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Progesterone-independent activation of rat brain progestin receptors by reproductive stimuli

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ABSTRACT

Activation of steroid hormone receptors by steroid hormones alters both the physiology and behavior of animals. Steroid hormone receptors (*e.g.*, progestin receptors) can also be activated in the absence of steroid hormones by pharmacological treatment with neurotransmitters or neuropeptides. However, it is not known if progesterone-*in*dependent activation of brain progestin receptors occurs under natural, physiological, conditions. We report that increases in reproductive behavior and brain immediate early gene expression in female rats induced by mating stimuli can be blocked by prior treatment with progesterone antagonists in the absence of circulating progesterone. This suggests that progestin receptors are activated in a progesterone-independent manner by a physiologically relevant stimulus in female rats, thus implicating a novel pathway by which mating stimuli and other environmental influences could activate steroid receptors to influence neuronal response and behavior.

Estrous behavior in female rats is induced by the sequential actions of estradiol and progesterone (1,2). Estrous behavior is abolished by ovariectomy and is reinstated by the administration of exogenous estradiol and progesterone (2). Estradiol treatment alone can induce some aspects of estrous behavior; however, the effects of estradiol on sexual behavior are strongly facilitated by subsequent treatment with progesterone (3, 4). Many of these actions of steroid hormones on behavior and neuronal function are believed to be mediated through intracellular neuronal steroid receptors (5). Ligand binding to receptors results in a conformational change that allows the activated steroid-receptor complex to bind to a hormone response element located on DNA (6). Once bound to DNA, the steroid-receptor complex influences many aspects of neuronal function (7-12). Especially important within the context of sexual behavior is the fact that estradiol induces the synthesis of progestin receptors (13) in some estrogen receptor-containing neurons (14). Treatment with drugs that block the binding of estradiol or progesterone to their receptors prevents these hormones from facilitating sexual behavior (5), providing strong evidence that some of the effects of estradiol and progesterone on behavior are mediated by steroid receptors.

Recent studies suggest that estrogen (15) and progestin receptors (16,17) may also be activated *in vitro* in the absence of progesterone. Indeed, progestin receptors are activated *in vitro* by treatment with particular neurotransmitters (17) or neuropeptides (18). More importantly, intracerebroventricular infusion of dopaminergic agonists increases sexual receptivity in estradiol-primed rats, and treatment with progesterone antagonists or antisense oligonucleotides directed at the progestin receptor mRNA blocks this facilitation (19). Similarly, infusion of gonadotropin-releasing hormone (GnRH) into the brain of estrone-primed rats increases sexual behavior (20), and this increase is blocked by a progesterone antagonist (21). These studies suggest the tantalizing idea that progesterone-*in*dependent activation of progestin receptors occurs *in vivo* after treatment with particular neurotransmitters or neuropeptides. Furthermore, this activation may influence sexual behavior and/or other physiological processes.

In order to test the hypothesis that progesterone-independent activation of progestin receptors occurs under physiological conditions, we relied on a second mode of facilitation of sexual behavior, that is, sexual behavior induced by social interaction. Repeated testing or exposure of estradiol-primed female rats to males increases sexual receptivity (22). Similarly, the vaginal-cervical stimulation (VCS) received by the female, either from a male or a mechanical probe plus flank-perineum stimulation, increases lordosis in estradiol-primed female rats (23). As both dopamine (24,25) and GnRH (26) are released during mating stimulation, and there is evidence that each activates progestin receptors (19,21), we hypothesized that mating stimuli increase lordosis *via* progesterone-independent activation of progestin receptors. Thus, increases in lordosis observed following repetitive mating or VCS in estradiol-primed female rats should be blocked by a progesterone antagonist.

