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5-Hydroxytryptamine$_{1B}$ Receptors Modulate the Effect of Cocaine on c-*fos* Expression: Converging Evidence Using 5-Hydroxytryptamine$_{1B}$ Knockout Mice and the 5-Hydroxytryptamine$_{1B/1D}$ Antagonist GR127935

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SUMMARY

Serotonergic transmission has been suggested to modulate the effects of cocaine. However, the specific receptors and brain structures underlying this phenomenon have not been identified. To test the possible contribution of the 5-hydroxytryptamine$_{1B}$ (5-HT$_{1B}$) receptor, we studied the induction of the immediate-early gene c-*fos* elicited by cocaine in knockout mice lacking this receptor. 5-HT$_{1B}$ knockout mice display a markedly reduced effect of cocaine on c-*fos* induction in different brain structures, most notably in the striatum. In addition, the administration to wild-type mice of the 5-HT$_{1B}$ receptor agonist RU24969 results in a striatal induction of c-*fos* expression very similar to that induced by cocaine in its time course, cellular and anatomical distribution, and pharmacology. Here, we also report the ability of a 5-HT$_{1D}$ receptor antagonist, GR127935, to antagonize 5-HT$_{1B}$ receptors *in vivo*. Finally, when administered to wild-type mice, GR127935 reduces the increase in striatal c-*fos* expression elicited by cocaine. These converging lines of evidence obtained with the knockout mice and 5-HT$_{1B/1D}$ antagonist indicate that cocaine acts as an indirect agonist of 5-HT$_{1B}$ receptors *in vivo* and demonstrate that activation of 5-HT$_{1B}$ receptors contributes to the cellular responses elicited by cocaine.

Cocaine is a potent and widely abused psychostimulant that exerts its behavioral and neurophysiological effects through inhibition of the dopamine, 5-HT (serotonin), and norepinephrine reuptake systems (1). A large body of evidence has demonstrated that the reinforcing and locomotor activating effects of cocaine are mediated by the mesostriatal and mesolimbic dopamine pathways (2). However, a significant contribution of serotonergic neurotransmission in mediating the behavioral and neurophysiological effects of cocaine has also been suggested. Agents that activate dopamine or norepinephrine systems, or both, but are devoid of strong serotonergic activity are neither intensively abused by humans nor avidly self-administered by animals (3). Global alterations of 5-HT transmission in rodents result in a variety of changes in cocaine-induced behaviors (4–6). In humans, depletion of the 5-HT precursor tryptophan attenuates both the euphorigenic and anxiogenic effects of intranasal cocaine (7) and reduces cue-induced craving for cocaine (8).

5-HT transmission in the ventral midbrain has a positive modulatory effect on the functional activity of the mesotelencephalic dopaminergic system (9, 10). Although a variety of 5-HT receptors might be involved in this phenomenon, several lines of evidence point toward a prominent role of ventral midbrain 5-HT$_{1B}$/5-HT$_{1D\ \beta}$ receptors (the 5-HT$_{1B}$ receptor is the rodent homologue of the human 5-HT$_{1D\beta}$ receptor; for a review, see Ref. 11). These receptors are the most abundant 5-HT receptors in the ventral midbrain (12, 13), and their stimulation results in disinhibition of dopaminergic neurons in the substantia nigra and VTA (14, 15) and in facilitation of dopamine release in the CPu and NAc (9, 16).

The immediate-early gene c-*fos* is induced in the central nervous system by many external stimuli and drug treatments and can be used as a molecular marker of neuronal activation (17). Cocaine has been shown to induce c-*fos* in many brain regions (18, 19), including the CPu and the NAc, in which a stimulatory effect of the 5-HT$_{1B}$ receptor has been suggested. Induction of c-*fos* has been postulated to mediate

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; GABA, γ-aminobutyric acid; VTA, ventral tegmental area; CPu, caudate putamen; NAc, nucleus accumbens; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)-tetralin; IR, immunoreactivity; PBS, phosphate-buffered saline; S-CM-G-^{125}I-TNH$_2$, serotonin-*O*-carboxymethyl-glycyl-^{125}I-tyrosinamide; NMDA, *N*-methyl-D-aspartate.

changes in gene expression that lead to long term adaptations of the central nervous system to external stimuli (17). It is therefore possible that cocaine-elicited induction of c-*fos* and related proteins contribute to the long term neurochemical and behavioral changes induced by cocaine (20).

