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A Progestin Antagonist Blocks Vaginocervical Stimulation-Induced Fos Expression in Neurones Containing Progestin Receptors in the Rostral Medial Preoptic Area

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Abstract

Vaginocervical stimulation (VCS) has a variety of effects on the brain, physiology and behaviour. Previous work demonstrated that a progestin antagonist blocked neuronal response to VCS (i.e. Fos expression) in the absence of progesterone in some neurones, and suggested that some of the effects of VCS on the brain are mediated by ligand-independent activation of progestin receptors (PRs). Although it had been reported previously that some of the cells in which VCS induces Fos expression also contain PRs, it had not been determined if a progestin antagonist blocked Fos expression in these particular neurones. The purpose of this experiment was to determine if a progestin antagonist decreases Fos expression specifically in cells that also express PRs in the preoptic area and ventromedial hypothalamus. As has been shown previously, VCS increased Fos-immunoreactive (ir) expression in the particular areas studied. In the rostral medial preoptic area, VCS increased Fos expression in cells that coexpressed PRs, as well as in cells that do not. However, in the caudal medial preoptic area, VCS only increased Fos expression in cells that did not coexpress PRs. Injection of the progestin antagonist, RU 486, decreased Fos expression in the rostral, but not caudal medial preoptic area, and it decreased Fos expression only in cells that coexpressed PR-ir. In contrast to a previous report, in the present study, the progestin antagonist did not inhibit VCS-induced Fos expression in the ventromedial hypothalamic area. The results of this experiment suggest that the progestin antagonist inhibits VCS-induced Fos expression in some neurones by blocking PRs, and they provide further support for the idea that VCS influences neuronal response in some cells by ligand-independent activation of PRs in those cells.

Genital stimulation that accompanies copulatory behaviour affects the reproductive behaviour and physiology of rats and many other species. For most responses, the principal component is vaginocervical stimulation (VCS), which can be applied by a male rat or by an experimenter with a glass or plastic probe. Behaviourally, in the short term, VCS induces lordosis (1), and it prolongs lordosis responses (2). At longer latencies, it may also increase rejection (3, 4) and cause abbreviation of the period of sexual receptivity (5–9), especially when mating stimulation is paced by the female (10). In a testing situation in which the female is allowed to pace the rate of mating, VCS results in longer latencies to return to the males (11). VCS also alters pain perception (12, 13), induces LH release (14) and induces the twice daily surges of prolactin that result in pseudopregnancy (15, 16).

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 neurones. 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 (MPO), bed nucleus of stria terminalis, posterodorsal portion of the medial amygdala, ventromedial hypothalamic area (VMH), midbrain central grey and other areas related to reproduction (17–20).

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Some of the effects of mating stimulation on the brain seem to be due to VCS-induced neuronal changes that are dependent upon activation of progestin receptors (PRs). Progestin antagonists block both VCS-induced Fos expression in some cells and facilitation of sexual behaviour by mating stimulation, suggesting that functional PRs are essential for these responses (21). Since the activation of PRs by these stimuli, similar to the activation of PRs by agonists of the D_1/D_5 subtype of dopamine receptors (22, 23), occurs in the absence of circulating progesterone, this process has been referred to as ligand-independent or progesterone-independent activation of PRs (21).

Although it has been reported that some of the cells in which VCS induces Fos expression also contain PRs (24), and it was hypothesized that progestin antagonists block VCS-induced Fos expression by acting directly on PRs in these cells, this has not been demonstrated. The purpose of the present study was to determine if a progestin antagonist decreases Fos expression specifically in cells that also contain PRs in two brain areas: MPO and the ventrolateral aspect of the ventromedial hypothalamus (VMHvl).

Materials and methods

Animals

Female Sprague-Dawley rats, weighing 175–200 g (Charles River Breeding Laboratories, Inc, Wilmington, MA, USA) were grouped-housed for one week in a 14 : 10 h light : dark cycle, with food and water available *ad libitum*. Procedures were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts, Amherst. All rats were then ovariectomized after i.p. injections of a cocktail of xylazine (5 mg/kg), ketamine (26 mg/kg) and acepromazine (0.9 mg/kg). Two weeks later, rats were injected s.c. with 2 μg oestradiol benzoate (EB). Forty-eight hours later, a 1 ml plastic syringe attached to a force gauge was used to apply pressure of 300 g to the cervix (VCS^+) or control perineal region (VCS^-) for 2 s, twice per minute for 10 min. One hour before VCS, the VCS^+ animals were injected s.c. with either RU 486 (5 mg) or 0.1 ml sesame oil vehicle, and the VCS^- animals were injected with oil vehicle (n = 6–7 per group).

