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D_1-dopamine receptors activate c-*fos* expression in the fetal suprachiasmatic nuclei

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ABSTRACT **The existence of an activatable dopamine system within the hypothalamic suprachiasmatic nuclei (SCN), the site of a biological clock, was investigated in rats during fetal life. *In situ* hybridization studies revealed that D_1-dopamine receptor mRNA was highly expressed in the fetal SCN and not expressed in other hypothalamic regions. Cocaine injected into pregnant rats or directly into rat fetuses on day 20 of gestation selectively activated c-*fos* gene expression in the fetal SCN; cocaine did not induce c-*fos* expression elsewhere in the fetal brain or in the maternal SCN. This cocaine-induced activation of c-*fos* expression in fetal SCN was mediated in part through D_1-dopamine receptors, as the cocaine-induced activation was partially blocked by the D_1-dopamine receptor antagonist SCH 23390. In addition, the selective D_1-dopamine receptor agonist SKF 38393 induced high levels of c-*fos* expression in the fetal SCN. The presence of an activatable dopamine system within the fetal SCN provides a mechanism through which maternal signals could entrain the fetal biological clock and through which maternally administered psychotropic drugs could alter normal development of the circadian timing system.**

The hypothalamic suprachiasmatic nuclei (SCN) function as a biological clock that generates circadian rhythms in mammals (1–3). Retinal pathways transmit sensory information about the daily light–dark cycle from the eye to the SCN. This photic information entrains the biological clock so that circadian rhythms are expressed in proper relationship to each other and the 24-hr day. An entrained biological clock plays a major role in optimizing the efficiency of biological systems and better prepares the body to cope with stress, injury, and disease.

In fetal rats, the SCN are functioning as a biological clock soon after SCN neurons have migrated to their final cellular position (4, 5). At this stage of development, retinal pathways to the SCN have not yet matured. Instead, the mother transduces photic information into circadian signals that entrain the timing (phase) of the fetal biological clock to prevailing environmental light–dark conditions. It appears that multiple maternal signals are capable of entraining the fetus and that these signals normally operate in concert (4, 5). Specifically, periodic feeding can entrain the fetuses of SCN-lesioned pregnant rats (6), and pharmacological doses of the pineal hormone melatonin can entrain the fetuses of SCN-lesioned pregnant hamsters (7). Unknown are the cellular and molecular mechanism(s) through which these signals normally act to entrain the fetal SCN.

As part of our ongoing efforts to delineate functionally relevant neurotransmitter systems within the fetal SCN that may participate in maternal–fetal entrainment, we discovered a high level of D_1-dopamine receptor gene expression in the biological clock of fetal rats. We further report that activation of D_1-dopamine receptors in the fetal SCN induces c-*fos* gene expression. This activation may have important consequences for normal circadian development.

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MATERIALS AND METHODS

Animals. Timed-pregnant Sprague–Dawley rats (Zivic–Miller) were shipped to us between days 8 and 15 of gestation (day 0 = day of insemination). Animals were housed one or two per cage in a light–dark cycle consisting of 12 hr of light per day, with lights on from the hours of 0700 to 1900 eastern standard time.

***In Situ* Hybridization Histochemistry.** Animals were killed by decapitation. Heads (fetuses) or brains (adults) were frozen in cooled (−20°C) 2-methylbutane and stored at −80°C until sectioning. Coronal 15-μm sections were cut in a cryostat, thaw-mounted onto slides coated with Vectabond (Vector Laboratories), and air-dried. Slides were then refrozen and stored at −80°C.

Antisense and sense complementary RNA (cRNA) probes were generated by digestion of plasmids with the appropriate restriction endonuclease, followed by *in vitro* transcription with T7, T3, or SP6 RNA polymerase (Promega) in the presence of uridine 5′-[α-[^{35}S]thio]triphosphate (New England Nuclear). The *in situ* hybridization method for slide-mounted sections was essentially identical to that previously described (8). The specificity of the hybridizing signals was determined by incubation of adjacent sections with sense cRNA probes generated from the same plasmids; this resulted in only background levels of hybridization. The plasmid containing a portion of the rat D_1-dopamine receptor cDNA fragment was obtained from J. S. Fink (Massachusetts General Hospital, Boston). The plasmid containing the mouse c-fos cDNA was obtained from M. E. Greenberg (Harvard Medical School, Boston).

For image analysis, section(s) with the most intense hybridization signal in the SCN were selected from each fetal brain; sections were examined at 45- to 90-μm intervals with each probe. The optical density (OD) of the fetal SCN and adjacent hypothalamus were determined using the Drexel University Image Processing Center "Brain Software Package" run on an IBM-AT computer. The relative OD of SCN (OD of SCN/OD of adjacent hypothalamus) was then calculated. The values obtained by image analysis of film autoradiograms were linearly related to grain density in emulsion autoradiograms ($R = 0.84$; $P < 0.01$).