A second, independent way to determine if mating stimuli activate brain progestin receptors is to examine neuronal response following mating stimuli. Using immunocytochemistry for the detection of immediate early gene proteins, such as Fos, it is possible to identify neurons that respond to physiologically-relevant stimuli, such as estradiol and progesterone treatment (11,12), as well as VCS (27), in female rat brain. We have recently found that treatment with either progesterone (28) or VCS (29) increases Fos-expression within progestin receptor-containing neurons of estradiol-primed female rats. As either progesterone or VCS increases Fos expression within progestin receptor-containing neurons, and each appears to activate progestin receptors, we hypothesized that VCS may also increase Fos expression in some neurons by activating progestin receptors in a progesterone-independent manner. To test this hypothesis, we examined VCS-induced Fos expression in estradiol-primed rats following treatment with either a progesterone antagonist or oil vehicle.

Materials and Methods

Repeated mating paradigm.

All female Sprague-Dawley rats were ovariectomized and adrenalectomized to remove peripheral sources of progesterone. Two weeks later, they were injected with 2 μg estradiol benzoate followed forty-eight hours later by either the progesterone antagonist RU 486 (5 mg; n = 7) or oil vehicle (n = 9). One hour later, all rats received manual stimulation of the vagina and cervix by a plastic probe plus flank-perineum stimulation during the dark phase of the illumination cycle. Stimulation was administered with a 1 cc plastic syringe plunger attached to a force gauge (FDN5, Wagner Instruments, Greenwich, CT, USA) for 10 minutes. Each stimulation consisted of 200 g of force on the vagina and cervix for 2 sec followed by 1 minute of no stimulation. Thus, each animal received 10 stimulations. During VCS, the rat's flank and perineum were manually palpated by the experimenter during insertion of the probe, and lordosis was scored. Lordosis quotient represents the number of lordosis responses divided by the number of mounts by the male or palpations by the experimenter. Fifteen minutes after VCS, each rat was placed in an

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arena with a sexually active male rat for 15 min, or until ten mounts were received, followed by 15 min of nonexposure in its home cage. As sexual behavior that occurs due to estradiol alone (in the absence of progesterone) does not appear to depend upon progestin receptors (30), rats with lordosis quotients greater than 80 at the first mating test (one oil-treated and two RU 486-treated rats) were excluded from further testing and all analysis. Following 15 min of nonexposure, each female was then placed back into the test arena with a sexually active male for 15 min, or until ten mounts were received, followed by another 15 min of nonexposure. This mating paradigm was repeated four times. Fifteen minutes after the last mating test, the rats' flanks and perineum were manually palpated by the experimenter, and lordosis quotients and ratings were scored . Efficacy of adrenalectomy technique was assessed by a corticosterone ^{125}I radioimmunoassay kit (ICN Biomedicals, Inc.) in separate groups of animals 30 min after they received VCS in a manner identical to that used in the behavior tests. Levels of corticosterone in ovariectomized/adrenalectomized rats that received VCS were below the detectability of the assay (less than 25 ng/ml; n = 6). In contrast, corticosterone levels in ovariectomized control rats that received VCS were 473 ng/ml ± 125 (n = 4). Data were analyzed by either a two-way repeated measures analysis of variance followed by a Student-Newman-Keuls multiple comparisons method or a Fisher-Exact test when appropriate. The software used for statistical analysis was SigmaStat Statistical Analysis System 1.01 (Jandel Scientific, Corta Madera, CA, USA). The anesthetic/analgesia procedures used were described previously (11). All of these procedures were approved by the University of Massachusetts Institutional Animal Care Committee.

VCS-induced Fos paradigm.

Adult female rats were ovariectomized two weeks prior to the experiment. All rats were injected with 2 μg estradiol benzoate followed forty-eight hours later by either VCS (VCS+) or control perineal stimulation (VCS-) applied manually by the experimenter. One hour before stimulation, VCS+ rats received an injection of either RU 486 (5 mg; n = 8) or oil vehicle (n = 6) subcutaneously, while VCS- control rats (n = 3) received an injection of oil vehicle. One hour following stimulation, the rats were perfused, and the brain sections were immunostained for Fos protein. The number of Fos-immunoreactive (Fos-IR) cells was analyzed using computer-assisted image analysis (Image 1.55; W. Rasband, NIH; ref 11). Statistical analysis was done by a one-way analysis of variance followed by a Student-Newman-Keuls multiple comparisons method. Another set of rats was ovariectomized and adrenalectomized to remove peripheral sources of progesterone, and received VCS one hour following injection of either RU 486 (5 mg; n = 6) or oil vehicle (n = 6) subcutaneously.