Perfusions

One hour after the end of VCS, all animals received a lethal dose of sodium pentobarbital (89 mg/kg), and they were perfused with 0.9% physiological saline (25 ml) for 1 min followed by 4% paraformaldehyde (25 ml/min) for 10 min After the brains were removed from the cranium, they were placed into 0.1 M sodium phosphate buffer (pH 7.2) containing 20% sucrose for 48 h. Thirty-five μm sections from the preoptic area to the midbrain region were cut on a freezing microtome, and the sections were placed into a cryoprotectant solution and stored at −20 °C.

Double-label immunofluorescence for PR-immunoreactive (ir) and Fos-ir

For all animals, a set of one in every four brain sections through the preoptic area and the hypothalamus was selected. They were removed from cryoprotectant and rinsed three times for 5 min each in Tris-buffered saline (0.05 M TBS, pH 7.6). Sections were placed into 1% H_2O_2, 20% normal goat serum and 1% bovine serum albumin for 20 min. Sections were then incubated for 2 days at 4 °C in solution containing the PR antibody (MAB 462, Chemicon, Tremecular, CA, USA; 1 : 1000 dilution) and the rabbit polyclonal fos antibody (AB5, 1 : 50 000) in a buffer containing TBS, 0.1% gelatin, 0.02% sodium azide, 0.5% Triton X-100, 1% normal goat serum (TBS-gel). Following three washes in TBS-gel buffer, the sections were incubated in a solution containing the fluorescein isothyocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (to reveal Fos-ir; Jackson Immunoresearch, West Grove, PA, USA; 10 μg/ml) for 90 min at room temperature. The sections were then rinsed three times for 5 min each in TBS-gel, and incubated in a solution containing the rabbit IgG (Jackson Immunoresearch; 1 : 1000) for 60 min at room temperature. The sections were rinsed three times for 5 min each in TBS-gel, and incubated in a solution containing the cyanine-3 conjugated goat anti-mouse secondary antibody (to reveal PR-ir; Jackson Immunoresearch; 7 μg/ml) and the FITC-conjugated goat anti-rabbit secondary antibody (to reveal Fos-ir; Jackson Immunoresearch; 10 μg/ml) for 90 min at room temperature. After a final three rinses for 5 min each in TBS, the sections were mounted onto slides, air-dried and coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA).

Double-label immunofluorescent controls

In order to control for nonspecific immunofluorescent staining, cross-immunoreactivity and 'bleed through' of the fluorochromes, other sections were incubated either in solutions in which the primary antibodies were omitted, but the secondary antibodies were present, or in solutions in which only one primary antibody was present, but both secondary antibodies and the IgGs were present. No nonspecific immunofluorescent staining, cross-immunostaining or 'bleed through' was observed (data not shown).

Quantification of double-labelled immunofluorescent cells

Using the NIH Image computer analysis system (developed at the National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/), digitized pictures of the same microscopic field were captured with two pass-band filters, specific for FITC and cyanine 3. The images were superimposed, and the numbers of single- and double-labelled cells were counted by eye.

Neuroanatomical areas to be analysed were chosen based on previous studies indicating areas rich in VCS-induced Fos expression (19) and oestradiol-induced PR expression (24, 25) or in which a progestin antagonist decreases VCS-induced Fos expression (24). This included the rostral medial preoptic area (rMPO), caudal medial preoptic area (cMPO), medial ventrolateral aspect of the ventromedial hypothalamus (mVMHvl) and caudal ventrolateral aspect of the ventromedial hypothalamus (cVMHvl) (Fig. 1). The quantification was performed with a ×20 objective, and only cells in which an immunoreactive cell nucleus was clearly distinguishable were counted.

