Research report

A dopamine antagonist blocks vaginocervical stimulation-induced neuronal responses in the rat forebrain

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Abstract

During mating in rats, the male provides vaginocervical stimulation (VCS) to the female via intromissions. VCS, provided manually, mimics many aspects of mating, including facilitation of lordosis, induction of sexual receptivity, abbreviation of the period of sexual receptivity, and induction of twice-daily prolactin surges, which result in pseudopregnancy. VCS also induces the expression of Fos, the protein product of the immediate early gene c-fos, which has been used as a marker for neurons that are responsive to mating stimuli. Because VCS induces the release of dopamine in the forebrain, as well as phosphorylation of DARPP-32, a phosphoprotein associated with activation of the D₁ subtype of dopamine receptor, we tested the hypothesis that VCS induces Fos expression by acting on the D₁ class of dopamine receptors. Injection of SCH 23390, an antagonist of the D₁ class of dopamine receptors, virtually eliminated VCS-induced Fos expression without affecting constitutive levels of Fos-Immunoreactivity (Fos-IR) in all brain areas in which VCS induced Fos expression. In a follow-up experiment, expression of a second immediate early protein, egr-1, was blocked as well, suggesting that these results are not specific to Fos. Therefore, the results are consistent with the idea that VCS induces dopamine release, causing activation of D₁ dopamine receptors, which in turn, results in neuronal response, as seen by both Fos and egr-1 expression.

1. Introduction

In female rats, sexual behaviors and reproductive physiology are regulated by ovarian steroids via their effects on the central nervous system [8,39]. The expression of sexual behaviors during the estrous cycle is dependent on the sequential release of estradiol followed by progesterone and is linked to the time of ovulation [8,9,45]. Removal of endogenous sources of estradiol and progesterone via ovariecotmy eliminates the expression of sexual receptivity in female rats [9]. Although rats respond to treatment with estradiol alone [14], after ovariecotmy or combined ovariecotmy/adrenalecotmy, the sequential administration of estradiol and progesterone allows for the use of lower doses of estradiol [53], and it restores the full complement of sexual behaviors.

The genital stimulation that accompanies penile intromissions from the male rat or provided manually by an experimenter (vaginocervical stimulation: VCS) affects the reproductive behavior and physiology of rats as well as other species. For instance, VCS alters pain perception [13,26], and induces LH release [38] and the twice daily surges of prolactin that then result in pseudopregnancy [21,48]. Behaviorally, in the short term, VCS provided by the experimenter or a male rat induces lordosis [47], and it prolongs lordosis responses [15]. In the longer term, it decreases lordosis response, increases rejection [22,43], and it causes abbreviation of the period of sexual receptivity [4,6,23,28,46], especially when mating stimulation is paced by the female [16], and it results in longer return latencies in a pacing situation [12].

In studies of the neural substrate underlying these
responses to VCS, immunostaining for the protein products of immediate early genes (e.g., c-fos and egr-1) has been used to identify responsive neurons. VCS, provided either by a probe or by intromissions from a male rat increases expression of immediate early proteins in a variety of neuroanatomical areas, including the medial preoptic area (mPOA), bed nucleus of stria terminalis (BNST), post-erodorsal portion of the medial amygdala (MePd), ventromedial hypothalamic area (VMH), midbrain central gray and other areas related to reproduction [17,41,44,49].

It is likely that dopamine mediates at least some of the effects of VCS on the forebrain. Dopamine is released in the forebrain of female hamsters [25] and rats [34,40,50] as a result of sexual behavior, in some cases, linked specifically to intromissions. Infusion of dopamine or a dopamine agonist into the mPOA and arcuate-ventromedial area of ovariectomized, estradiol-primed rats, facilitates lordosis [18]. Although VCS is not necessary for female rodents to display lordosis in response to appropriate stimulation, the levels of dopamine in the nucleus accumbens of hamsters increase only when intromission is allowed [25]. This suggests that dopamine release is sensitive to the vaginal and cervical stimulatory components of stimulation from the male.