Fos-immunocytochemistry.

Sections were incubated in a rabbit polyclonal *c-fos* antiserum directed against the N-terminal of the Fos protein (0.01 μg/ml; SC 52, Santa Cruz Biotechnology, Inc., Lot# H024, Santa Cruz, CA, USA) diluted in TBS (pH 7.6, 4°C) containing 0.1% gelatin, 0.02% sodium azide, 0.5% Triton X-100 and 1% normal goat serum for 2 days at 4°C. Following three rinses in TBS, sections were incubated in a secondary serum (3 μg/ml of biotinylated goat anti-rabbit IgG; Vector Laboratories, Burlingame, CA, USA) diluted in TBS (pH 7.6) containing 1% normal goat serum for 90 minutes at room temperature. Sections were then incubated with DH:biotinylated horseradish peroxidase H complex (1:100 in TBS (pH 7.6); Vectastain Elite Kit; Vector Laboratories, Burlingame, CA, USA) for 90 minutes. Following three additional rinses in TBS (pH 7.6) of five minutes each, sections were treated with 0.05% diaminobenzidine and 0.05% H_2O_2 in TBS (pH 7.6) for five minutes.

Results

Effects of the progesterone antagonist RU 486 on sexual behavior.

While rats receiving manual VCS plus flank-perineum stimulation showed increases in lordosis at the time of stimulation, there was no difference in lordosis quotients between rats pretreated with RU 486 or oil vehicle. However, when placed in an arena with a sexually active male 15 min after VCS, RU 486-treated rats showed a decrease in sexual receptivity compared to oil-treated controls (Fig 1). In addition, while subsequent mating tests further facilitated sexual receptivity in oil-treated controls by the third and fourth test, this increase was completely inhibited in RU 486-injected rats (Fig 1). When stimulated manually by the experimenter following the last mating test, RU 486-treated rats again showed significantly lower levels of lordosis than oil-treated controls (Fig 1).

Effects of the progesterone antagonist ZK 98299 on sexual behavior.

We repeated the experiment using the Type I progesterone antagonist, ZK 98299. As predicted, female rats receiving oil vehicle (n = 5) showed facilitation of sexual receptivity by the third and fourth mating test. However as with RU 486, females receiving ZK 98299 (n = 5) showed no such facilitation.

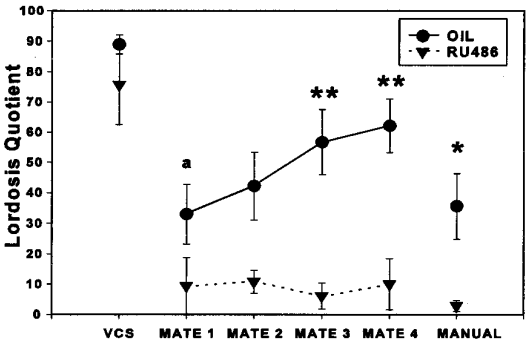

Figure 1. Effects of the progesterone antagonist RU 486 on sexual behavior of estradiol-primed female rats following VCS and repeated testing with a male. VCS = manual vaginal-cervical stimulation. Mate = 15 min mating test with a male. Manual = manual stimulation of flanks and perineum by the experimenter.

Effects of the progesterone antagonist RU 486 on Fos expression in ovariectomized rats.

