

**Immunohistochemical controls.** Additional sections from each rat were processed in both the ER + PRV-ir and the AR + PRV-ir IHC assays, as above, but without any primary antibodies (zero primary antibody negative controls) resulting in no labeling. Other sections were used as controls to test for cross-reactions between primary and secondary antibodies. The
cross-reaction control sections were processed as above except that they were incubated with only one of the primary antibodies and both secondary antibodies. Specifically, (i) RB132 was incubated without 1D5, (ii) 1D5 was incubated without RB132, (iii) the porcine anti-PRV antibody was incubated without PG-21 and (iv) PG-21 was incubated without the porcine anti-PRV antibody.

Imaging and quantification

For each brain section, digital serial photomicrographs were made at sufficiently high resolution to visualize individual labeled neurons (Fig. 1). These photomicrographs were then melded together via a computer imaging program (Photoshop version 6.0) to make one image. The resulting composite photomicrograph was then magnified and section boundaries and major landmarks were traced onto it, as were the locations of cells staining positive for PRV-ir (with and without AR-ir or ER-ir). These tracings, with plotted cell locations, were mapped onto matching standard rat brain atlas plates by superimposing the tracings over the atlas plates and transferring the positions of the labeled cells to the plates. Cells were then counted visually from these atlas plates using the regional boundaries and nomenclature of Swanson [60].

RESULTS

Immunohistochemical cross-reaction controls

The IHC cross-reaction controls produced single-labeling only, indicating that each primary antibody reacted normally with the appropriate secondary antibody and did not cross-react with the other secondary antibody and that the secondary antibodies did not cross-react with each other. No dual-labeled or inappropriately single-labeled neurons were found in any of these control tissue sets.

PRV infections
PRV successfully infected 11 of 20 rats. Because there was considerable individual variation in the extent of PRV-ir neuronal labeling among the eleven rats that developed infections, the four cases most heavily labeled by PRV-ir, but showing no lysis at any level of the neuroaxis, were mapped and quantified in their entirety (Tables 1 and 2). The remainder of the PRV-ir labeled cases (N = 7 rats) were not quantified because PRV-ir neuronal labeling was sparse (e.g., fewer than 5 PRV-ir neurons in the MPO).

In the diencephalic area of the brain, PRV-ir was localized in several hypothalamic nuclei, notably the anterior hypothalamus (AHA), bed nucleus of stria terminalis (BST), suprachiasmatic nucleus (SCN), hypothalamic paraventricular nucleus (PVH), median preoptic area (MEPO), medial preoptic area (MPO), medial preoptic nucleus (MPN), retrochiasmatic hypothalamus, lateral hypothalamus (LH), dorsomedial hypothalamus (DM), ventromedial hypothalamus (VMH) and posterior hypothalamus (PH). Other areas exhibiting PRV infections were the premammillary nucleus (PM), arcuate nucleus (ARC), ventral tegmental areas, zona incerta (ZI), periaqueductal gray (PAG), central nucleus of the amygdala (CEA) and medial amygdala (MEA).

**Distribution of PRV-ir and ER-ir Labeling.** Examples of ER-ir single-labeled neurons (Fig. 2A), PRV-ir single-labeled neurons (Fig. 2B) and PRV + ER-ir dual-labeled neurons (Fig. 2C) were found in all mapped cases. Injection of PRV into the prostate gland produced selective labeling in the brain (Fig. 3 and Fig. 4). PRV-ir labeling, with and without ER-ir, was consistently present in the MPO (including the MPN), the BST, PVH, ZI and in a continuum of cells in the perifornical area that extended through the LH to the PH. Sparser and/or inconsistent PRV-ir labeling also was noted in the CEA, MEA, VMH and PAG.
**Distribution of PRV-ir and AR-ir Labeling.** AR-ir single-labeled neurons (Fig. 2D), PRV-ir single-labeled neurons (Fig. 2E) and PRV + AR-ir dual-labeled neurons (Fig. 2F) were found in all mapped cases. The distribution of PRV-ir labeling, with or without AR-ir, matched very closely the distribution described above for PRV-ir, with or without ER-ir, even though different anti-PRV primary antibodies were used and is depicted for two cases (Fig. 5 and Fig. 6). To facilitate comparisons between the distribution of PRV + AR-ir labeling and that of PRV + ER-ir, Figs. 3 and 5 are from one animal and Figs. 4 and 6 are from a different animal. PRV-ir labeling, with and without AR-ir, was consistently present in the MPO (including the MPN), BST, PVH, ZI and in a continuum of perifornical cells in the LH to the PH, and less consistently present and/or sparser in the CEA, MEA, VMH and PAG.