Northern Blot Analysis. Total cellular RNA was isolated using guanidium thiocyanate, and poly(A)$^+$ RNA was isolated using an oligo(dT) column. Poly(A)$^+$ RNA was fractionated on a 1% agarose/formaldehyde gel and transferred to GeneScreen (New England Nuclear). Blots were hybridized sequentially with ^{32}P-labeled probes (specific activity, $>10^9$ cpm/μg) generated from the mouse c-fos cDNA and the

Abbreviations: SCN, suprachiasmatic nuclei; cRNA, complementary RNA; GD, gestational day.

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mouse actin cDNA and washed at high stringency as described (8). The final washing of the blots was in 0.2× standard saline citrate/0.1% SDS at 65°C for 40 min. Blots were exposed at −80°C to x-ray film with an intensifying screen.

Drugs. SCH 23390 and SKF 38393 were purchased from Research Biochemicals (Natick, MA). Cocaine was purchased from Sigma.

RESULTS

D_1-dopamine receptor gene expression was mapped in fetal and maternal brains by *in situ* hybridization with a 201-base-pair antisense cRNA probe complementary to a fragment of the coding region of the rat D_1-dopamine receptor. Analysis of film autoradiographs showed that D_1-dopamine receptor mRNA is highly expressed throughout the fetal SCN on gestational days (GDs) 18 and 20 and is not apparent in other hypothalamic regions (Fig. 1); GD 18 is the earliest age the SCN can be consistently delineated by light microscopy. D_1-dopamine receptor mRNA levels in the fetal SCN were higher than those found in the adult SCN or fetal striatum but were comparable to the high levels of expression in adult striatum (Fig. 1). In contrast to the fetal hypothalamus, D_1-dopamine receptor mRNA expression is more widespread in the hypothalamus of adult rats, with D_1-dopamine receptor mRNA being expressed in the supraoptic and paraventricular nuclei to a similar degree as in the SCN (ref. 9; D.R.W. and S.M.R., unpublished data).

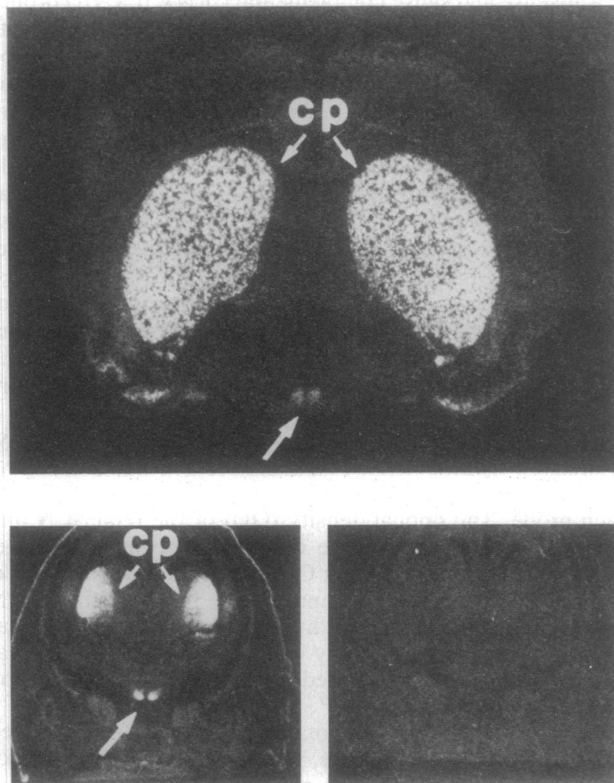

FIG. 1. Dark-field photomicrographs of film autoradiograms depicting D_1-dopamine receptor mRNA in the maternal and fetal SCN. (*Upper*) D_1-dopamine receptor mRNA distribution in a coronal section of maternal rat brain through the SCN (arrows) and caudate putamen (CP). (*Lower left*) D_1-dopamine receptor mRNA distribution in a coronal section of fetal brain on GD 18. (*Lower right*) Adjacent section of fetal rat brain hybridized with a sense-strand (control) cRNA probe. Comparable results were obtained with other GD 18 (n = 4) and GD 20 (n = 6) fetuses.