To test the hypothesis that 5-HT_{1B} receptors play a role in mediating the actions of cocaine, we studied how inactivation of these receptors affects the induction of c-*fos* elicited by cocaine. No 5-HT_{1B}-selective antagonists have been developed so far (for a review, see Ref. 13). However, knockout mice that lack a specific neurotransmitter receptor constitute a new and complementary approach to pharmacological techniques. We have applied this approach successfully to the field of 5-HT receptors by generating mice that lack the 5-HT_{1B} receptor (21). These mice have become a useful tool for probing 5-HT_{1B} receptor-mediated behaviors and physiological effects (21–23). Here, we show that 5-HT_{1B} knockout mice display a markedly reduced effect of cocaine on c-*fos* induction and that the 5-HT_{1B} agonist RU24969 induces c-*fos* in brain areas in which knockout mice showed a reduced induction by cocaine. Finally, we report the ability of a 5-HT_{1D} receptor antagonist, GR127935, to antagonize 5-HT_{1B} receptors *in vivo* and reduce the induction of c-*fos* elicited by cocaine. The converging lines of evidence obtained with the knockout mice and the 5-$HT_{1B/1D}$ antagonist demonstrates that cocaine acts as an indirect agonist at 5-HT_{1B} receptors *in vivo* and that activation of 5-HT_{1B} receptors contributes to the cellular responses elicited by cocaine.

Materials and Methods

Animals. All experiments were performed on adult wild-type and 5-HT_{1B} -knockout 129/Sv-ter inbred mice. The 5-HT_{1B} mutant mice were generated as previously described (21). Both the wild-type and mutant mice were bred at the New York State Psychiatric Institute animal facility and identified by genomic Southern analyses of tail biopsies (21). Mice were housed four to a cage with food and water available *ad libitum* and maintained in a temperature-controlled environment on a 12-hr/12-hr light/dark cycle with lights on at 7:00 a.m.

Drug treatment. Drugs were administered intraperitoneally (1.0 ml/kg). Drugs were dissolved in isotonic saline or another appropriate vehicle just before use. For *in vivo* pharmacology studies, specific antagonists were administered 30 min before the injection of cocaine or RU24969. Haloperidol, GBR12909, 8-OH-DPAT, SCH 23390, MK-801, and Baclofen were purchased from Research Biochemicals (Natick, MA); cocaine was purchased from Sigma Chemical (St. Louis, MO); RU24969 was a gift from Rousell Uclaf (Romainville, France); GR127935 was a gift from Glaxo Group Research Limited (Greenford, UK); and WAY-100635 was a gift from Wyeth Ayerst (Princeton, NJ).

Northern blot analysis. Animals were killed by rapid decapitation 45 min after injection. Brains were removed and rinsed in ice-cold PBS for 1 min before dissection. Striatum and cerebellum were dissected, and total RNA was prepared using the Ultraspec RNA kit by Biotecx (Houston, TX). Twenty micrograms of total RNA was electrophoresed on a 1% agarose-formaldehyde gel, and the RNA was transferred to Hybond N^+ nylon membranes (Amersham International, Buckinghamshire, UK). cDNA probes for rat c-*fos* (kindly provided by Dr. T. Curran, Roche Institute of Molecular Biology) and mouse β-actin (Stratagene, La Jolla, CA) were labeled with [^{32}P]dCTP by using a random primer kit by Boehringer-Mannheim Biochemicals (Indianapolis, IN) to a specific activity $\geq 7 \times 10^8$ cpm/μg. Absorbance measurements of autoradiograms were performed to estimate mRNA levels.