**Quantification of Dual Labeling.** For each of the four mapped cases, the total counts of PRV-ir neurons and the percentages of those that also expressed ER-ir are presented (Table 1) as well as the total counts of PRV-ir neurons and the percentages that were AR-ir (Table 2). In the MPO, MPN, BST, and PVH there was a high degree of both ER-ir and AR-ir labeling in PRV-ir neurons.

**DISCUSSION**

Inoculation of the prostate with PRV labeled the central origins of the autonomic outflow circuits that are commonly seen after PRV injection of other peripheral autonomic targets, such as the PVH and VMH [57], but, most importantly, PRV-ir also was expressed in several additional brain areas that previously have been shown to mediate male mating behavior, specifically the MPO, BST, ZI and PAG. Moreover, in these areas and also in the PVH, the majority of PRV-ir cells also expressed either ER-ir or AR-ir. Thus, it appears that the neural circuitry underlying prostatic control includes steroid receptor-containing diencephalic neurons.
located in brain regions that mediate male sexual behavior and that may be involved with the terminal consummatory aspects of mating. This is consistent with the hypothesis that a steroid-responsive neural network governs both male copulatory behavior and the prostate gland. Moreover, the present findings reinforce earlier reports that mating-induced Fos-ir is colocalized with steroid receptors in neurons in these same brain areas [23].

Male mating behavior is thought to be controlled by a supraspinal neuronal network that integrates exogenous and endogenous cues and the brain areas implicated in previous research that are important for male mating correlate well with brain areas labeled by PRV-ir in the present study. In this study, we labeled a diencephalic circuit by inoculating the prostate gland with PRV and many PRV-ir neurons in the MPO, MPN, BST, ZI and PAG expressed steroid receptors. The distribution of labeling of the neurons projecting to the prostate gland in this study agrees well with previous studies where PRV was injected into the prostate gland [48, 71] or prostate gland and urinary bladder [44]. To our knowledge however, and most importantly, this is the first study demonstrating the presence of gonadal steroid receptor-ir in the neurons comprising the circuit from the brain to the prostate gland. Moreover, the PRV-ir circuit exhibits certain features suggesting it may function in reproductive behavior, specifically that it contains steroid receptors in brain areas known to mediate mating and is connected with peripheral structures involved in mating.

It is universally accepted that the MPO plays a central role in the expression of vertebrate male sexual behavior. Lesions of the MPO or of the MPN reduces [11, 26, 35, 59] and stimulation of the MPO facilitates [17, 18, 37, 64] male mating behavior. Lesions of the BST and/or the stria terminalis (st; a fiber tract connecting the amygdala with the hypothalamus) interrupts male mating to a lesser extent than do lesions of the MPO, typically by affecting the
cadence of mating, resulting in increased frequencies of behaviors preceding ejaculation and longer latencies until ejaculation occurs [19, 21, 62, 70]. Lesions of the ZI virtually eliminate male mating behavior [36]. Studies that combined PAG lesions with MPO stimulation suggest that the descending pathway for male sexual behavior passes through the PAG from the MPO [38]. The structures implicated functionally in the control of male sexual behavior also were double-labeled in the present study with PRV- and AR- or ER-ir.