We next examined whether D_1-dopamine receptors in the fetal SCN can be activated. In the striatum of adult rats, pharmacological activation of D_1-dopamine receptors induces c-*fos* gene expression (10, 11). The products of immediate-early genes like c-*fos* act as third messengers, inducing long-term alterations in gene expression in response to acute neurochemical input (12). For our studies, we administered cocaine to pregnant rats on GD 20 and examined c-*fos* expression in the fetal and maternal brains 40 min after injection. We used cocaine because it acts on monoamine release and reuptake at presynaptic terminals (13, 14), so that only receptors receiving an endogenous monoaminergic input should be affected by cocaine. c-*fos* gene expression was examined in fetal and maternal brains by *in situ* hybridization using a 1.8-kilobase antisense cRNA probe generated from the mouse c-fos cDNA.

Maternally administered cocaine activated c-*fos* gene expression in the fetal SCN (Fig. 2) in a dose-dependent manner (Fig. 3*A*; $P < 0.0001$, Kruskal–Wallis analysis of variance). Remarkably, c-*fos* expression was selectively increased in the SCN; analysis of film autoradiographs of fetal brain atlases revealed that cocaine did not induce high levels of c-*fos* expression elsewhere in the fetal brain (sections examined at 90-μm intervals; data not shown). The induction of c-fos mRNA in the fetal SCN appeared to be the result of a direct action of cocaine within the fetus, because cocaine (30 mg/kg, i.p.) administered directly to rat fetuses delivered by cesarean section on GD 20 also induced c-*fos* expression in SCN (Fig. 3*B*). The ability of cocaine to induce c-*fos* expression in the fetal biological clock was not dependent on the time of day of administration; cocaine induced c-*fos* expression in the SCN of fetuses from dams injected on GD 20 at night between the hours of 0200 and 0500 ($P < 0.001$, Mann–Whitney U test; data not shown) to the same degree as cocaine induced c-*fos* expression when administered during the day between the hours of 1400 and 1700. As expected (10, 11), cocaine activated c-*fos* expression in the maternal striatum (data not shown). There was no induction of c-fos mRNA in the maternal SCN after either day- or nighttime injections of cocaine ($P > 0.05$; data not shown).

To examine whether cocaine induces c-*fos* expression in the fetal SCN through D_1-dopamine receptors, the D_1-dopamine receptor antagonist SCH 23390 (0.2 mg/kg, i.p.) was administered 30 min prior to cocaine treatment. The antagonist reduced the cocaine-induced induction of c-fos mRNA in the fetal SCN by 50% ($P < 0.001$, Mann–Whitney U test; Fig. 3*C*) and prevented induction of c-fos mRNA in maternal striatum (data not shown; as reported in ref. 10). In addition, the D_1-dopamine selective agonist SKF 38393 (10 mg/kg, i.p.) induced high levels of c-*fos* gene expression in the fetal (but not maternal) SCN (Fig. 3*D*). Northern blot analysis of fetal anterior hypothalami containing SCN revealed a hybridizing transcript of 2.6 kilobases after SKF 38393 treatment that was not apparent in the hypothalami of vehicle-treated animals (Fig. 4).

DISCUSSION

The results clearly show that an activatable dopamine system exists within the fetal biological clock. Cocaine induction of c-*fos* expression in the fetal SCN occurs in part through D_1-dopamine receptor activation, as shown with the D_1-dopamine antagonist and agonist studies. The inability of SCH 23390 to completely block cocaine-induced c-*fos* expression may be due to inadequate antagonist levels in fetal brain, a direct action of cocaine on D_1-dopamine receptors, or the existence of other transmitter systems in the fetal SCN that can be activated by cocaine.

The functional consequences of D_1-dopamine-receptor-mediated c-*fos* induction in fetal SCN may be substantial. In