The Fos antiserum concentration was lowered to detect little or no Fos-IR in unstimulated controls; therefore, Fos-IR was virtually absent in all brain regions examined in VCS- controls. However, the number of Fos-IR neurons was increased dramatically in VCS+ animals within the medial amygdala (MeA), medial preoptic nucleus (MPO), dorsomedial hypothalamus (DM), bed nucleus of the stria terminalis (BSTM), anterior-periventricular thalamic nucleus (PVA) and the caudal ventromedial nucleus of the hypothalamus (cVMHVL; Figure 2A). Treatment with RU 486 decreased VCS-induced Fos-IR only in areas containing high levels of progestin receptors, such as the MPO and the VMH (Figure 2A). However, VCS-induced Fos-IR was unaffected in areas containing little to no progestin receptor-immunoreactivity, such as the MeA and PVA.

Effect of progesterone antagonists on Fos expression in ovariectomized/adrenalectomized rats.

The reduction of VCS-induced Fos-IR by RU 486 was independent of adrenal progesterone, as RU 486 decreased VCS-induced Fos-IR in the MPO, BSTM, and cVMHVL of ovariectomized/adrenalectomized estradiol-primed female rats (Figure 2B). We also obtained similar

results in the MPO and the BSTM of ovariectomized/adrenalectomized estradiol-primed female rats using the Type I progesterone antagonist, ZK 98299.

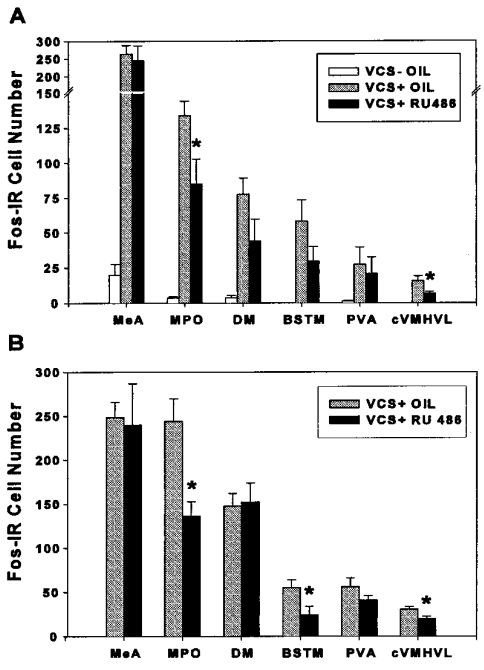

Figure 2. (A) Number of Fos-IR cells (Mean + SEM) per section for each area from estradiol-primed ovariectomized rats receiving manual vaginal-cervical stimulation (VCS+) or control perineal stimulation (VCS-). Rats received either 5 mg RU 486 or oil vehicle one hour prior to stimulation, and they were perfused one hour after stimulation. VCS+ significantly increased Fos-IR in all areas examined when compared to VCS- controls. (B) Number of Fos-IR cells per section for each area from estradiol-primed ovariectomized-adrenalectomized rats receiving manual vaginal-cervical stimulation and either 5 mg RU 486 or oil vehicle.

