D₁ Dopamine Receptor Agonist (SKF-38393) Induction of Fos Immunoreactivity in Progestin Receptor-Containing Areas of Female Rat Brain

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Abstract

Injection of dopamine or dopamine receptor subtype agonists facilitates the expression of lordosis in estrogen-primed female rats. The D₁ receptor specific agonist, SKF-38393, facilitates lordosis in estradiol-primed female rats via a process that requires progestin receptors. Based on these data, neuronal response to the D₁ receptor agonist SKF-38393 was assessed by expression of the immediate early gene protein, Fos. In the first experiment we examined the modulation of Fos expression by D₁ agonists in progestin receptor-containing areas of estradiol-primed female rat brain. In the second experiment we examined if there are progestin receptor-containing cells that respond to stimulation of D₁ receptors with increased Fos expression. Ten to 14 days following ovariectomy and stereotaxic surgery, animals were injected with 5 µg estradiol benzoate. Forty eight h later they were injected intracerebroventricularly with 100 ng of SKF-38393 or saline. One h following injection animals were perfused, and brain sections immunostained for Fos protein. Results from the first experiment suggest that SKF-38393 increased the total number of Fos immunoreactive cells in the mid-ventromedial hypothalamic nucleus/ventrolateral portion (VMHVL), the caudal VMHVL, the paraventricular hypothalamic nucleus and the caudate putamen. In the medial preoptic area, the rostral VMHVL and the arcuate hypothalamic nucleus, there was a significant increase in the number of darkly stained Fos-immunoreactive cells following the SKF-38393 treatment. In the second study, SKF-38393 increased the number of progestin receptor-containing cells which contained Fos immunoreactivity in the caudal VMHVL. The results suggest potential sites of action for the facilitation of sexual behavior by centrally administered D₁ agonists.

In female rats, the neuroendocrine and hormonal events which take place during the estrous cycle tightly regulate the timing of physiological as well as behavioral events leading to optimal reproductive efficiency. The behavioral events associated with behavioral estrus in ovariectomized rats can be mimicked using a sequential hormonal paradigm of estradiol followed by progesterone (1-3). While estradiol induces some of the behaviors associated with female sexual behavior, both estradiol and progesterone are required to achieve the full complement of sexual behaviors (1-3). Furthermore, progesterone has the additional property of facilitating the effects of estradiol, such that lower doses of estradiol can be used (1 3).

A number of neurotransmitters and neuropeptides can substitute for progesterone in facilitating female rat sexual behavior (4-19). One neurotransmitter that has been extensively studied in the facilitation of sexual behavior is dopamine. Systemic and central administration of dopamine or dopamine agonists facilitate female rat sexual behavior under some hormonal conditions (10, 11, 19-23). The dopaminergic facilitation of sexual behavior can be blocked using dopamine antagonists (10, 11, 19, 21).

With the advent of more specific dopaminergic agonists and antagonists, it has been possible to examine whether activation of specific subsets of dopaminergic receptors such as the D₁ and D₂ receptor subtypes facilitate female rat sexual behavior. For example, dopamine facilitated sexual behavior can be blocked using specific antagonists to D₂ receptors (11) suggesting that D₂ receptors play a role in facilitation of sexual behavior. Interestingly, the effects of D₂ agonists on sexual behavior appear to depend on the state of receptivity of the animal. In non-receptive rats treated with low levels of estradiol, D₂ agonists facilitate sexual behavior (11, 21). However in sexually receptive animals treated with high levels of estradiol or estradiol benzoate/progesterone, D₂ activation inhibits sexual behavior (11, 21, 24).

Recently, the role of brain D₁ receptors in the facilitation of female rat sexual behavior has been more closely examined (10, 19, 25). These experiments suggest that stimulation of D₁ receptor subtypes in the brain facilitates sexual behavior under some hormonal conditions (10, 19, 25). Of particular interest was the observation that the D₁ agonist facilitation can be blocked by progestin receptor antagonists (10, 19, 25), antisense oligonucleo-

tides to progestin receptor mRNA (10, 19, 25) and antisense oligonucleotides to the dopamine D_1 receptor (19). These data suggest that dopamine D_1 agonists facilitate female rat sexual behavior through stimulation of the D_1 receptor and that this facilitation occurs via a progestin receptor-mediated event.

Increases in the expression of the Fos protein—a product of the immediate early gene, c-fos—have been observed following the administration of progesterone in estradiol-primed female rats (26, 27). Furthermore, progesterone has been shown to increase the expression of Fos in a population of cells which contain progestin receptors (28). Based on these observations we used Fos immunocytochemistry to determine if a D_1 dopamine agonist (SKF-38393; injected i.c.v.) modulates Fos expression. The purpose of using Fos in this series of experiments was not to identify all cells that respond to the D_1 agonist (not every cell stimulated by the D_1 agonist is expected to exhibit increases in Fos expression) but to determine if there are populations of cells which may be potential sites of interaction between dopamine and progestin receptors. We first examined the modulation of Fos expression by D_1 agonists in progestin receptor-containing areas of estradiol-primed female rat brain. We then examined if there are progestin receptor-containing cells that respond to stimulation of D_1 receptors with increased Fos expression. Specific brain areas that were examined for changes in Fos immunoreactivity induced by the dopamine agonist included those associated with female rat sexual behavior as well as the caudate putamen (CPu), which has high levels of dopamine receptors (Fig. 1).

Results

Experiment 1: SKF-38393 induction of Fos immunoreactivity in female rat forebrain

Intracerebroventricular infusion (i.c.v.) of SKF-38393 caused a significant increase in the total number of Fos immunoreactive (Fos-Ir) cells in the mid-ventromedial hypothalamic nucleus/ventrolateral portion (mVMHVL) compared to saline treated animals (23.3 ± SEM 4.5 vs 8.0 ± SEM 0.9; Fig. 2 and 3). The total number of Fos-Ir cells also increased in the caudal VMHVL (cVMHVL)(21.3 ± 1.9 vs 14.0 ± 1.9) and paraventricular hypothalamic nucleus (Pa) following SKF-38393 (42.5 ± 4.0 vs 26.3 ± 4.9). Finally, SKF-38393 more than doubled the number of Fos-Ir cells in the CPu (113.5 ± 7.4 vs 51.8 ± 12.4; Fig. 2 and 4).