Immunohistochemistry. Two hours after the last injection (with the exception of the RU24969 time course experiment; see Fig. 2C), mice were anesthetized with a ketamine/xylazine mixture and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer over 10 min. The brains were postfixed in 4% paraformaldehyde for 2 hr at room temperature and placed in 30% sucrose in PBS for 48 hr at 4°. Sections (30 μm) were cut in a freezing microtome and collected in PBS. Free floating sections were pretreated with 3% H_2O_2 in PBS and incubated overnight at 4° in affinity-purified primary antibody raised against the c-*fos* *N*-peptide (AB-2; Oncogene Sciences, Mineola, NY) diluted 1:500 in PBS containing 0.3% Triton X-100, 10% normal goat serum (GIBCO, Grand Island, NY), and 1% bovine serum albumin (Boehringer-Mannheim). After three PBS washes, sections were carried through standard avidin-biotin immunohistochemical protocols using an Elite Vectastain kit (Vector Laboratories, Burlingame, CA). Chromogen reaction was performed with diaminobenzidine (Sigma) and 0.003% H_2O_2 for 10 min. The sections were mounted on chromalum-coated slides and coverslipped with Aqua-PolyMount (Polysciences, Warrington, PA). Omission of the primary antibody or preadsorption with the *N*-peptide (Oncogene Sciences) resulted in the absence of labeling.

Cell counting and data analysis. Counting of immunopositive nuclei was performed using a computerized image analysis system (MCID, Imaging Research, St. Catherine's, Ontario, Canada) attached to a microscope (Leica Diaplan,Wetzlar, Germany). The counting was performed in a semiautomated fashion with shading error acquired in a non-sample-containing part of the preparation to correct for uneven distribution of light and form and shape factors suitable for the used magnification (100×).

The number of c-*fos*-immunoreactive cells in the CPu is presented as the mean number of cells counted in anterior, middle, and posterior CPu in three 30-μm coronal sections of four to six animals per treatment and genotype. The three different anteroposterior levels correspond to the F 1.0, F 0.4, and F −0.2 levels (24). The counting of the other brain areas studied was performed in sections corresponding to the F 1.0 level, where F is the distance to bregma in millimeters (in frontal sections).

Receptor autoradiography. Mice were decapitated while under chloral hydrate anesthesia, and the brains were rapidly removed and frozen by immersion in isopentane refrigerated with liquid nitrogen. Horizontal brain sections (10 μm thick) were thaw-mounted on gelatin-coated slides and stored at −20° until use. Autoradiography was performed as previously described (25). Incubations were carried out in Kreb's solution containing 10 μM pargylline and 0.01% ascorbic acid. Total binding to 5-$HT_{1B/1D}$ sites was determined with 0.3 nM S-CM-G-^{125}I-TNH_2 (2000 Ci/mmol; Immunotech S.A., Marseilles, France). Homothetic sections were incubated in the same conditions but in the presence of 100 nM CP93129 (5-hydroxy-3[4–1,2,5,6-tetrahydropyridyl]-4-azaindole) to displace binding to 5-HT_{1B} sites and in the presence of 10^{-5} M 5-HT to determine nonspecific binding of the radioligand. After 1 hr of incubation (20°), the sections were rinsed (twice for 1 min) in cold distilled water and dried under a stream of warm air. Sections were exposed to film (Hyperfilm [^{3}H], Amersham) for 5 days. The ligands used for displacements were obtained from the following sources: CP93129 was a gift from Pfizer, sumatriptan was a gift from Glaxo, and 4{2-(4-[3-(trifluoromethyl)phenyl]-1-piperazinil)-ethyl}benzeneamine was a gift from Dr. H. Gozlan (Faculté de Médecine, Pitié Salpêtrière, Paris, France).