Although at least five subtypes of the dopamine receptor are expressed in the brain [11,32], much of the focus in the work on hormone–neurotransmitter interactions in sex behavior is on the closely related D1 and D3 dopamine receptors. Administration of D1 agonists in estradiol-primed rats [1,30] induces sexual receptivity in response to mounts by male rats with levels of lordosis comparable to rats receiving estradiol and progesterone. Intracerebral administration of a D1 antagonist or oligonucleotides antisense to D1 mRNA blocks behaviors induced by D1 agonists [30], which suggests that the effects of the drugs are specific to the D1 and D3 subtypes of receptors.

Infusion of the D1 subtype dopamine agonist, SKF 38393, increases Fos expression in many brain areas, including the VMHVL, paraventricular nucleus of the hypothalamus (PVN), caudate–putamen, mPOA, and arcuate nucleus [35]. VCS induces phosphorylation of DARPP-32 (dopamine- and cAMP-regulated phosphoprotein), a protein that is phosphorylated in part after the activation of the D1 subtype dopamine receptors, suggestive of the involvement of the D1 subtype dopamine receptor in the mediation of the effects of VCS [36].

In sum, mating simulation, including VCS, causes dopamine release, and an increase in expression of immediate early proteins and phosphorylation of DARPP-32 in some neurons. This suggests the hypothesis that VCS induces immediate early protein expression via the activation of D1 dopamine receptors. To test this idea, we attempted to block VCS-induced expression of two immediate early proteins by administering the D1 dopamine antagonist, SCH 23390, prior to VCS.

2. Materials and methods

2.1. Animals

Female Sprague–Dawley rats weighing 125–150 g were obtained from Charles River Breeding Laboratories (Wilmington, MA 01888-1000, USA). Upon arrival, animals were housed four rats per cage in stainless steel cages on a 14:10 light:dark cycle (lights on at 20.00 h and off at 10.00 h). Temperature was maintained at approximately 21°C with food and water available ad libitum.

2.2. Surgery

Seven to 10 days after arrival, rats were ovariecotomized via a ventral route after i.p. injections of a cocktail of xylazine (5 mg/kg), ketamine (26 mg/kg), and acepromazine (0.9 mg/kg). After surgery, rats were allowed to recuperate for 12 days. They were then handled on four separate occasions for at least 3 min each during this time to ensure minimal Fos-IR induction related to stress at the time of experimentation.

2.3. Experiments

2.3.1. Experiment 1: SCH 22390 decreases VCS-induced Fos-IR in localized regions of the female rat forebrain

Twelve days following surgery, rats were injected s.c. with 2 µg estradiol benzoate (EB) dissolved in 0.1 ml sesame oil. The animals were divided into three groups (n=4–6): (1) VCS− and saline vehicle; (2) VCS+ and saline vehicle; and (3) VCS+ and the dopamine D1 subtype receptor antagonist, SCH 23390 (Sigma Chemical, St. Louis, MO, USA). Forty-eight hours after EB administration, rats received i.p. injections of either SCH 23390 (1 mg/kg) or saline solution at physiological pH. After 15 min the animals received VCS (VCS+), which was administered for 10 min at 1 min intervals for 2 s with an optimal pressure of 150 g with a 1 ml plastic syringe plunger attached to a force gauge (FDN5, Wagner Instruments, Greenwich, CT, USA). Other animals received control stimulation to the perineum (VCS−). All treatments were performed in the animal rooms under red light. One hour after receiving VCS or control stimulation, the animals were perfused.

2.3.2. Experiment 2: Constitutive levels of Fos-IR are not reduced by SCH 23390

As in Experiment 1, rats were injected with 2 µg EB dissolved in sesame oil, 48 h prior to experimentation. The animals were divided into the following four groups (n=4–6 per group):

1. VCS− and vehicle
Fifteen minutes after rats were injected with either vehicle or SCH 23390, VCS was applied at 30 s intervals (rather than 1 min as in Experiment 1) for 2 s each, with a pressure of 150 g. One hour later, animals were perfused. In Experiment 2a, sets of sections from Groups 1, 2 and 3 were immunostained for Fos-IR, and they were analyzed to determine if the lower dose of the D₁ antagonist blocked VCS-induced Fos expression.