PRV-ir labeling also occurred in some brain areas that traditionally have not been associated with male mating. Heavy PRV-ir occurred in the all neuroanatomical subdivisions of the PVH and in a continuum of perifornical cells through the LH and PH. This is not unexpected because these brain areas are involved in control of numerous autonomic targets and many sexual responses have an autonomic component, among them, cardiovascular responses, blood vessel dilatation and constriction, glandular emissions, secretions from seminal vesicles, contraction of the prostate and vas deferens and other reproductive organs [42]. Interestingly, mating-induced Fos-ir has been reported in the PVH of male rats [4, 27, 33, 67]. Moreover, lesions of the parvocellular PVH are associated with a “decrease in seminal emission at the time of ejaculation” [1] and there are suggestions that the PVH might be involved in the supraspinal control of ejaculation [67]. Moreover, some PVH neurons project to the spinal nucleus of bulbocavernosus [65, 66], a spinal cord area that regulates penile reflexes [6] and several studies have suggested that the descending oxytocinergic neurons of the PVH play a role in male sexual behavior [2, 10, 30]. Many PRV-ir neurons in the PVH showed ER-ir or AR-ir, consistent with a role in regulation of mating behavior and it is noteworthy that the anti-ER antibody used in this study recognizes ERα, which is the ER subtype suggested as being important in male sexual behavior [47, 53]. Although the PVH contains appreciable quantities of ERα [20], ERβ has been
reported to predominate in the PVH [58] but this study does not address the question of whether ERβ colocalizes with PRV-ir.

The MEA and the central tegmental field (also known as the posterior subparafascicular nucleus of the thalamus; CTF/SPFp) showed little or no PRV-ir. This was unexpected because both areas are known to have direct reciprocal connections to the MPO and both express mating-induced Fos-ir colocalized with steroid hormone receptors [23-25]. We expected to see dense PRV-ir in steroid-sensitive neurons of the MEA because the posterodorsal nucleus of the MEA is specifically activated by ejaculation [14] and because previous studies from our laboratory demonstrated that E2 implants to the MEA promote mounting and intromission [28], whereas inhibiting the conversion of E2 from T in the amygdala [29] causes severe deficits in mating. The amygdala, however, also appears to have additional roles to play in the expression of male mating such as processing pheromonal and chemosensory cues vital for mating [46]. These sensory and integrative functions, which are hormone dependent, suggest that the amygdala interfaces with brain areas that mediate the motor aspects of mating and might modulate activity in the MPO. Yet, cells in the amygdala were not reliably PRV-labeled in this study and it is unlikely that, given more time, cells in the amygdala would have become PRV infected because the 7 day survival period employed in this study should have been sufficiently long to permit PRV to progress throughout the forebrain. Instead, it is more likely that the amygdala simply is not a major element in the PRV-ir circuit described here. Although the PRV-ir circuit described here might only control prostatic functions, if it additionally plays a behavioral role, then these findings may have implications for the organization of the neural circuits underlying male mating behavior. That is, conceivably the neural circuits mediating appetitive aspects of mating (e.g., mounting and intromission) or sensory feedback from ejaculation are independent of those
mediating consummatory aspects of mating (e.g., the ejaculatory pattern) where neurons in the MEA are contained in the former circuit(s) and the PRV-ir cells identified here are contained in the latter circuit(s). Such dissociation between sexually relevant neural circuits previously has been reported in female rats in which “different sexual stimuli may be integrated through distinct ER-containing circuits in the rostral MPO and postrodorsal medial amygdala” [22]. In this regard, it is noteworthy that E₂ stimulation of the male rat amygdala promotes mounting and intromission only and does not maintain ejaculatory performance [28]. This is consistent with mating-induced Fos-ir expression in the MEA of male hamsters at sexual satiety, suggesting that this brain area responds to sensory feedback from ejaculation, but is not contributing the motor control of ejaculation [13].