Discussion

The results of this study support the hypothesis that progestin receptors may be activated in a progesterone-independent manner *in vivo*, and that this novel form of progestin receptor activation may play a functional role in the regulation of female sexual behavior. While manual VCS plus flank-perineum stimulation reliably induced lordosis at the time of stimulation, there was no difference in lordosis quotients between rats pretreated with the progesterone antagonist RU 486 or oil vehicle. The lack of immediate effect of RU 486 on VCS-induced lordosis suggests that there may be a neuronal component activated by VCS that is not affected by prior treatment with progesterone antagonists. However, when the female rats were then placed in an arena with a sexually active male 15 min after VCS, RU 486-treated rats showed lower levels of sexual receptivity than the oil-treated controls (Fig 1). In subsequent mating tests, sexual receptivity increased by the third and fourth mating test in the oil-treated controls. In contrast, this increase was completely inhibited in RU 486-injected rats (Fig 1). Following the last mating test, rats were stimulated manually by the experimenter. Again progesterone antagonist-injected rats showed significantly lower levels of lordosis than those controls receiving oil vehicle (Fig 1). Thus, while VCS increases, and repetitive exposure to the male further facilitates, sexual receptivity in female rats, this facilitation is completely eliminated by treatment with a progesterone antagonist. Therefore, it appears that mating stimuli may activate progestin receptors in a progesterone-independent manner in the absence of progesterone to affect behavior. To determine if the behavioral consequences of blocking progestin receptors were unique to the Type II progesterone antagonist RU 486, we repeated the experiment using the Type I progesterone antagonist, ZK 98299 (2 mg) (31). Female rats receiving oil vehicle showed facilitation of sexual receptivity by the third and fourth mating test. However as with RU 486, the mating tests failed to facilitate sexual behavior in the ZK 98299-injected rats. Thus, mating stimulation facilitates sexual receptivity in estradiol-primed ovariectomized/adrenalectomized female rats, and this facilitation is blocked by treatment with progesterone antagonists. This suggests that the increase in sexual receptivity that occurs in response to either somatosensory information provided by VCS or other stimuli associated with mating may be due to progesterone-independent activation of neuronal progestin receptors.

Another way to examine if mating stimuli activate brain progestin receptors is to examine neuronal response following mating stimuli. As either progesterone and VCS increases Fos expression within progestin receptor-containing neurons, and both appear to activate progestin receptors to affect sexual behavior, we hypothesized that some of the VCS-induced Fos expression may occur *via* activation of progestin receptors. Thus, a component of the VCS-induced Fos expression would be reduced by a progesterone antagonist.

The Fos antiserum concentration was intentionally titrated so that little to no Fos-IR was present in unstimulated animals; therefore, Fos-IR was virtually absent in all brain regions examined in VCS- controls. However, the number of Fos-IR neurons was increased dramatically in VCS+ animals within MeA, MPO, DM, BSTM, PVA and the cVMHVL (Figure 2A). Prior treatment with the progesterone antagonist RU 486 decreased neuronal response (*i.e.*, Fos-IR) to VCS only in areas containing high levels of progestin receptors, such as the MPO and the VMH (Figure 2A). However, VCS-induced Fos-IR was unaffected in areas containing little to no progestin receptor-immunoreactivity, such as the MeA and PVA. While this does not prove that the progesterone antagonist blocked VCS-induced responses in neurons containing progestin receptors, it is suggestive of this possibility.

To exclude the possibility that VCS causes release of progesterone from the adrenal glands, and that RU 486 is blocking progesterone-induced Fos expression rather than Fos induced by afferent input, we repeated the experiment in ovariectomized/adrenalectomized female rats. The reduction of VCS-induced Fos-IR by RU 486 was independent of adrenal progesterone, as RU 486 decreased VCS-induced Fos-IR in the MPO, BSTM, and cVMHVL of ovariectomized/adrenalectomized estradiol-primed female rats (Figure 2B). We also obtained similar results using the Type I progesterone antagonist, ZK 98299. These results suggest that the VCS-induced Fos expression in some cells is dependent upon the availability of unoccupied progestin receptors. These studies are consistent with the idea that some information provided by VCS or mating-stimulation activates neuronal progestin receptors in the absence of progesterone. As mating stimuli increase dopamine release in the VMH (24) and nucleus accumbens (25), and dopamine agonists may activate progestin receptors, perhaps mating stimuli activate progestin receptors by a dopaminergic system. It is also possible that other neurotransmitters or neuropeptides, such as GnRH, that are released during interaction with a male, activate steroid receptors.

The present results suggest that brain progestin receptors may be activated by behaviorally-relevant stimuli in a progesterone-independent manner. These findings suggest the exciting idea of a cellular process by which numerous environmental signals, such as

photoperiodic, chemosensory, and somatosensory information, could influence steroid hormone receptor-dependent processes in the absence of circulating hormones. The resulting consequences of steroid receptor activation by environmental signals may produce changes in a wide variety of physiological, emotional, and behavioral events.

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