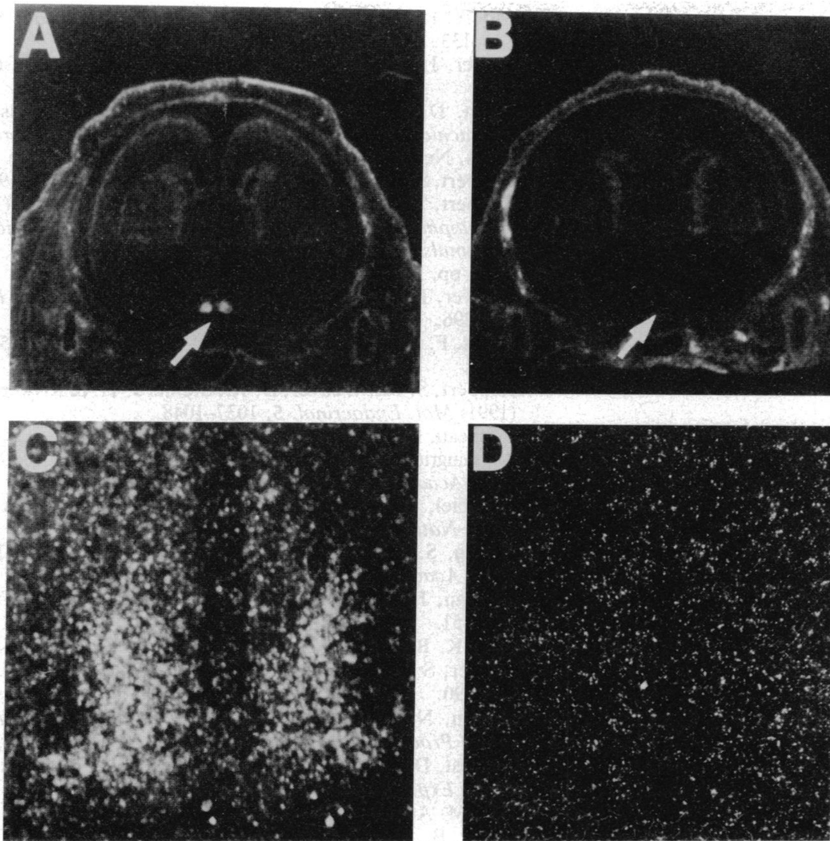

FIG. 2. Cocaine activates c-*fos* expression in fetal SCN. (*A* and *B*) Low-power dark-field photomicrographs of film autoradiograms of coronal sections of fetal brain (GD 20) depicting c-fos mRNA 40 min after maternal injection of cocaine (30 mg/kg) (*A*) or saline vehicle (*B*). Arrows depict location of SCN. (*C* and *D*) Dark-field photomicrographs of emulsion autoradiograms depicting c-fos mRNA in fetal SCN after maternal injection of cocaine (*C*) or vehicle (*D*). (The width of *A* and *C* are 1.0 cm and 1.2 mm, respectively.)

the adult SCN, light exposure at night induces expression of c-fos mRNA and Fos protein (15–20) and causes phase shifts in circadian rhythms (1–3). Furthermore, the quantitative features of light-induced c-*fos* expression and light-induced phase shifts are very similar, suggesting that Fos may be a molecular component of the photic entrainment pathway for mammals (19, 20). We have recently shown by Western blot analysis that Fos protein is expressed in the fetal hypothalamus (unpublished data). Thus, an activatable fetal dopamine system may represent an important unexpected mechanism for maternal signals to entrain the fetal SCN. Indeed, a dopamine system in the fetal hypothalamus may represent a