Test for effects of RU 486 on epitope availability

To determine whether RU 486 binding to PRs interferes with binding of the MAB 462 antibody to the receptor, groups (n = 4–5) of ovariectomized rats were injected s.c. with oil vehicle or 2 μg EB. Forty-eight hours later, the EB groups were injected s.c. with oil, 5 mg progesterone or 5 mg RU 486. The rats were perfused 1 h later, and their brains were immunostained for PR-ir as described, except that a Vectastain ABC Elite-diaminobenzidine technique was used that was similar to that described previously (25). PR-ir cells were counted within a field of the rMPO using a ×20 objective.

Statistical analysis

One-way analysis of variance was performed to determine statistical differences in the means of single and double-labelled cells among groups. Statistically significant results were followed by a Fisher's LSD test for pair-wise, multiple comparisons. Results were considered statistically significant if $P < 0.05$. In one case, a Kruskal–Wallis one-way analysis of variance on signed ranks was used because the data did not pass the normality test for analysis of variance.

Results

In the control experiment to determine if RU 486 has an effect on the ability of the MAB 462 antibody to bind to PRs, analysis of variance revealed a highly statistically significant effect of treatment on number of PR-ir cells (Table 1) ($P < 0.001$). However, this was entirely attributable to the increase in the number of PR-ir cells in all groups treated with oestradiol. In a second analysis of variance, contrasting only the three groups receiving EB treatment, there was no treatment effect. Therefore, neither progesterone nor RU 486 had any influence on PR-immunostaining under these conditions.

There was a statistically significant effect of treatment on Fos-ir expression in each neuroanatomical area ($P < 0.005$).

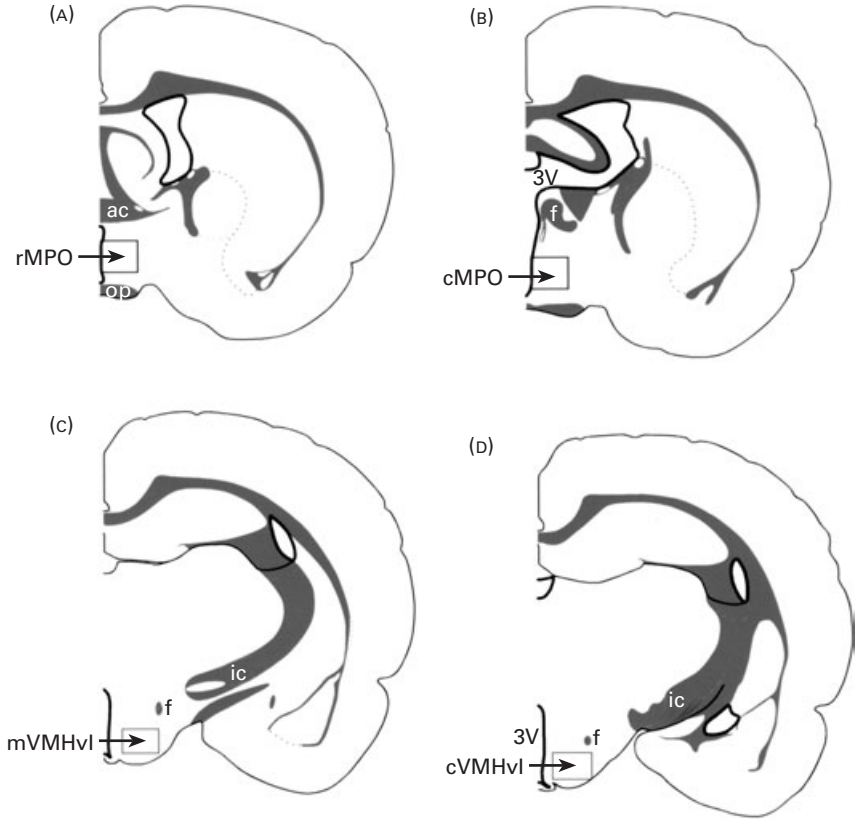

FIG. 1. Sections indicating the specific brain sections in which cells were counted for the rostral medial preoptic area (rMPO) (A; level 20), caudal medial preoptic area (cMPO) (B; level 22), medial ventrolateral aspect of the ventromedial hypothalamus (mVMHvl) (C; level 28) and caudal ventrolateral aspect of the ventromedial hypothalamus (cVMHvl) (D; level 30). Drawings are adaptations. Panel numbers refer to level of section in Swanson (44).