While the total number of Fos-Ir cells did not change in the medial preoptic area (MPA), the rostral VMHVL (rVMHVL) or the arcuate hypothalamic nucleus (Arc) following the SKF-38393 treatment, there were significant increases in the number of darkly stained cells. In other words, SKF-38393 increased the number of cells which contained higher maximum optical densities (MOD). MOD was determined by measuring the darkest point of each cell (the highest gray level) and determining the mean for all cells within a given image. Specifically, SKF-38393 induced a statistically significant increase the number of Fos-Ir cells compared to saline treatment in the MPA (129.3 ± 13.0 vs 68.3 ± 16.7), the rVMHVL (5.5 ± 1.0 vs. 2.0 ± 0.8) and the Arc (13.3 ± 2.6 vs. 6.0 ± 1.5) (Table 1). The image analysis thresholds (relative gray levels of > 100 for the MPA, > 100 for the rVMHVL and > 200 for the Arc) were set post hoc based on analysis of frequency distribution histograms for each area (described in the computer-assisted image analysis description in methods section).

SKF-38393 did not induce any increase in intensity of Fos-immunostaining in the medial preoptic nucleus/central (MPO), the dorsomedial hypothalamic nucleus (DM), the medial amygdaloid nucleus (Me) or the rVMHVL combined with the ovarian steroid hormone receptor area that extends toward the fornix (rVMHVL-ORA).

Experiment 2: SKF-38393 induction of Fos immunoreactivity in progestin receptor immunoreactive (PR-Ir) cells

We examined D_1 agonist induced Fos immunoreactivity in progestin receptor containing cells in the rVMHVL-ORA, cVMHVL, the MPO (Fig. 5) and the Arc based on where progesterone induces Fos in progestin receptor containing cells (26, 27). Of these areas, the only one in which SKF-38393 significantly increased the number of PR-Ir cells expressing Fos was the cVMHVL (15.0 ± 2.48 vs 7.5 ± 2.25) (Fig. 6). There was no change in the number of PR-Ir cells expressing Fos in the rVMHVL-ORA or in the MPO, which is consistent with our observations that overall Fos immunostaining did not change in these areas. However, there also was no change in the number of PR-Ir cells expressing Fos in the Arc, an area which showed changes in the number of darkly stained Fos-Ir cells. This suggests that the increase in Fos expression in the Arc may be occurring in cells which either do not contain progestin receptors or which contained low, undetected levels of progestin receptors.

Discussion

Dopamine and dopaminergic drugs have been implicated in the facilitation and inhibition of female rat sexual behavior. Interestingly, the effects of dopaminergic drugs on sexual behavior are influenced by the hormonal state of the animal. In female rats treated with low levels of estrogens, administration of dopamine and dopamine agonists such as apomorphine facilitate sexual behavior (11, 21 23). However, dopamine and dopamine agonists suppress sexual behavior in female rats which have been made sexually receptive by either the administration of high doses of estrogens or by administration of estrogens followed by progesterone (11, 22, 24). In addition to facilitating and inhibiting the onset of sexual behavior, dopamine may also play a role in the events which take place during and after mating. For example, in female rats and hamsters, mating stimulation elicits the release of dopamine from nerve terminals in the CPu (29, 30), nucleus accumbens (30) and the ventromedial hypothalamus (31).