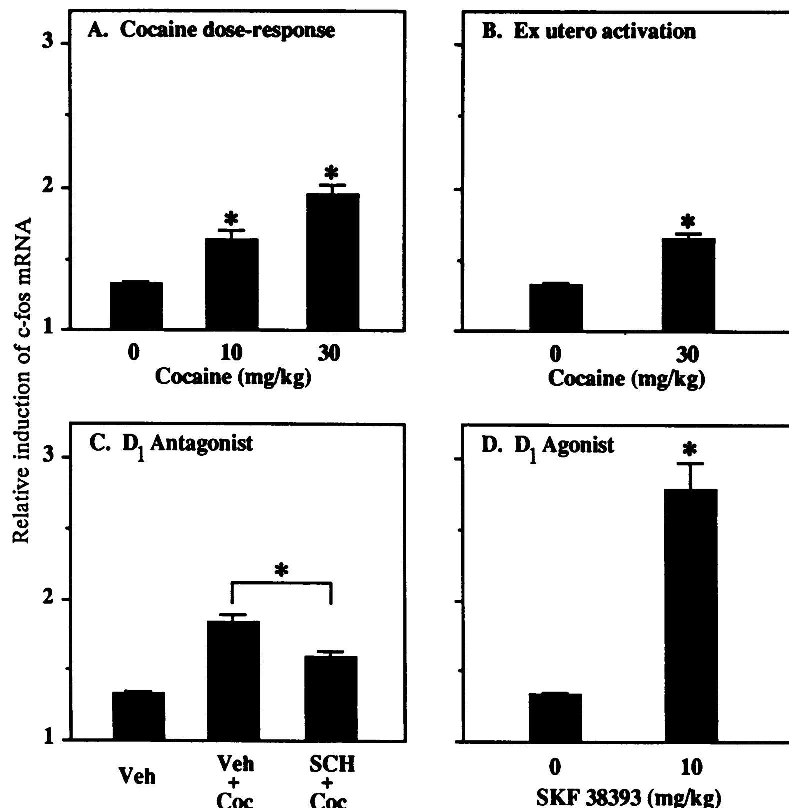

FIG. 3. Relative induction of c-*fos* gene expression in the fetal SCN. (*A*) Cocaine dose–response experiment. Pregnant dams (GD 20) were treated with cocaine (10 or 30 mg/kg) or saline vehicle. (*B*) *Ex utero* activation. Fetuses delivered by cesarean section on GD 20 were treated with cocaine (30 mg/kg) or saline vehicle. (*C*) D_1-dopamine receptor antagonist. Dams (GD 20) were treated with the D_1-dopamine antagonist SCH 23390 (0.2 mg/kg, SCH) or vehicle (Veh) 30 min before injection of cocaine (30 mg/kg, Coc). (*D*) D_1-dopamine receptor agonist. Dams (GD 20) were treated with the D_1-dopamine receptor agonist SKF 38393 (10 mg/kg) or saline vehicle. Fetal brains were collected 40 min after injection. In *A–D*, bars represent the mean ± SEM of 6–15 fetuses (3–4 fetuses per dam). The relative OD of the SCN in representative sections hybridized with the sense (control) c-fos probe was 1.02 ± 0.01 (n = 24) and did not vary by treatment. The experiments in *A*, *C*, and *D* were performed, hybridized, and processed simultaneously, and the data from the single vehicle control group is replotted in each of these panels. *, Significant difference from control of group indicated, $P <$ 0.001, Mann–Whitney U test.

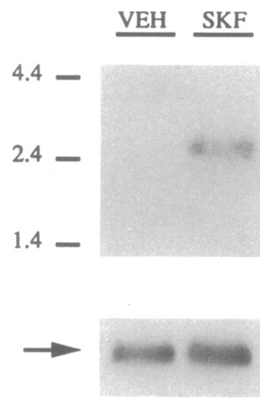

FIG. 4. (*Upper*) Northern blot analysis of c-*fos* induction in fetal hypothalamus by SKF 38393. Fetal hypothalami (10 per batch) containing SCN were collected 40 min after injection of SKF 38393 (10 mg/kg) or vehicle (VEH) into dams and processed for poly(A)$^+$ RNA. Each lane contained 3 μg of RNA. Locations of RNA size markers are indicated on the left in kilobases. (*Lower*) Hybridization pattern using an actin probe to verify equal loading of lanes (arrow).

final common pathway by which entraining information reaches the fetal SCN. As previously mentioned, the two identified entraining signals are rhythmic food ingestion and timed injections of melatonin (6, 7). The rhythmic ingestion of food by the mother could provide a rhythmic precursor pool of tyrosine, driving rhythmic fetal brain dopamine production (21, 22); maternally derived melatonin could directly modulate the release of dopamine in the fetal SCN, as demonstrated in other systems (23, 24). Melatonin receptors are expressed in the fetal SCN (25, 26).

There are two distinct differences in c-*fos* inducibility in SCN between fetuses and adults. In adult rodents, the photic induction of Fos appears to be mediated through *N*-methyl-D-aspartate receptors (27), whereas c-*fos* expression in the fetus is induced through D_1-dopamine receptors. In addition, c-*fos* induction in adults only occurs at times when light causes phase shifts (15–20), whereas c-*fos* induction in the fetus does not appear to vary over the course of the day. Further comparison of the nature and consequences of the induction of c-*fos* and other immediate early genes between fetuses and adults may help advance our understanding of the molecular basis of entrainment.

The c-*fos* response to D_1-dopamine receptor stimulation in the fetal SCN is apparent on GD 18 (data not shown) when the nuclei are morphologically immature and virtually devoid of synapses (28–30). Nonetheless, tyrosine hydroxylase-positive neurons and fibers have been reported in the SCN region of the fetal brain (31–33) and could provide a source of dopamine for D_1-receptor activation within the SCN. These tyrosine hydroxylase-positive neurons and fibers disappear around birth and are infrequent in the adult SCN (34). Thus, developmental changes in the dopaminergic system within the SCN may underlie the differential response to dopaminergic stimulation in the fetus and adult.

The discovery of a functional dopamine system within the fetal SCN suggests that the fetal biological clock may be particularly susceptible to the effects of maternally administered psychotropic agents, including drugs of abuse. For example, repeated cocaine use during pregnancy may cause repeated phase shifts in the developing biological clock resulting in "fetal jet lag." This alteration in circadian function might, in turn, contribute to the long-term behavioral abnormalities associated with fetal cocaine exposure (35–38). In broader terms, the fetal SCN may prove to be a model system for examining the neurochemical and subsequent behavioral effects of psychotropic agents that act through D_1-dopamine receptors.

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