TABLE 1. Mean (±SEM) Number of PR-ir Cells Counted in a Section of the rMPO After Injection s.c. of Ovariectomized Rats with Oil Vehicle or Oestradiol Benzoate (EB) Followed by Oil Vehicle, 5 mg Progesterone or 5 mg RU 486.

Oil vehicle	25.5±3.12
EB+oil vehicle	119.4±7.25
EB+5 mg progesterone	108.8±3.43
EB+5 mg RU 486	124.0±7.71

Consistent with earlier reports, VCS induced Fos expression in all regions studied (Fig. 2). Furthermore, consistent with a previous report that RU 486 blocked VCS-induced Fos expression in the MPO, RU 486 decreased the number of cells expressing Fos-ir in the rMPO by 32.6% ($P=0.03$).

Although treatment was without effect on the number of PR-ir cells in either part of the VMHvl, there was a trend of a decrease in the number of PR-ir cells after RU 486 treatment in the rMPO. Although there was also a statistically significant overall treatment effect ($P<0.05$) in the cMPO, this was not due to VCS or progestin antagonist treatment decreasing the number of PR-ir cells (Table 2). Rather, the group receiving VCS and the progesterone antagonist had a significantly fewer PR-ir cells than the VCS group ($P=0.015$).

There was a statistically significant treatment effect on the number of cells that coexpress Fos and PR-ir in the rMPO [$F(2,16)=9.94$, $P<0.002$], mVMH [Kruskal–Wallis, $H=7.378$ (2), $P<0.02$] and cVMH [$F(2,14)=14.81$, $P<0.001$], but not the cMPO (Fig. 3). This confirms and extends our earlier finding (24) that many of the cells that are responsive to VCS also contain PR-ir. In fact, RU 486 treatment eliminated the effect of VCS on these cells, decreasing the number of cells coexpressing Fos-ir and PR-ir by 53% in the rMPO (Fig. 3) ($P<0.002$), but not in other areas.

Discussion

As has been reported earlier, VCS induced Fos expression in the MPO and VMHvl. Also consistent with earlier work, in which RU 486 blocked VCS-induced Fos expression in some cells in the medial MPO (24), we observed a decrease in the number of Fos-ir cells in the rMPO. In contrast, RU 486 did not block VCS-induced Fos expression in the cMPO, nor in the mVMHvl, nor the cVMHvl (a site at which RU 486 was reported to reduce Fos expression in the earlier study). No definitive explanation is apparent for the failure to detect a decrease in Fos expression in the cVMHvl after RU 486 treatment, as has been observed earlier (21). However, the number of cells expressing Fos-ir after VCS in this region is much smaller than in the rMPO, and the previously reported effects in the cVMHvl are more modest than the rMPO (21). Although these results demonstrate conclusively

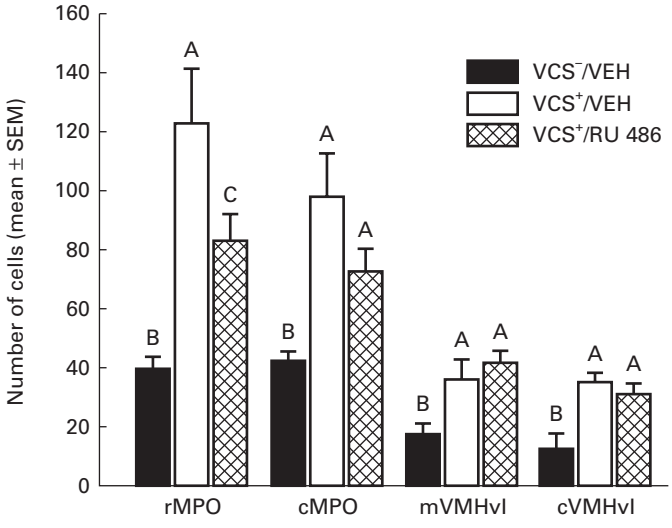

FIG. 2. Number of Fos-ir cells (mean ± SEM) in the rostral medial preoptic area (rMPO), caudal medial preoptic area (cMPO), medial ventrolateral aspect of the ventromedial hypothalamus (mVMHvl) and caudal ventrolateral aspect of the ventromedial hypothalamus (cVMHvl) samples after control stimulation (perineal region) (VCS^-) preceded by vehicle (VCS^-/VEH), vaginocervical stimulation (VCS^+) preceded by vehicle (VCS^+/VEH) or VCS^+ preceded by RU 486 (VCS^+/RU 486). Bars with different letters over them denote statistically significant differences between groups.