It appears that at least some of the facilitatory effects of dopamine may be due to a central site of activation. This is supported by observations in which direct infusion of dopamine, or dopamine agonists into the MPO (22) or VMH (19, 22) areas or i.c.v. infusions of apomorphine (10, 25) facilitate sexual behavior in estrogen-primed rats. It appears that activation of D_1 subtype receptors may play an important role in central nervous system facilitation of sexual behavior. For example, i.c.v. injection of various dopamine D_1 agonists facilitated sexual behavior in estradiol-primed female animals (10, 19, 25). On the other hand, i.c.v. infusions of the D_2 agonist quinpirole did not affect sexual behavior (10, 25). The fact that the facilitation by the centrally administered D_1 agonist was blocked by progesterone antagonists (10, 19, 25), by administration of an antisense oligonucleotide directed toward the progestin receptor mRNA (10, 19, 25) and by antisense oligonucleotides directed towards D_1 recep-
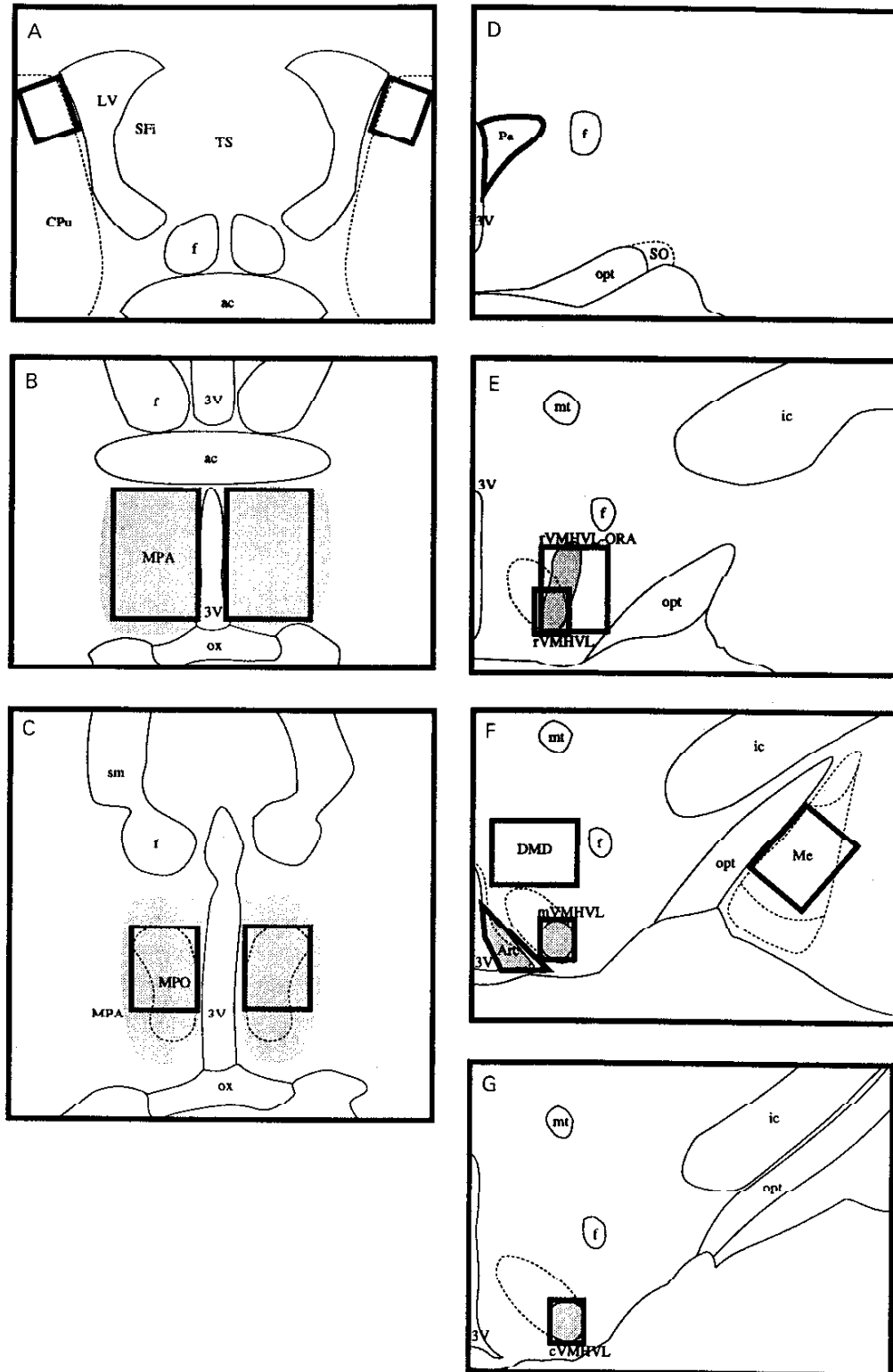
FIG. 1. Composite drawings of sections that were analyzed from the (A) CPu, (B) the MPA, (C) the MPO, (D) the Pa, (E) the rVMHVL-ORA and rVMHVL, (F) the DM, Arc, mVMHVL, Me and (G) the cVMHVL. Shaded areas represent areas containing PR-Ir cells within and continuous with the areas analyzed. *Abbreviations*: 3V, 3rd ventricle; ac, anterior commisure; Arc, arcuate hypothalamic nucleus; CPu, caudate putamen; DM, dorsomedial hypothalamic nucleus; f, fornix; ic, internal capsule; LV, lateral ventricle; Me, medial amygdaloid nucleus; MPA, medial preoptic area; MPO, medial preoptic nucleus; mt, mammillothalamic tract; opt, optic tract; ox, optic chiasm; Pa, paraventricular hypothalamic nucleus; rVMHVL-ORA, the ovarian receptor-containing area associated with the rVMHVL that extends dorsolaterally towards the fornix; SFi, septofimbrial nucleus; sm, stria medullaris thalamus; SO, supraoptic nucleus; TS, triangular septal nucleus; VMHVL, ventromedial hypothalamic nucleus, ventrolateral.
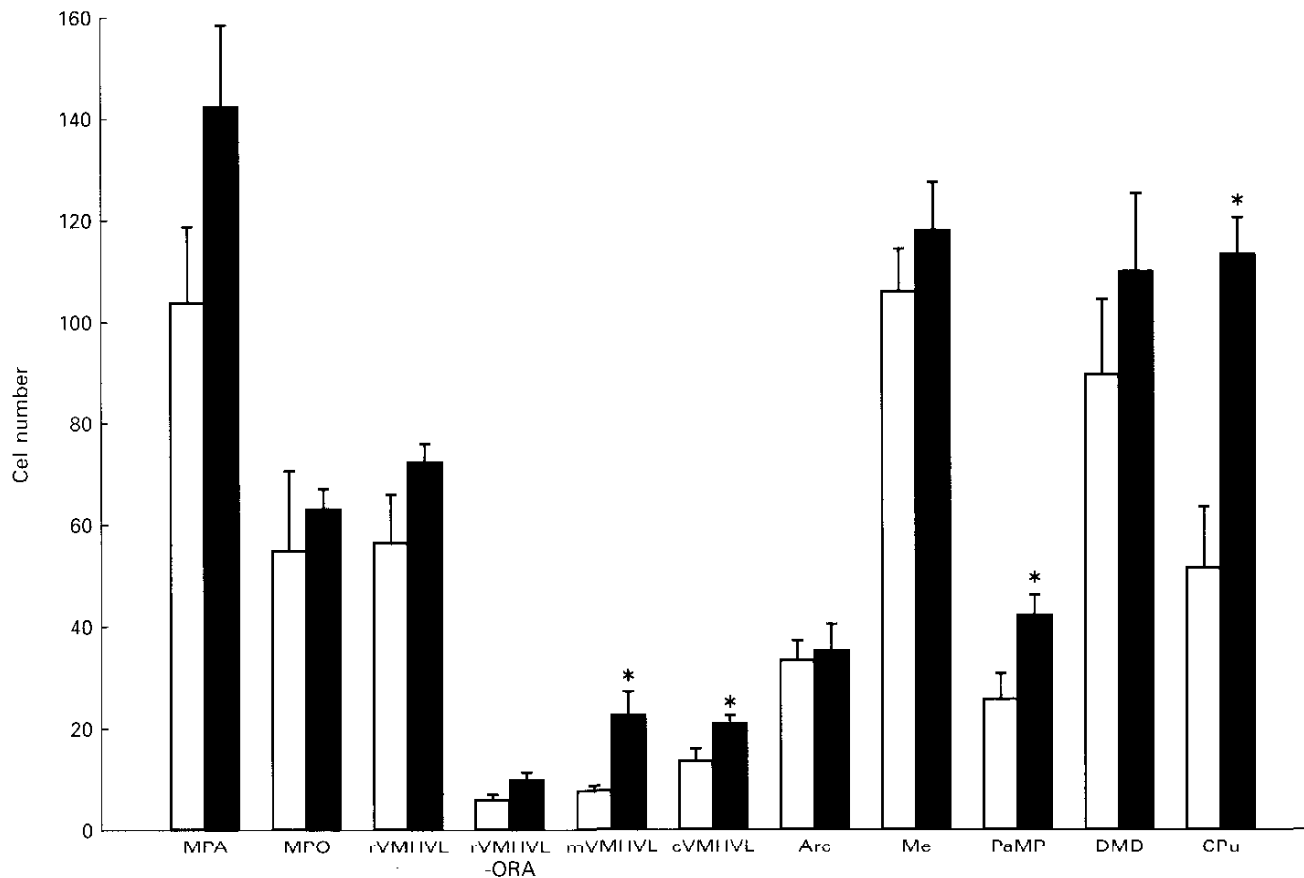
FIG. 2. Effects of a D_1 agonist (SKF-38393) on Fos-Ir cell number in female rat brain. Open bars = Saline vehicle (3 µl i.c.v.), Solid bars = 100 ng SKF-38393 i.c.v. See Fig. 1 for nomenclature. *P < 0.05 compared to saline vehicle.

tor subtypes (19) suggests that the pathway by which the D_1 agonist activates sexual behavior involves the D_1 receptor and includes a progestin receptor-mediated component.