Results

Mice lacking the 5-HT_{1B} receptor display a reduced effect of cocaine on striatal c-*fos* induction. The induction in c-*fos* IR triggered by a 30 mg/kg dose of cocaine was studied in wild-type and 5-HT_{1B} knockout mice. In wild-type mice, cocaine produced a marked induction in the number of

c-*fos* IR cells in the CPu (20-fold induction compared with saline injected mice) and moderate (2–6-fold) inductions in the NAc, cingulate cortex, piriform cortex, and lateral septum (Table 1) These findings are in good agreement with that reported in rats (18, 19). In 5-HT$_{1B}$ knockout mice, the induction in c-*fos* IR elicited by cocaine in the CPu was markedly lower than that in wild-type mice (60% reduction in the number of IR cells compared with wild-type mice) (Table 1; Fig. 1, A and B). 5-HT$_{1B}$ knockout mice also showed a moderate but significant reduction (21%) in the number of c-*fos* IR cells in the cingulate cortex compared with wild-type mice (Table 1). In other analyzed regions, NAc, piriform cortex, and lateral septum, there was no difference found between wild-type and 5-HT$_{1B}$ knockout mice in the induction of c-*fos* IR (Table 1). A dose-response curve revealed that the reduced effect of cocaine in the CPu of knockout mice was also evident at 10, 20, and 40 mg/kg doses, although less prominent at 10 mg/kg than at higher doses (Fig. 1C). In all other structures except for the cingulate cortex, no difference between wild-type and knockout mice was found at any of the assayed doses (data not shown).

We also compared the striatal induction of c-*fos* by Northern blot to determine whether the difference found at the protein level reflects a change in transcript level. Given previous reports of induction of c-*fos* mRNA by cocaine in cerebellum (26), we also compared the effect of cocaine in this structure. In 5-HT$_{1B}$ knockout mice, the level of striatal c-*fos* mRNA after a 30 mg/kg intraperitoneal dose of cocaine corresponded to 46% of that showed by wild-type mice (Fig. 1D). No difference in the level of induction of c-*fos* transcripts was found in the cerebellum (Fig. 1D).

To test the possibility that 5-HT$_{1B}$ knockout mice are deficient in striatal c-*fos* inducibility, we compared in wild-type and knockout mice the effect of two nonserotonergic drugs that have been shown to induce c-*fos* expression in the CPu in rats: the dopamine D$_2$ receptor antagonist haloperidol and the dopamine-selective uptake blocker GBR-12909 (27, 28). Haloperidol, which induces c-*fos* in both the dorsomedial and the lateral striatum, produced a similar increase of c-*fos* IR in the CPu of wild-type and knockout mice (Fig. 1C). Furthermore, GBR-12909, which induces striatal c-*fos* IR in a pattern very similar to the one induced by cocaine, also had a comparable effect in the CPu of wild-type and knockout mice (Fig. 1C).