In Experiment 2b, additional sets of sections from Groups 1, 2 and 4 were immunostained with a higher concentration of Fos antiserum in order to increase immunostaining for Fos in the absence of VCS. The sections were analyzed to determine if the antagonist decreases constitutive Fos expression.

2.3.3. Experiment 3: VCS-induced egr-1 expression and inhibition by a D₁ antagonist

Additional sets of brain sections from Groups 1, 2 and 3 from Experiment 2 were immunostained for a second immediate early protein (egr-1). If neurons in the eVMHVL showed response when another immediate early protein was used, then it would be possible to determine if the dopamine antagonist blocks the expression of the second protein as well.

2.4. Perfusion

All animals were anesthetized with an i.p. injection of a lethal dose of sodium pentobarbital (89 mg/kg) and chloral hydrate (425 mg/kg). The brain was then first flushed with 25 ml phosphate-buffered saline at pH 7.2, and then fixed with 2% acrolein (in 0.1 M sodium phosphate buffer at pH of 7.2) as previously described [3]. The flow-rate for fixation was maintained at 25 ml/min for 14 min at 100 mmHg of pressure. The brain was then removed, blocked and stored for 48 h in 0.1 M sodium phosphate buffer at a pH of 7.2 containing 20% sucrose at 4°C. The brain was cut into 40 μm sections from the rostral preoptic area to the mammillary bodies on a freezing rotary microtome. The sections were stored in cryoprotectant at −20°C until they were immunostained.

2.5. Immunocytochemistry

A series of one in every four sections from each animal was selected. The sections were removed from cryoprotectant and rinsed three times with 0.05 M Tris-buffered saline (TBS with pH 7.6) at 5 min/rinse. To ensure removal of residual aldehydes, tissue was pretreated with 1% sodium borohydride for 10 min. Following the pretreatment the tissue was rinsed another three times in 0.05 M TBS at 5 min/rinse. Sections were then incubated with 1% H₂O₂, 20% normal goat serum, 1% bovine serum albumin in TBS for at least 20 min to reduce nonspecific staining and endogenous peroxidase activity. Sections were then incubated with either the polyclonal primary antiserum to Fos (Ab-5, a rabbit antiserum, Cal Biochem, San Diego, CA, USA, diluted to a concentration of 1:250,000 or 1:60,000) or to egr-1 (SC-110, a rabbit antiserum, Santa Cruz Biotechnology, Santa Cruz, CA, USA, diluted to a concentration of 1:1000), 1% normal goat serum, 0.5% Triton X-100 in 0.05 M TBS with NaN₃, 1% gelatin and Triton X-100 for 72 h at 4°C. Subsequent to application of primary antibody, sections were rinsed in a modified Tris buffer made of 0.1% gelatin, 0.02% sodium azide, 0.5% Triton X-100 and 1% normal goat serum, three times at 5 min/rinse. Sections were then incubated in a secondary biotinylated goat-anti-rabbit antiserum at 3 mg/ml (Vector Laboratories, Inc., lot # 20167, Burlingame, CA, USA), 1.5% normal goat serum in modified Tris-buffered saline buffer for 90 min. Tissue was then rinsed twice with the modified TBS buffer and once with standard 0.05 M TBS prior to incubation in the avidin-biotinylated complex solution, Vectastain ABC Peroxidase Elite Series Kit (Vector Labs), at a concentration of 1:100 in 0.05 M TBS for 90 min. Tissue was again rinsed three times in TBS buffer prior to application of 0.05% diaminobenzidine, 0.05 M TBS and 3% H₂O₂. Sections were treated with diaminobenzidine for 3 min, after which they were rinsed three times.

2.6. Computer-assisted image analysis

Sections were mounted onto subbed glass slides and coverslipped with DePex mounting medium (BDH Laboratory Supplies, Poole, Dorset, UK). One carefully matched section per area per rat was analyzed using computer-assisted imaging software, public domain NIH image (developed by the National Institute of Health, available at http://rsb.info.nih.gov/nih-image/). In order to capture pictures, an MTI CCD 72 camera (DAGE MTI Inc., Michigan City, IN, USA) was mounted onto a Leitz Dialux microscope (Ernst Leitz, Wetzlar, Germany). Prior to computer-imaging the microscope was adjusted for Köhler illumination at ×100 magnification. The microscope and camera were focused onto a black dot present on a coverslipped, subbed slide. The camera gain and black level were adjusted so that the gain level was between 2 and 10, and the black level between 250 and 254. This calibrated the camera, so that as wide a range as possible of gray levels was used. Once the camera had been calibrated, all sections for a particular area were captured.
during the same session. Cells were defined as an area between 10 and 200 pixels at a magnification of ×100.