Based on these findings, we examined whether D_1 receptor induction of Fos expression occurs in progestin receptor-containing areas and cells. In experiment 1, we examined several areas which are associated with female sexual behavior including the MPA, MPO, VMHVL, rVMHVL-ORA, DM, and Arc for changes in Fos immunoreactivity. Of these areas, all but the DM have high levels of estrogen-induced progestin receptors in rats (32, 33) and guinea pigs (34, 35). In all of the progestin receptor-containing areas except for the MPO and the rVMHVL-ORA we found increases in either Fos-Ir cell number or maximum optical density following D_1 receptor activation.

The observation that some areas exhibit changes in total cell number while other areas exhibit changes in the number of darkly stained cells, does not necessarily suggest that these areas respond in a qualitatively different manner to the stimuli. The differential staining in response to the D_1 agonist may be due to technical issues associated with differences in the titration curve of primary antibody in different areas. For example, one may titrate the primary antibody so that control groups show very little staining. Under these conditions, any increases in immunostaining due to treatment become readily apparent as changes in

cell number. As primary antibody concentrations increase, changes in absolute cell number shift towards changes in optical density. We have recently demonstrated that changes in optical density as measured by computer assisted image analysis are comparable to changes in cell number when analyzed using lower primary antibody concentrations (26). While it might be desirable to titrate the antibody concentration for every area analyzed, it would be impractical due to the enormous amounts of tissue that would be required. The compromise is to examine changes in absolute cell number as well as changes in the number of darkly immunostained cells. Finally, it is also possible that the differences in the intensity of the response to the D_1 agonist may be due to different rates of diffusion of the agonist to the various areas.

In experiment 2, areas within the MPO, rVMHVL-ORA, cVMHVL and Arc were analyzed to determine whether SKF-38393 increased Fos expression in progestin receptor-containing cells. Of the four areas analyzed, the cVMHVL was the only one in which SKF-38393 increased the number of progestin receptor-containing cells expressing Fos. These data suggest that some progestin receptor-containing cells respond to D_1 receptor stimulation. However, there are probably progestin receptor-containing cells that were stimulated by the D_1 agonist but which did not exhibit changes in Fos expression. Therefore, the progestin receptor-containing cells within the cVMHVL are

TABLE 1. Fos-Ir Cell Number at Various Gray Scale Thresholds following the Administration of Vehicle or Dopamine D₁ Agonist (SKF-38393)

		Total cell #	>50	>100	>150	>200
				Gray scale thresholds (0–255)		
MPA	V	104.3 ± 15.1	104.3 ± 15.1	*68.3 ± 16.7*	25.3 ± 8.7	5.8 ± 2.5
	SKF	142.3 ± 16.5	142.3 ± 16.5	*129.3 ± 13.0***	43.8 ± 4.1	11.5 ± 2.1
MPO	V	54.8 ± 15.8	54.8 ± 15.8	25.0 ± 4.5	4.5 ± 1.0	1.5 ± 0.6
	SKF	63.3 ± 3.9	63.3 ± 3.9	28.8 ± 5.6	6.8 ± 1.5	0.5 ± 0.5
rVMHVL-ORA	V	57.0 ± 9.2	57.0 ± 9.2	45.8 ± 5.3	22.0 ± 2.1	8.8 ± 2.3
	SKF	72.3 ± 4.3	72.3 ± 4.3	57.8 ± 4.8	27.3 ± 4.2	12.0 ± 1.6
rVMHVL	V	6.3 ± 0.9	6.3 ± 0.9	*2.0 ± 0.8*	*1.0 ± 0.6*	0.5 ± 0.5
	SKF	9.8 ± 1.7	9.8 ± 1.7	*5.5 ± 1.0***	*3.3 ± 0.9**	0.5 ± 0.3
mVMHVL	V	*8.0 ± 0.9*	*8.0 ± 0.9*	*5.3 ± 0.6*	*2.0 ± 0.9*	0.8 ± 0.5
	SKF	*23.3 ± 4.5***	*23.3 ± 4.5***	*16.8 ± 3.8***	*6.5 ± 1.8**	2.3 ± 0.9
cVMHVL	V	*14.0 ± 1.9*	*14.0 ± 1.9*	10.0 ± 1.9	2.0 ± 0.9	0.3 ± 0.3
	SKF	*21.3 ± 1.9***	*21.3 ± 1.9***	15.0 ± 2.0	3.3 ± 1.1	0.0 ± 0.0
Arc	V	33.8 ± 4.0	33.8 ± 4.0	33.5 ± 3.9	19.0 ± 3.9	*6.0 ± 1.3*
	SKF	35.8 ± 5.1	35.8 ± 5.1	35.8 ± 5.1	24.5 ± 2.5	*13.3 ± 2.6***
Me	V	106.3 ± 8.6	106.3 ± 8.6	95.5 ± 8.6	51.8 ± 10.0	25.3 ± 8.3
	SKF	118.3 ± 9.3	118.3 ± 9.3	100.0 ± 14.6	52.8 ± 14.2	25.5 ± 8.9
Pa	V	*26.3 ± 4.9*	*26.3 ± 4.9*	21.5 ± 5.6	9.0 ± 4.2	2.3 ± 1.3
	SKF	*42.5 ± 4.0***	*42.5 ± 4.0***	*42.0 ± 4.1***	13.0 ± 3.8	2.5 ± 0.9
DM	V	90.0 ± 14.9	89.3 ± 15.1	55.0 ± 23.0	30.5 ± 21.8	12.0 ± 10.7
	SKF	109.8 ± 15.6	109.8 ± 15.6	78.3 ± 22.2	33.0 ± 13.0	11.0 ± 5.0
CPu	V	*51.8 ± 12.4*	*51.8 ± 12.4*	*32.8 ± 9.8*	19.5 ± 9.2	10.3 ± 6.5
	SKF	*113.5 ± 7.4***	*113.5 ± 7.4***	*99.3 ± 7.7***	37.5 ± 7.4	7.8 ± 2.4

Data represented as mean ± SEM **$P < 0.05$ two-tailed t-test. *$P < 0.05$ one-tailed t-test.
Bold italics are used to more easily identify significant results.

binding sites, D₁ receptor immunoreactivity, D₁ receptor mRNA and localization of dopaminergic terminal fields. For example, the medial aspect of the caudate putamen exhibited a large increase in Fos immunoreactivity following SKF-38393. This is consistent with studies showing very high levels of D₁ binding (36, 37), immunoreactivity (38, 39) and receptor mRNA (19, 40, 41) as well as dopaminergic terminal fields (42) within this area.