A series of recent studies has defined a spinal ejaculation generating center in the lumbar spinal cord [13, 61], highlighting a set of neurons that are specifically activated by ejaculation. PRV studies have shown that these cells receive input from the prostate and bulbospongious muscle, supporting the idea that a spinal generating center coordinates emission and ejaculation [68, 69]. These lumbar neurons send ascending projections rostrally through the spinal cord and terminate in the CTF/SPFp, that has reciprocal connections to the MPO [24, 25]. The MPO is heavily reciprocally connected to the BST and MEA; thus, these ascending spinal neurons are in a position to transmit ejaculatory information to the brain. Although the lack of PRV-ir labeling in the CTF/SPFp is surprising, it is noteworthy that CTF/SPFp neurons are activated (i.e., express mating-induced Fos-ir) in response to ejaculation [13], which suggests that some genital/somatosensory feedback information is conveyed to the CTF/SPFp by a route that is independent of the motor circuits controlling prostatic functions. Thus, at least some neurons in
the CTF/SPFp, similar to those in the MEA, may be components in a neural circuit that is
independent of the PRV-ir circuit described here.

Mating behavior does not always end in ejaculation. Not all mounting behavior leads to
intromission and not all mounts with intromission lead to ejaculation. Because gonadal steroid
hormones must act in the brain for mounting to be initiated, presumably a steroid-responsive
brain network integrates exogenous sensory cues with the motor output needed to initiate mating
behavior (e.g., mounting and intromission). Activity in this network may then lead to activity in
independent circuits that, in all probability, synchronize both the autonomic motor neurons and
also the skeletal motor neurons necessary for ejaculation. Perhaps primary somatosensory
feedback is constantly being received and integrated in the brain which, in turn, signals
peripheral structures, coordinating mating postures and insemination. Such central-peripheral-
central circuits and their mechanisms of hormonal regulation are not yet fully understood but
Fos-ir labeling patterns and hormone manipulation studies have predicted a circuit for the
initiation of mating made up of steroid-responsive neurons. The PRV-ir labeling patterns
observed in this study provides evidence consistent with this idea. Collectively, we have
identified a steroid receptor-containing, supraspinal neuronal circuit that may contribute to
consummatory motor aspect of mating and that may synchronize autonomic and behavioral
reproductive responses.

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FIGURE CAPTIONS

FIGURE 1
Method for preparing maps of the distribution of pseudorabies virus-immunoreactivity (PRV-ir) in the male laboratory rat brain: High resolution photomicrographs of sections were melded together via computer and tracings were made of the positions of major landmarks and labeled cells. The tracings were superimposed onto standard rat atlas plates and the positions of PRV-ir cells were transferred to the atlas plates.

FIGURE 2
Examples of neuronal labeling: A. estrogen receptor immunoreactive (ER-ir) single-labeled neurons (blue arrows), B. pseudorabies virus-immunoreactive (PRV-ir) single-labeled neuron (red arrow), C. PRV + ER-ir dual-labeled neuron (green arrow), D. androgen receptor immunoreactive (AR-ir) single-labeled neuron, E. PRV-ir single-labeled neuron and F. PRV + AR-ir dual-labeled neuron. Scale bar: 10 µm.

FIGURE 3
Distribution of pseudorabies virus-immunoreactivity (PRV-ir) and estrogen receptor immunoreactivity (ER-ir) in one case: Open circles represent PRV-ir single-labeled neurons and filled circles represent PRV + ER-ir dual-labeled neurons.

FIGURE 4
Distribution of pseudorabies virus-immunoreactivity (PRV-ir) and estrogen receptor immunoreactivity (ER-ir) in a second case: Open circles represent PRV-ir single-labeled neurons and filled circles represent PRV + ER-ir dual-labeled neurons.
FIGURE 5
Distribution of pseudorabies virus-immunoreactivity (PRV-ir) and androgen receptor immunoreactivity (AR-ir) in one case: Open circles represent PRV-ir single-labeled neurons and filled circles represent PRV + AR-ir dual-labeled neurons.