2.7. Neuroanatomical areas analyzed

Neuroanatomical areas to be analyzed were chosen based on previous studies indicating areas rich in VCS-induced Fos expression [2,49]. The areas examined were the rostral ventrolateral aspect of the ventromedial hypothalamic area (rVMHVL), mid ventrolateral aspect of the ventromedial hypothalamic area (mVMHVL), caudal ventrolateral aspect of the ventromedial hypothalamic area (cVMHVL), medial preoptic area (mPOA), rostral preoptic area (rPOA), bed nucleus of the stria terminalis (BNST) and the posterodorsal medial amygdala (MePd) (Fig. 1).

2.8. Statistical analysis

The cell counts were tested for statistical significance using SigmaStat version 2.0 (copyright 1992–1997 Jandel Corporation, Cort Madera, CA, USA). One-way ANOVAs were performed followed by Student’s Newman–Keul’s test for pairwise contrasts. Effects were considered statistically significant if \( P < 0.05 \).

3. Results

3.1. Experiment 1

Consistent with previous studies, VCS induced Fos-IR in

Fig. 1. Drawings indicating (boxes) the neuroanatomical areas in which Fos-IR was quantified in Experiments 1 and 2: rPOA, rostral preoptic area; mPOA, medial preoptic area; BNST, bed nucleus of the stria terminalis; rVMHVL, rostral ventrolateral aspect of the ventromedial hypothalamus; mVMHVL, mid ventrolateral aspect of the ventromedial hypothalamus; cVMHVL, caudal ventrolateral aspect of the ventromedial hypothalamus; MePd, posterodorsal medial amygdala; ac, anterior commissure; f, fornix; op, optic tract; ic, internal capsule; 3V, third ventricle. Drawings are adapted from Brain Maps: Structure of the Rat Brain, 2nd edn., L.W. Swanson, Elsevier, Amsterdam, 1998.
localized regions of the female rat brain associated with reproduction. These areas included the rPOA, mPOA, and the MePd. No significant induction of Fos-IR by VCS was observed in other areas analyzed (i.e. the mVMHVL, eVMHVL, rVMHVL and the BNST). Administration of the dopamine antagonist, SCH 23390 (1.0 mg/kg), resulted in a significant decrease in VCS-induced Fos-IR in all areas in which VCS induced Fos-IR (Figs. 2 and 3).

3.2. Experiment 2a

In this experiment, a pattern of more frequent stimulation (twice per minute) was used in order to determine if this would result in Fos expression in additional neuroanatomical areas that were unresponsive in Experiment 1, and an attempt was made to block this with the dopamine antagonist. ANOVA revealed a significant treatment effect in all areas. Consistent with the results from Experiment 1, VCS induced a statistically significant increase in Fos expression in all areas studied, with the exception of the rVMHVL (in which there was a strong trend). Administration of the lower dose of the dopamine antagonist, SCH 23390 (0.5 mg/kg, i.p.), also decreased VCS-induced Fos-IR in each of these areas (Fig. 4).

3.3. Experiment 2b

Although in Experiments 1 and 2a, administration of the dopamine antagonist suppressed VCS-induced Fos expression, it is possible that the antagonist blocked constitutive Fos expression, not specifically Fos expression that is induced by VCS. In order to test this idea, the antibody was titrated to a higher concentration, which resulted in a greater number of Fos-IR cells in animals that did not receive VCS (constitutive Fos expression). VCS induced a statistically significant increase in Fos expression in all areas studied with the exception of the cVMHVL, in which there was a strong trend. SCH 23930 was without effect on constitutive levels of Fos-IR in any of these areas (i.e., Fos expression in the absence of VCS; Fig. 5).