The observation that SKF-38393 increases Fos expression in the CPu is also consistent with observations that SKF-38393 increased Fos mRNA in the CPu of mice (43) and that the D₁ agonist A-77636 increased Fos immunoreactivity in the CPu of rats (44). Both of these studies found that the density of Fos expression was greatest in the medial aspect of the CPu, the area in which Fos expression was measured in the current experiment. Interestingly, there is evidence that D₁ induction of Fos can be greatly enhanced when the CPu has been sensitized to D₁ receptor stimulation by lesioning of the nigrostriatal pathway (45-47). While the animals in the current experiment did not receive nigrostriatal lesions, they were exposed to estrogens which have been shown to modulate CPu D₁ receptor levels (48). Therefore, it is possible that the estradiol used in this experiment may have increased responsiveness of some CPu cells to SKF-38393.

The hypothalamic areas in which the D₁ agonist increased Fos expression (MPA, Pa, VMHVL, Arc) also have relatively high levels of D₁ receptor binding, immunoreactivity and mRNA as well as staining for dopaminergic terminal fields. D₁ binding has been reported in the MPA (37), the VMH (36), and the Arc (36). Dense immunostaining for D₁ receptors can be found in the Pa and the Arc (39). D₁ receptor mRNA has been reported in the Pa (40, 41), the VMH (19, 40), and the MPA (40). However, in the MPA, D₁ mRNA distribution is somewhat less

clear, as some have also reported no clear labeling of D₁ mRNA containing cells (41). Finally, staining for dopaminergic terminal fields has been reported in the MPA (42, 49, 50) and the Pa (42, 49, 50). It therefore appears that all of the sites which respond to i.c.v. infusions of the D₁ agonist with increased Fos expression contain either D₁ binding sites, immunostaining for D₁ receptors, or both.

Of the areas in which Fos expression did not change following D₁ receptor stimulation, only the Me has been shown to contain D₁ binding sites (36, 37) and labeling for D₁ receptor mRNA (40, 41). It is possible that the placement of the injection site of the D₁ agonist into the third ventricle was ineffective in stimulating D₁ receptors in the Me. Nonetheless, it would be unlikely that D₁ stimulation would increase Fos expression in the Me via a progestin receptor-mediated event, as progesterone itself does not seem to increase Fos expression in this area (26).

It is possible that the D₁ agonist may directly increase Fos expression in the MPA, VMH, Arc and Pa as all of these areas contain either D₁ binding sites, immunostaining for D₁ receptors or both. On the other hand, D₁ agonist-induced changes in Fos expression do not in themselves imply direct stimulation of D₁ receptors in that area. That is, 'direct' activation of cells in one area such as the VMH, may lead to an 'indirect' increase in Fos in a projection site such as the MPA. Therefore, it is likely that the D₁ agonist induces Fos expression, both directly and indirectly.

In summary, i.c.v. administration of a dopamine D₁ receptor agonist (SKF-38393) increased Fos expression in areas of the hypothalamus associated with female sexual behavior including the MPA and the VMHVL. More importantly, in the cVMHVL, the number of PR-Ir cells containing Fos increased in response to the D₁ agonist injection. The present results are consistent

FIG. 3. Representative photomicrograph of Fos immunoreactivity in the mVMHVL (A) 1 h following vehicle treatment and (B) 1 h following 100 ng SKF-38393. Magnification bar = 200 μm.
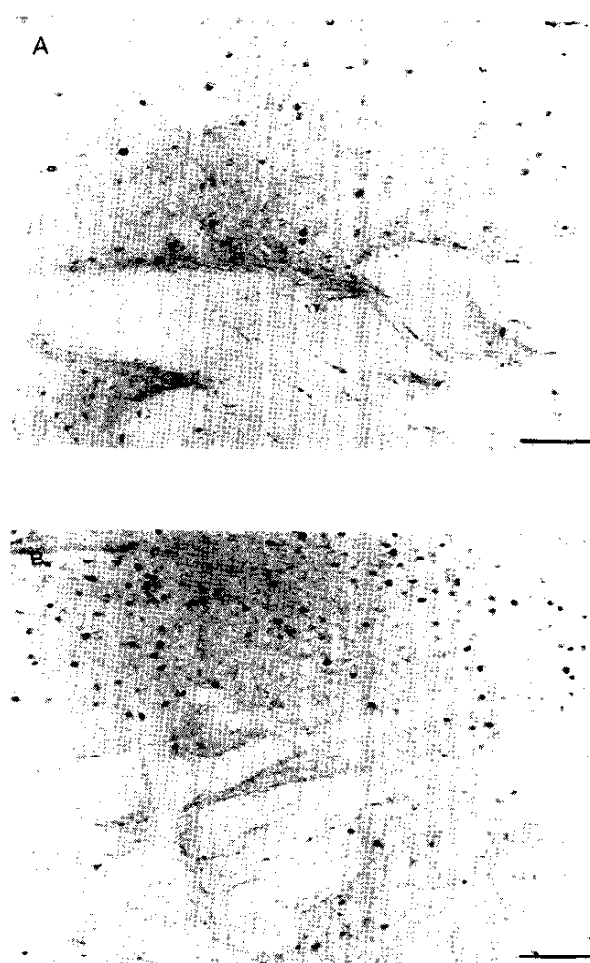

FIG. 4. Representative photomicrograph of Fos immunoreactivity in the CPu (A) 1 h following vehicle treatment and (B) 1 h following 100 ng SKF-38393. Magnification bar = 100 μm.

most likely a subset of possibly several progestin receptor-containing groups of cells that respond to D_1 agonist stimulation. Nevertheless, the increase in Fos expression in progestin receptor-containing cells in the cVMHVL is consistent with the finding that D_1 activation of sexual behavior appears to act via a progestin receptor mediated pathway (10, 25).