Activation of 5-HT$_{1B}$ receptors induces striatal c-*fos* expression. We then tested 5-HT$_{1B}$ agonists to determine whether they were able to increase c-*fos* expression in the brain regions in which knockout mice show a reduced effect of cocaine. The 5-HT$_{1A/1B}$ agonist RU24969 at a behaviorally active dose (5 mg/kg) (21) produced a very strong (28-fold) induction in the number of c-*fos* IR cells in the CPu of wild-type, whereas no effect was detected in knockout mice (Fig. 2, A and B), indicating that this effect requires the activation of 5-HT$_{1B}$ receptors. Similar to the induction elicited by cocaine, the induction of c-*fos* IR induced by RU24969 is localized to the dorsomedial part of the CPu, with the most intensely stained nuclei located near the ventricle and corpus callosum (Fig. 2A). The time course of the increase of c-*fos* IR induced by RU24969 in the CPu is as follows: the induction is apparent 1 hr after injection, peaks at 2–5 hr, and returns to basal levels after 24 hr (Fig. 2C). Light counterstaining with cresyl violet confirmed that the c-*fos* IR induced by RU24969 in the CPu is neuronal, and most of the labeled cells seem to correspond to the medium-sized spiny neurons of the striatum (data not shown). RU24969 (5 mg/kg) also produced a moderate induction of c-*fos* IR in the cingulate cortex (6-fold) and NAc (4.5-fold). No induction of c-*fos* IR was found in the piriform cortex or lateral septum after treatment with RU24969. The lowest assayed dose of RU24969 able to increase the number of c-*fos* IR cells in the CPu above basal levels was 2 mg/kg, and higher doses increased the number of IR cells in a dose-dependent manner (Fig. 2D). In knockout mice, RU24969 was unable to induce c-*fos* IR at any dose. Because RU24969 displays similar affinities for 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors, the fact that RU24969 had no effect in knockout mice suggests that stimulation of 5-HT$_{1A}$ receptors alone is not able to induce striatal c-*fos* IR. It is possible, however, that a simultaneous activation of 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors is required for the induction of c-*fos* IR in the CPu of wild-type mice by RU24969. To study the relevance of 5-HT$_{1A}$ receptor activation in the effect of RU24969, experiments were performed with different 5-HT$_{1A}$-selective agonists and antagonists (Fig. 3). The 5-HT$_{1A}$-selective antagonist WAY-100635 (13), when coadministered with RU24969, did not antagonize the effect of RU24969 on striatal c-*fos* IR, ruling out an involvement of 5-HT$_{1A}$ receptors in this phenomenon. As anticipated, the 5-HT$_{1A}$-selective agonist 8-OH-DPAT (13) at a behaviorally active dose (3 mg/kg) failed to induce c-*fos* IR in the CPu.

RU24969 and cocaine share common effector pathways. Pharmacological studies were performed to determine whether RU24969 increases striatal c-*fos* IR through activation of the same receptors and neurotransmitter pathways as cocaine. The effect of cocaine on striatal c-*fos* expression is

TABLE 1

Comparison of cocaine-elicited induction of c-*fos* IR in wild-type and 5-HT$_{1B}$ knockout mice in different brain structures

Values represent mean ± standard error number of c-*fos* IR cells counted (see Materials and Methods) in 30-μm coronal sections corresponding to the F 1.0 level (24). Saline (n = 3) and cocaine (n = 6) were administered for each genotype. Cocaine-treated mice received a 30 mg/kg intraperitoneal dose. Unpaired t test comparison revealed significant differences between genotypes in the CPu and cingulate cortex after cocaine treatment.

Structure	Saline		Cocaine	
	Wild-type mice	Knockout mice	Wild-type mice	Knockout mice
CPu	28.6 ± 2.1	26.2 ± 3.0	549.2 ± 45.3	221.1 ± 37.4[a]
NAc	11.3 ± 1.9	10.4 ± 1.8	47.7 ± 8.8	47.5 ± 13.5
Cingulate cortex	31.4 ± 3.6	33.7 ± 3.0	188.5 ± 10.5	148.8 ± 16.5[b]
Piriform cortex	27.1 ± 4.1	27.4 ± 4.7	74.6 ± 10.6	83.6 ± 11.5
Lateral septum	79.7 ± 5.4	70.5 ± 9.5	163.4 ± 11.2	175.5 ± 12.4

[a] $p < 0.001$.
[b] $p < 0.01$.