Fig. 2. Photomicrograph depicting Fos-IR in the posterodorsal medial amygdala (A) after VCS− (B) or VCS+ preceded by an injection of either saline vehicle (C) or SCH 23390 (D).
Fig. 3. Numbers of Fos-IR cells (mean±S.E.M.) in forebrain areas after VCS− or VCS+ preceded by an injection of either saline vehicle or SCH 23390. Asterisks above bars indicate statistically significant difference between VCS+ and other groups.

Fig. 4. Numbers of Fos-IR cells (mean±S.E.M.) in forebrain areas after VCS− or VCS+ preceded by an injection of either saline or SCH 23390. Asterisks above bars indicate statistically significant difference between VCS+ and other groups.
3.4. Experiment 3

VCS induced expression of egr-1 in all areas studied in this experiment (except for the mVMHVL, in which there was a strong trend. Like the experiments on Fos-IR, SCH 23390 completely blocked VCS-induced egr-1 expression in each of these areas (Fig. 6). However, in the POA, the antagonist did not reduce egr-1 expression to the level of controls.

4. Discussion

In all experiments, administration of the D₁ dopamine receptor antagonist, SCH 23390, significantly decreased VCS-induced immediate early protein expression in all areas in which VCS induced its expression. These results suggest that the stimulation of the D₁ subtype dopamine receptor is a necessary component for the induction of both Fos and egr-1 expression subsequent to VCS in these areas.

In contrast to the higher doses of SCH 23390 used in pilot experiments, which resulted in some immobility, the doses used in this study were without obvious effects on motor function. Because individual drugs do not always discriminate between similar subtypes of receptors, and we cannot exclude the possibility of blockade of the closely related D₂ (D₁₉) receptors, we refer here to blockade of the D₁ class of receptors.

The purpose of Experiment 2b was to ascertain whether SCH 23390 selectively reduced VCS-induced Fos-IR or non-specifically reduced constitutive levels of Fos-IR (Fos expression observed in the absence of VCS). In order to test the possibility that the antagonist reduced levels of Fos expression below constitutive levels, a higher concentration of antibody was used, so that the procedure was less selective for VCS-induced Fos expression. The D₁ antagonist did not significantly reduce levels of Fos-IR in the VCS animals in any area. This suggests that the D₁ subtype dopamine receptor antagonist selectively reduced VCS-induced Fos, rather than non-specifically reducing basal levels of Fos expression.

Finally, in Experiment 3 the antagonist blocked the expression of another immediate early protein, egr-1, which was induced by VCS in all areas studied. This suggests that VCS induction of immediate early proteins is mediated, at least in part, by D₁ dopamine receptors.

In the course of doing these experiments, which were designed to assess the necessity of dopamine receptors in the effects of VCS on neuronal response, a number of interesting observations about VCS induction of immediate early proteins in different brain areas were made. In the first experiment, we found that VCS induced Fos-IR in the rPOA, mPOA and MePd. Although this is consistent with previous work, in contrast to previous reports, we did not observe VCS-induced Fos expression in the VMHVL or the BNST. The lack of induction of Fos expression in those areas was likely due to insufficient stimulation; VCS was given for 2 s every minute. Other work supports the idea that the number of stimulations is important for the induction of immediate early protein-IR [42,44]. For
example, Fos-IR is induced in the MePd in a graded manner after five to 15 intromissions [44].

In Experiment 2, in which we increased the rate of stimulation to twice per minute (which also increased the number of stimulations), VCS induced Fos-IR in the rPOA, mPOA and MePd, as was shown previously, but also in the BNST, mVMHVL and cVMHVL. This induction of Fos-IR is consistent with previous studies (e.g., [3,49]). In Experiment 2b, in which the sensitivity of the immunocytochemical procedure was increased, Fos expression was seen in the rVMHVL as well (but only a trend was observed in the cVMHVL, because of higher variance in one group).

Finally, brain sections were immunostained for another immediate early protein that has been used to study mating-related changes in neuronal response, egr-1 [44]. In this case, immediate early protein response was seen in both levels of the POA and the rVMHVL and cVMHVL, suggesting that VCS does in fact induce neuronal response in each of these areas, and the failure to detect VCS-induced Fos in some areas in previous experiments was likely due to sensitivity of the particular procedure and perhaps excessive variance in some cases. Although the antagonist completely blocked VCS induction of this second protein in the VMHVL, inhibition in the POA was not as extensive as with Fos.