TABLE 2. Mean (±SEM) Number of Progestin Receptor (PR)-Immunoreactive Cells in Samples of Four Brain Areas.

	VCS^-/VEH	VCS^+/VEH	VCS^+/RU 486
rMPO	178.0±16.1	183.2±18.5	144.5±26.2
cMPO	148.3±13.8	115.8±15.6	89.3±16.2
mVMHvl	40.6±7.1	44.0±9.4	37.6±7.6
cVMHvl	106.3±17.5	99.7±20.5	78.8±12.6

VCS^-, control stimulation; VCS^+, vaginocervical stimulation; VEH, vehicle; rMPO, rostral medial preoptic area; cMPO, caudal medial preoptic area; mVMHvl, medial ventrolateral aspect of the ventromedial hypothalamus; cVMHvl, caudal ventrolateral aspect of the ventromedial hypothalamus.

that RU 486 blocks VCS-induced Fos expression in PR-containing cells in the rMPO, the negative results in the other areas do not exclude the possibility of blockade of Fos expression in populations of PR-ir cells elsewhere.

Because RU 486 is a glucocorticoid antagonist, as well as a progestin antagonist, we cannot exclude the possibility that glucocorticoid receptors are involved in the inhibition of VCS-induced Fos expression by this drug. However, VCS-induced Fos expression can be blocked by other progestin antagonists (21), and other experiments have demonstrated conclusively that the process of ligand independent activation, at least in some cases, occurs through PRs (23). Nevertheless, potential involvement of glucocorticoid receptors in inhibition of VCS-induced Fos expression by RU 486 cannot be excluded at this time.

As was reported earlier (24), many of the cells in the rMPO in which VCS increases Fos expression also contain PR-ir. In this area, in which RU 486 reduced the number of cells expressing VCS-induced Fos by approximately 50%, the progestin antagonist completely eliminated this increase in Fos-ir cells that coexpress PR-ir. This suggests that, while VCS induces Fos expression both in cells that express PR-ir and in those that do not, the progestin antagonist blocks Fos expression only in cells coexpressing PR-ir.

There is an interesting contrast between the results in the rMPO and those in the cMPO. In the rMPO, VCS induces Fos expression in cells that coexpress PRs, as well as in cells that do not coexpress PRs. RU 486 reduces Fos expression only in those cells that coexpress PRs. In contrast, in the cMPO, VCS induces Fos expression only in cells that do not coexpress PRs. In this area, RU 486 did not inhibit VCS-induced Fos. In both the mVMHvl and the cVMHvl, VCS induces Fos expression in both classes of cells, those that coexpress PR-ir and those that do not coexpress PR-ir. Although we did not observe a significant reduction in Fos-ir cells in either of these regions, it should be noted that the numbers of Fos^+/PR^+ are quite small. Therefore, we cannot exclude the possibility of an effect of RU 486 in those regions, especially since we have previously reported a small effect of RU 486 on VCS-induced Fos expression in the cVMHvl.

The presence or absence of PR-ir, similar to any immunocytochemically identified protein, is relative, rather than all-or-none. In fact, many more cells contain PRs than we typically immunolabel, because our immunocytochemical procedure is usually titrated so that we only label the most intensely immunostained cells (26). These presumably represent mostly the cells in which oestradiol induces PRs, resulting in considerably higher concentrations than in cells in which oestradiol is without effect. We have, for example, shown that while many cells contain PRs, those in which oestradiol induces the increased expression of PR-ir, using a technique titrated in this way, also contain ERs (27). Therefore, the colocalization described here presumably reflects cells which contain a relatively high concentration of PRs.