FIGURE 6
Distribution of pseudorabies virus-immunoreactivity (PRV-ir) and androgen receptor immunoreactivity (AR-ir) in a second case: Open circles represent PRV-ir single-labeled neurons and filled circles represent PRV + AR-ir dual-labeled neurons.
FIG 3B
FIG 4B
FIG 5B
## TABLE 1
PRV-ir and ER-ir

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<th>SUMMARY (Mean ± SEM)</th>
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<td></td>
<td>Total cell #</td>
<td>% ER-ir</td>
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<td>BRAIN AREA:</td>
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<td>MPO @ -0.46</td>
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<tr>
<td>ZI @ -1.78</td>
<td>0.3 ± 0.3</td>
<td>0.0 ± 0.0%</td>
</tr>
<tr>
<td>ZI @ -2.45</td>
<td>11.7 ± 3.2</td>
<td>49.7 ± 18.8%</td>
</tr>
<tr>
<td>VMH @ -2.45</td>
<td>4.3 ± 0.9</td>
<td>58.3 ± 30.0%</td>
</tr>
<tr>
<td>PAG @ -4.45</td>
<td>1.5 ± 1.5</td>
<td>25.0 ± 25.0%</td>
</tr>
<tr>
<td>PAG @ -4.60</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>PAG @ -5.65</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CASE #:</td>
<td>SUMMARY (Mean ± SEM)</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>----------------------</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Total cell #</td>
<td>% AR-ir</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRAIN AREA:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPO @ -0.46</td>
<td>12.7 ± 4.1</td>
<td>44.7 ± 6.8%</td>
</tr>
<tr>
<td>MPO @ -0.51</td>
<td>17.3 ± 6.2</td>
<td>42.7 ± 4.8%</td>
</tr>
<tr>
<td>MPN @ -0.46</td>
<td>8.0 ± 2.1</td>
<td>43.7 ± 14.8%</td>
</tr>
<tr>
<td>MPN @ -0.51</td>
<td>4.0 ± 2.1</td>
<td>30.3 ± 21.1%</td>
</tr>
<tr>
<td>BST @ -0.46</td>
<td>14.7 ± 7.8</td>
<td>64.7 ± 17.7%</td>
</tr>
<tr>
<td>BST @ -0.51</td>
<td>26.0 ± 10.1</td>
<td>78.0 ± 6.5%</td>
</tr>
<tr>
<td>BST @ -1.08</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0%</td>
</tr>
<tr>
<td>PVH @ -1.08</td>
<td>4.5 ± 1.5</td>
<td>83.5 ± 16.5%</td>
</tr>
<tr>
<td>PVH @ -1.78</td>
<td>30.7 ± 6.4</td>
<td>42.0 ± 7.1%</td>
</tr>
<tr>
<td>CEA @ -1.78</td>
<td>6.7 ± 5.7</td>
<td>31.3 ± 15.8%</td>
</tr>
<tr>
<td>CEA @ -2.45</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0%</td>
</tr>
<tr>
<td>MEA @ -2.45</td>
<td>4.0 ± 4.0</td>
<td>31.5 ± 31.5%</td>
</tr>
<tr>
<td>ZI @ -1.78</td>
<td>1.0 ± 0.6</td>
<td>50.0 ± 28.9%</td>
</tr>
<tr>
<td>ZI @ -2.45</td>
<td>15.5 ± 11.5</td>
<td>62.0 ± 12.0%</td>
</tr>
<tr>
<td>VMH @ -2.45</td>
<td>2.5 ± 1.5</td>
<td>87.5 ± 12.5%</td>
</tr>
<tr>
<td>PAG @ -4.45</td>
<td>8.5 ± 8.5</td>
<td>41.0 ± 41.0%</td>
</tr>
<tr>
<td>PAG @ -4.60</td>
<td>4.5 ± 1.5</td>
<td>83.5 ± 16.5%</td>
</tr>
<tr>
<td>PAG @ -5.65</td>
<td>5.0 ± 2.8</td>
<td>31.8 ± 14.3%</td>
</tr>
</tbody>
</table>