A variety of techniques has been used to determine the distribution of particular dopamine receptor subtypes in the brain. Dopamine D₁ and/or D₃ receptor immunoreactivity, binding and/or mRNA [10,19,24,27,52,54] have been localized within the amygdala, preoptic area, and ventromedial hypothalamus and other limbic areas. Using a sensitive in situ hybridization technique, we have observed D₁ and/or D₃ receptor mRNA in each of the areas [7] studied in the present experiment. However, the levels of D₁ and/or D₃ receptor mRNA in these areas are generally considerably lower than other well-characterized target areas, such as the caudate–putamen and nucleus accumbens.

Another less direct indicator of the neuroanatomical location of relevant D₁ dopamine receptors is phosphorylation of DARPP-32. DARPP-32, a phosphatase-1 inhibitor [20], is phosphorylated after the activation of the D₁ subtype dopamine receptors, and it is suggestive of activation of D₁ receptors, although other factors can also lead to its phosphorylation [20]. VCS increases phosphorylation of DARPP-32-IR in the mPOA, cVMHVL, medial amygdala, and BNST [36]. This is consistent with the idea that the D₁ and/or D₃ subtype of dopamine receptor is present and is likely to be activated in those areas as a consequence of VCS.

A variety of studies have demonstrated that dopamine is released into forebrain sites as a consequence of various sexual stimuli. Dopamine is released into the ventral striatum of hamsters as a result of sexual behavior [34], and in the nucleus accumbens and BNST specifically as a result of intromissions in hamsters [25]. Likewise dopamine is released into the MPOA [33] and nucleus accumbens [40] of female rats as a result of perineal or vaginal

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**Fig. 6.** Numbers of Egr-1-IR cells (mean±S.E.M.) in the POA and VMH after VCS− or VCS+ preceded by an injection of either saline vehicle or SCH 23390. Different letters above bars indicate statistically significant differences between groups.
stimulation, and there is some evidence that if female rats are allowed to pace their copulatory interactions, dopamine release in the nucleus accumbens and striatum is greater than if they are not allowed to pace [37]. Dopamine, as well as norepinephrine, is released into the ventromedial hypothalamic area in female rats during mating [51].

It will be essential to determine the site of origin of the dopaminergic neurons, the stimulation of which results in immediate early protein expression in the areas studied here. The mesolimbic dopaminergic neurons of the substantia nigra (A9) and ventral tegmentum (A10) project to many of these areas [5]. Furthermore, as discussed earlier, intromissions in hamsters result in dopamine release in the nucleus accumbens and BNST; the source of this dopamine is believed to be cell groups A9 and A10 [25]. While not directly studied, it is likely that the source of dopamine responsible for the VCS-induced immediate early protein expression in the present studies originates in these cell groups as well.

Although the use of a dopamine antagonist tells us that dopaminergic neurotransmission mediates the effects of VCS in some neurons, systemic injection does not inform us of the specific sites of the relevant dopamine receptors involved in this process. It was beyond the scope of the present experiments, which were designed to determine only if D₁ dopamine receptors are involved, but it will be essential to determine the site(s) of the relevant receptors. It is also necessary to determine if the stimulation of each area is direct, by dopamine in parallel dopaminergic projections from the midbrain to forebrain, if dopamine stimulation of D₁ receptors is just one step in a serial pathway, or some combination of the two.

Finally, these results have implications for work on ligand-independent activation of progestin receptors. Progesterone facilitates sexual behavior via a process that requires progestin receptors, and D₁ agonists can substitute for progesterone in the facilitation of lordosis by a process that requires functional PRs [1,29–31]. Furthermore, mating stimulation, including VCS, facilitates sexual behavior [4] and induces Fos expression in some neuroanatomical areas by a process that requires functional progestin receptors [3]. The fact that VCS requires D₁ dopamine receptor activation to influence neuronal responses suggests that mating stimulation induces ligand-independent activation of sexual behavior (and Fos expression) via a process that includes activation of D₁ dopamine receptors.

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References