It is interesting that in both experiment 1 and 2 the rVMHVL-ORA and the MPO did not show any changes in Fos-Ir as these are areas that contain many PR-Ir cells. However, we expect that only a small portion of PR containing cells contain D_1 receptors and would therefore be responsive to D_1 receptor stimulation. The lack of response in the rVMHVL-ORA and MPO may suggest that the PR containing cells in these areas are not D_1 receptor responsive. Alternatively, the cells in the rVMHVL-ORA and MPO may respond to D_1 receptor stimulation without changes in Fos expression. Finally, in experiment 1, the rVMHVL did show changes in Fos-Ir while the rVMHVL-ORA did not. This is probably due to addition of the larger, non-responsive -ORA to the calculations which would have diluted the small effect seen in the more restricted rVMHVL.

The fact that SKF-38393 increased Fos expression in the Arc in experiment 1, but it did not increase Fos expression in progestin receptor-containing cells (experiment 2), suggests that the increase

in Fos expression in the Arc may not occur in progestin receptor-containing cells. It is possible that changes in Fos expression in the progestin receptor cells in the Arc were not observed due to the sensitivity differences between the immunofluorescent technique as compared to diaminobenzidine. However, we sought to minimize this by adjusting our immunofluorescent technique so that the number of fluorescently stained Fos-immunoreactive cells was very similar to the number of Fos-immunoreactive cells detected with diaminobenzidine. It is also possible that progestin receptor-containing cells in the Arc do not respond to stimulation of D_1 receptors by increased Fos expression.

Interestingly, there is evidence that progesterone and SKF-38393 have similar effects on Fos expression in the MPA and the cVMHVL. SKF-38393 and progesterone (26, 27) each increase Fos expression in the MPA. Furthermore, SKF-38393 and progesterone (28) each increase Fos immunoreactivity in progestin receptor-containing cells in the cVMHVL. These data suggest that progesterone and SKF-38393 may each induce Fos expression in some of the same neurons in the MPA and the cVMHVL.

The induction of Fos by i.c.v. administration of SKF-38393 (experiment 1) is consistent with the distribution of D_1 receptor
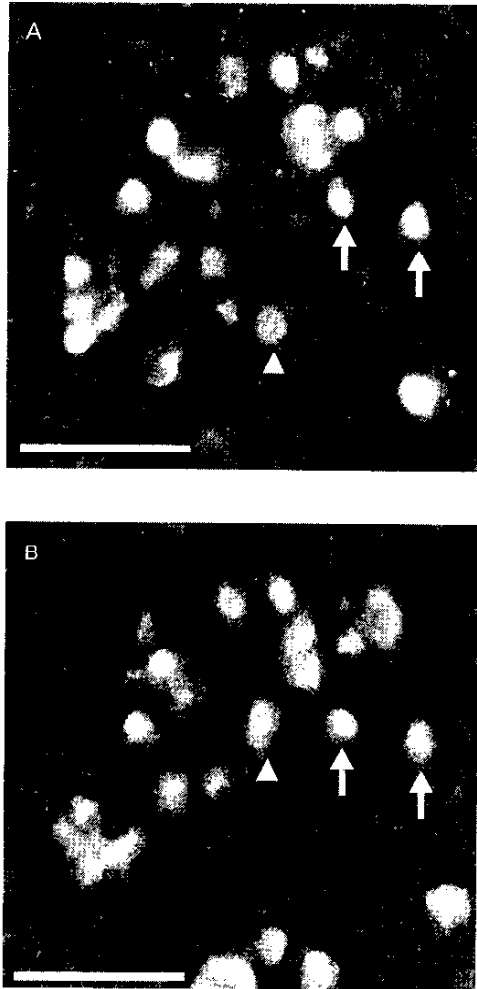
FIG. 5. Photomicrograph of (A) fluorescent PR-Ir cells and (B) fluorescent Fos-Ir cells in the same field within the MPO. Arrows point to examples of cells containing both (A) progestin receptor immunoreactivity and (B) Fos immunoreactivity. Arrowheads point to examples of cells containing only (A) progestin receptor immunoreactivity or (B) Fos immunoreactivity. Magnification bar = 50 μm.
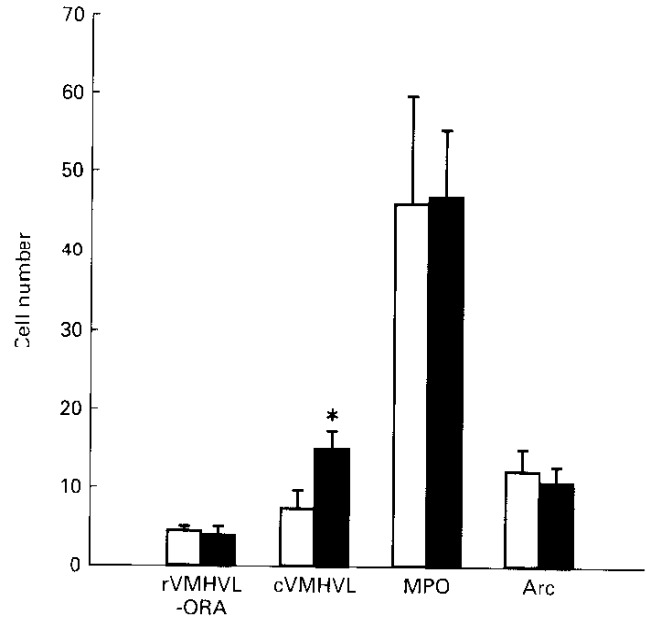

FIG. 6. Number of progestin receptor-containing cells that express Fos in female rat brain 1 h following vehicle or SKF-38393. Open bars = Saline vehicle (3 μl i.c.v.), Solid bars = 100 ng SKF-38393 i.c.v.. See Fig. 1 for nomenclature. *P < 0.05 compared to saline vehicle.

with data showing an increase in female sexual behavior following infusions of dopamine or dopamine agonists into the MPA and the VMH area (19, 22). They also suggest a neural substrate for the finding that intracerebral infusion of a D_1 agonist facilitates female sexual behavior via a progestin receptor-mediated event (10, 19, 25).