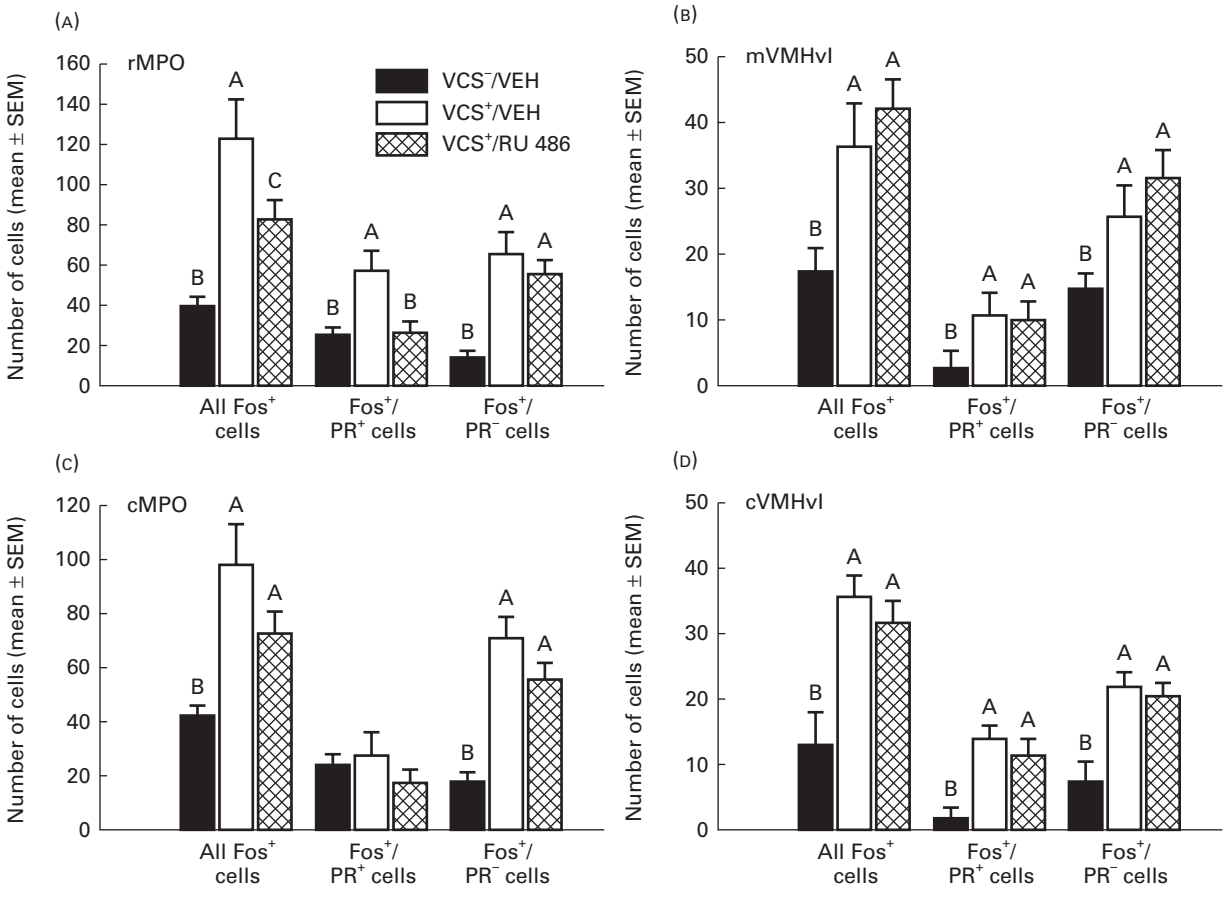

FIG. 3. Number of cells (mean ± SEM) expressing Fos-immunoreactivity (ir) (Fos^+ cells), Fos-ir and progestin receptor (PR)-ir (Fos^+/PR^+ cells) or Fos^+ without PR-ir (Fos^+/PR^- cells) in sections from the rostral medial preoptic area (rMPO) (A), caudal medial preoptic area (cMPO) (B), medial ventrolateral aspect of the ventromedial hypothalamus (mVMHvl) (C) or caudal ventrolateral aspect of the ventromedial hypothalamus (cVMHvl) (D) after control stimulation (VCS^-) preceded by vehicle (VCS^-/VEH), vaginocervical stimulation (VCS^+) preceded by vehicle (VCS^+/VEH) or VCS^+ preceded by RU 486 (VCS^+/RU 486). Bars with different letters indicate statistically significant differences between groups.