Materials and methods

Animals

Female CD VAF Sprague Dawley rats (175–200 gm) were obtained from Charles River Breeding Laboratories, Inc. (Kingston, NY, USA). Animals were group housed 4 per cage in stainless steel cages. Animals were maintained on a 14:10 light:dark cycle with lights on from 21.00 to 11.00 h and temperature maintained at approximately 22 °C. Food (Lab Diet 5001, Rodent Diet, PMI Feed, Inc., St. Louis, MO, USA) and water were available ad libitum. All animals were maintained under these conditions for 7–10 days before any experimental manipulations took

place. Following stereotaxic surgery, all animals were housed singly to prevent damage to cannulas by cage-mates. All other parameters remained constant.

Surgery

Ovariectomy: Seven to 12 days prior to experimentation rats were anesthetized with a combination of pentobarbital (31.0 mg/kg) and chloral hydrate (149.0 mg/kg). They were then ovariectomized to eliminate all endogenous gonadal steroids.

Stereotaxic surgery: Immediately following ovariectomy animals underwent stereotaxic surgery for implantation of the guide cannula. A 28 g guide cannula with a 30 g inner cannula (Plastics One, Roanoke, VA, USA) extending from the tip was placed into the brain using Bregma as a reference; AP −3.3 mm, ML 0.0 mm, and DV 8.5 mm. All coordinates were based on the rat stereotaxic atlas of Paxinos & Watson (51). These coordinates placed the tip of the guide cannula directly above the third ventricle. The infusion cannula was designed to extend 1 mm beyond the tip of the guide cannula within the third ventricle (Fig. 7). Animals were fitted with a removable dummy inner cannula to prevent contaminants from entering the guide cannula.

Experimental protocol

Following surgery, all animals were handled daily. This included removing and reinserting the dummy cannula to acclimate them to the injection procedure. Seven to ten days following surgery animals were injected subcutaneously with 5 μg estradiol benzoate (EB; 11.30 h). Forty-eight to 50.5 h later (11.30–14.00 h) rats were injected i.c.v. via the guide/injection cannula assembly with either 100 ng SKF-38393 in 3 μl sterile saline or the vehicle. Injections lasted 120 s, which included a 60 s injection period followed by a 60 s waiting period to ensure that the entire treatment volume had entered the third ventricle. The injection cannula was then removed and the dummy cannula was replaced. In order to minimize any non-specific Fos induction all injections were done in the animal's home room and cage under red light. One hour following i.c.v. injections (12.30–15.00) animals were perfused. In experiment one, brain sections were stained for Fos immunoreactivity using diaminobenzidine. In experiment two, adjacent sections from the same animals were stained for both Fos and progestin receptors using a double label immunofluorescent technique.
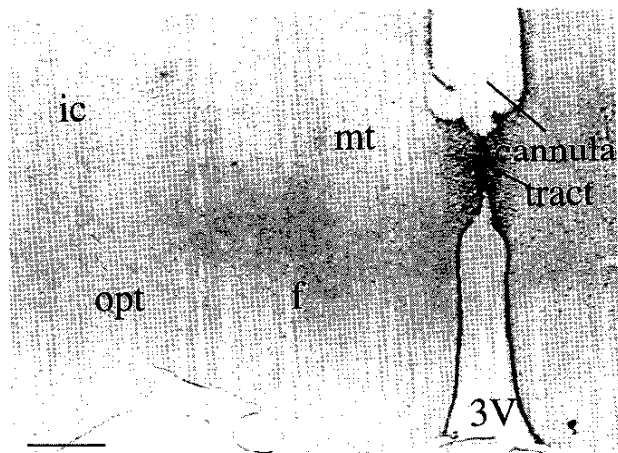
FIG. 7. Photomicrograph of cannula tract caused by guide and injection cannula. Magnification bar = 500 μm. See Fig. 1 for nomenclature.

Perfusion

All animals were injected with a lethal combination dose of pentobarbital (8.9 mg) and chloral hydrate (42.5 mg). When animals were deeply anesthetized, the thoracic cavity was exposed, the thoracic aorta and posterior vena cava clamped and 1 ml of saline containing 5,000 IU of heparin was injected into the left ventricle. Incisions were made in the right atrium and the left ventricle followed by insertion of a cannula through the left ventricle into the aorta. Twenty-five ml of physiological saline were flushed through the brain before the flow of 2% acrolein (in 0.1 M NaPO₄ buffer; pH 7.2) began. Perfusion pressure was maintained at approximately 100 mm Hg at a flow rate of 25 ml/min for 14 min. Following removal from the cranium, blocked brains were stored overnight at 4 °C in 0.1 M sodium phosphate buffer (pH 7.2 at 4 °C) containing 20% sucrose. Forty μm sections were cut through the hypothalamus using a freezing rotary microtome and were stored in cryoprotectant at −20 °C until preparation for immunocytochemistry.

Diaminobenzidine immunocytochemistry

For each animal, a set of one in four sections was removed from cryoprotectant and rinsed 3 times for 5 min each in 0.5 M Tris-buffered saline (TBS, pH 7.6, 22 °C). Sections were then pretreated in 1% sodium borohydride for 10 min to remove residual aldehydes. Following pretreatment and three additional rinses in TBS for 5 min each, sections were placed into 1% H₂O₂, 20% normal goat serum, and 1% bovine serum albumin for 20 min to reduce nonspecific staining and endogenous peroxidase activity. Sections were then incubated in rabbit polyclonal c-fos antiserum directed against the N-terminal of the Fos protein (0.05 μg/ml; SC 52, Santa Cruz Biotechnology, Inc., Lot# 1283, Santa Cruz, CA, USA) diluted in modified TBS (mTBS; pH 7.6, 4 °C) containing 0.1% gelatin, 0.02% sodium azide, 0.5% Triton X-100 and 1% normal goat serum for 3 days at 4 °C. Following 3, 5 min rinses with mTBS buffer (pH 7.6, 22 °C), tissue sections were incubated in secondary antiserum (3 μg/ml, biotinylated goat anti-rabbit, Vector, lot# D1117) diluted in mTBS (pH 7.6, 22 °C) for 90 min at room temperature. Following 2 washes in mTBS (pH 7.6, 22 °C) and one wash in TBS, the sections were incubated for 90 min at room temperature in Avidin DH: biotinylated horseradish peroxidase H (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA, USA). Following 3 washes in TBS. the sections were treated with diaminobenzidine (0.05% in TBS) in the presence of hydrogen peroxide (0.05%) for approximately 10 min and then rinsed twice in TBS. All Fos immunostaining was eliminated by omission of primary antibody from the immunocytochemical procedure.

Double-labeled fluorescent immunocytochemistry

For each animal, a set of 1 in 4 sections was removed from cryoprotectant and rinsed 3 times for 5 min each in TBS. Sections were pretreated in 1% sodium borohydride for 10 min to remove residual aldehydes. Following pretreatment and three additional 5 min rinses in TBS, sections were

placed into 1% H₂O₂, 20% normal goat serum, and 1% bovine serum albumin for 20 min to reduce nonspecific staining and endogenous peroxidase activity. Sections were incubated in a cocktail of: 1) rabbit polyclonal c-fos antiserum directed against the N-terminal of the Fos protein (3 μg/ml; SC 52, Santa Cruz Biotechnology, Inc., Lot# H024, Santa Cruz, CA, USA) and 2) mouse monoclonal progestin receptor antibody directed against the hinge region of both A and B progestin receptor isoforms (3 μg/ml; H928, StressGen, Biotechnologies Corp., Lot# 1.0, Victoria, BC, Canada) diluted in mTBS (pH 7.6, 4 °C) containing 0.1% gelatin, 0.02% sodium azide, 0.5% Triton X-100 and 1% normal goat serum for 3 days at 4 °C. Following 3, 5 min rinses with mTBS buffer (pH 7.6, 22 °C), tissue sections were incubated in a cocktail of fluorescently-labeled secondary antiserum: 1) Texas Red goat anti-rabbit serum (5 μg/ml; Jackson ImmunoResearch, Inc., West Grove, PA, USA) for detection of Fos immunoreactivity and 2) fluorescein isothiocyanate (FITC) goat anti-mouse serum (5 μg/ml; Jackson ImmunoResearch, Inc.) for detection of progestin receptor immunoreactivity diluted in mTBS (pH 7.6, 22 °C) for 90 min at room temperature. To increase the signal, the fluorescently-labeled secondary antiserum was bridged using the following procedure (32). Following 3 rinses, sections were placed in mTBS (pH 7.6, 22 °C) containing 1) rabbit (5 μg/ml, Sigma Chemical, St Louis, MO, USA) and 2) mouse (5 μg/ml, Sigma Chemical) immunoglobulins for 90 min at room temperature. Sections were then rinsed 3 times for 5 min each in mTBS (pH 7.6, 22 °C) and placed back into fluorescently-labeled secondary antiserum for 60 min. The bridging sequence was repeated 3 times. After bridging of the fluorescently-labeled secondary antiserum, sections were rinsed 3 times for 5 min each in TBS (pH 7.6). Sections were mounted onto glass slides with 0.25 M n-Propyl gallate in glycerol to reduce photobleaching (52). Fos immunostaining was eliminated, and progestin receptor immunostaining was unaffected by omission of the Fos primary antibody from the immunocytochemical procedure. Progestin receptor immunostaining was eliminated, and Fos immunostaining was unaffected following omission of the progestin receptor primary antibody from the immunocytochemical procedure. All staining was eliminated following the omission of both primary antibodies from the immunocytochemical procedure. Crossreactivity between primary and secondary antisera appeared negligible as no staining occurred when Fos primary antiserum was incubated with progestin receptor secondary antiserum and vice versa. Finally, it is clear from our observations that under the colocalization proceedures we observed numerous PR-Ir cells which contained no Fos-Ir and vice versa (see Fig. 5).

Areas analyzed

Eleven areas throughout the female rat forebrain were examined bilaterally for the induction of Fos immunoreactivity by SKF-38393 (Fig. 1). The areas included: the CPu, MPA, MPO, Pa, rVMHVL, rVMHVL-ORA, mVMHVL, cVMHVL, DM, Arc, and the Me. Bilateral measurements consisting of 600 μm × 800 μm areas were analyzed for the CPu, MPO, rVMHVL-ORA, DM and Me. The MPA areas measured 800 μm × 1,200 μm. The VMHVL areas analyzed consisted of bilateral 313 μm × 375 μm areas. The shapes of the Pa and the Arc areas that were analyzed were determined separately for each section based on phase contrast microscopy.

Computer-assisted image analysis

All computer-assisted image analysis was done using a Leitz Dialux 20 microscope (Ernst Leitz Wetzler GMBH, Germany) attached to a MTI CCD 72 camera (DAGE MTI, Michigan City, IN, USA) which was in turn connected to a Macintosh Quadra 700 (Apple Computer, Cupertino, CA, USA). The Image 1.55 program (W. Rasband, NIH, Bethesda, MD, USA) was used for all image processing and analysis.

Prior to each imaging session the following protocol was used. The microscope was adjusted for Kohler illumination and fitted with a Wratten gelatin filter (Eastman Kodak Company, Rochester, NY, USA), as this provided the best image transfer from the brown staining of diaminobenzidine to the gray scale used in image analysis. The microscope and camera were focused on a coverslipped slide with a black mark on it. The camera gain and black levels were adjusted so that the blank portion of the slide produced gray levels within the single digits while the black mark produced a gray level of 255. This effectively calibrated the camera to use the full range of gray levels available to the imaging program (0–255, with 0 being white and 255 being black). All images for an area were taken during the same session.

Cell like objects were excluded from analysis if they were less than 10 pixels or greater than 200 pixels in area at 10× magnification. The mean pixel density and the standard deviation was obtained for the area analyzed within each image. The density threshold for each image was set at 5 SDs above the mean for the area within the image. Total cell number and the average maximal optical density (MOD) for each image were analyzed. MOD was determined by measuring the darkest point of each cell (the highest gray level) and determining the mean for all cells within a given image. The MOD for each cell was invariably found within the nucleus of the cell. Using this technique, computer-assisted cell counts were indistinguishable from counts done by eye alone.

Data analysis and statistics

Data collected from image analysis were analyzed based on two criteria. The first criterion examined whether Fos-Ir cell number was affected by treatment. The second criterion examined whether optical density of Fos-Ir cells was influenced by the treatment. To analyze optical density, the MOD for each cell from an image was recorded. We then created a frequency distribution histogram for each image based on the MOD of each cell. From these histograms we set various optical density thresholds to analyze the data. These optical density thresholds included all cells with maximum optical densities greater than the gray level of 50, 100, 150 and 200. For the analysis of Fos induction two tailed Student *t*-tests were run to compare experimental groups to vehicle groups. One tailed Student *t* tests were used in making comparisons within the Fos/progestin receptor co-localization experiment. Data were considered statistically different at P < 0.05.

Acknowledgements

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