In the initial control experiment to determine if RU 486 interfered with the ability of the PR antibody to immunostain PRs, RU 486 was without effect. However, in the double-label experiment, there was a nonsignificant trend for RU 486 to decrease the number of PR-ir cells in the rMPO that must be considered in the interpretation of this experiment. While it is possible that this is due to a subtle change in epitope availability when some receptors are bound to RU 486, this is unlikely. Although we have reported that occupation of the receptor with progesterone decreases the immunostaining of the receptor using a different PR antibody than was used here (25), this was not the case with the MAB 462 antibody, as was seen in the control experiment. We cannot exclude the possibility of an interaction of VCS and RU 486 on PR epitope availability. It should be noted that the trend of a lower number of PR-ir cells in the rMPO after RU 486 treatment was due to only two rats with lower numbers of PR-ir cells. It is unlikely that the robust decrease in the number of cells coexpressing Fos-ir and PR-ir in the rMPO after RU 486 treatment is referable to the trend of a decrease in the number of PR-ir cells after treatment with the antagonist.

The present results lend further support to the idea that the intracellular signal conveying information about genital stimulation to some forebrain cells is mediated by ligand-independent activation of PRs. Mating stimulation that includes VCS (21), or VCS alone (8), induces sexual behaviour in ovariectomized-adrenalectomized rats primed with a dose of oestradiol that is insufficient to induce response by itself; the mating-induced facilitation of sexual behaviour can be blocked by progestin antagonists, suggesting that ligand-independent activation of PRs is essential for the response (21). Similarly, VCS-induced Fos expression in some forebrain neurones is blocked by treatment with progestin antagonists. Although it was hypothesized that the progestin antagonists acted directly on neurones containing PRs, it had not been shown that this was the case. The present study confirms that, at least in the area in which the response is most robust (rMPO), the progestin antagonist selectively blocks Fos expression in VCS-responsive neurones, which also coexpress PRs. This is consistent with, but not defeinitive proof of the idea that the induction of Fos expression in some neurones requires the presence of functional PRs in those neurones.

Although this experiment does not address directly the neurotransmitter involved in the induction of Fos expression by ligand-independent activation of PRs in response to genital stimulation, previous work suggests that dopamine is critically involved. First, dopamine is released in the forebrain of female hamsters (28) and rats (29–31) as a result of sexual behaviour; in some cases, linked specifically to intromissions (28). Second, VCS induces Fos expression in neuroanatomical areas, which contain D_1 and/or D_5 dopamine receptors (32–35) and VCS-induced Fos expression in all forebrain areas studied was blocked by administration of a D_1/D_5 antagonist (36). Third, VCS induces phosphorylation of DARPP-32, a phosphoprotein, which is essential for progesterone or dopamine-facilitated sexual behaviour (37), in the MPO and cVMHvl studied here, as well as other neuroanatomical areas (38). Finally, a D_1 dopamine agonist induces Fos expression in the MPO and VMHvl, and some of the responsive neurones coexpress PRs (37). It should be noted that evidence to support a role for dopamine in VCS-induced Fos expression in some neurones does not exclude a possible role of other neurotransmitters that are influenced by genital stimulation, such as norepinephrine (40–42), in this regulation.

The results of this study suggest that genital stimulation may influence neuronal function in a subset of neurones receiving input from genital areas only when PR expression is elevated. While the neuroendocrine function is not known, the results suggest that some neurones might respond to genital stimulation regardless of level of PRs, and other neurones might respond depending on the level of PRs. Since elevated PR expression in most neurones is dependent upon oestradiol priming, this might then provide a mechanism for selective gating of the afferent signal deriving from genital stimulation in specific neurones and during specific reproductive conditions. It should be noted that ligand-independent activation of PRs has been implicated in the process by which the spontaneous GnRH surge is regulated (43). Perhaps this process is also involved in induced ovulation in response to genital stimulation under some conditions. Alternatively, the PR-mediated, neuronal response to genital stimulation might participate in the timing of sexual receptivity under natural conditions.

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