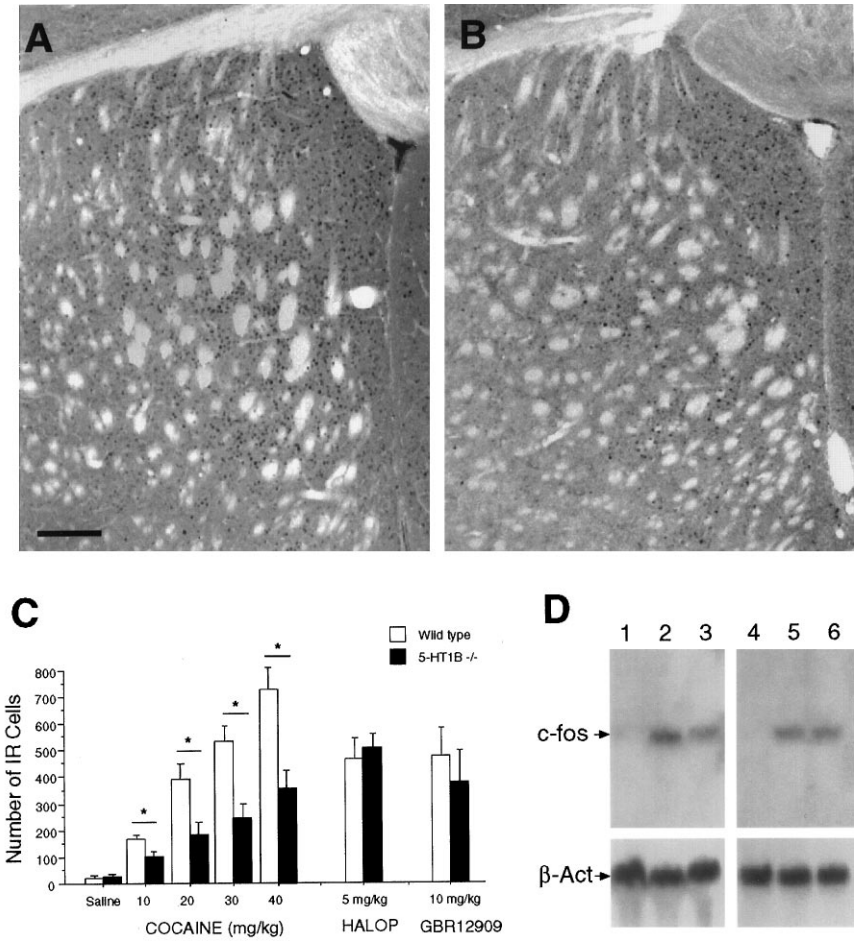

Fig. 1. Comparison of cocaine-, haloperidol-, and GBR12909-elicited induction of c-*fos* in the striatum of wild-type and 5-HT_{1B} knockout mice. Effect of a 30 mg/kg intraperitoneal dose of cocaine on c-*fos* IR in the anterior CPu of (A) wild-type and (B) 5-HT_{1B} knockout mice. *Scale bar*, 200 μm for both photographs. C, Effect of intraperitoneal doses of cocaine (10- 40 mg/kg), haloperidol (*HALOP*, 5 mg/kg), and GBR12909 (10 mg/kg) on c-*fos* IR in the CPu of wild-type and 5-HT_{1B} knockout mice. Values represent the mean number of c-*fos* IR cells counted in anterior, middle, and posterior CPu in three 30-μm coronal sections of four to six animals per treatment and genotype. Analysis of variance of the cocaine dose-response data revealed a significant effect of genotype ($p < 0.0005$), dose ($p < 0.0001$), and the interaction between dose and genotype ($p < 0.05$). *Post hoc* Scheffé's comparisons revealed significant differences between genotypes at all cocaine doses but not after haloperidol or GBR12909 treatments ($*$, $p < 0.005$). D, Northern blot analysis of the effect of cocaine on c-*fos* mRNA expression in striatum and cerebellum of wild-type and knockout mice. *Lanes 1–3*, striatal mRNA samples. *Lanes 4–6*, cerebellar samples. *Lanes 1* and *4*, wild-type mice injected with saline; the basal level of c-*fos* mRNA was undetectable in both wild-type and knockout mice (not shown). *Lanes 2* and *5*, wild-type mice administered a 30 mg/kg intraperitoneal dose of cocaine. *Lanes 3* and *6*, knockout mice administered a 30 mg/kg intraperitoneal dose of cocaine.

dependent on dopaminergic transmission and can be blocked by the D_1 receptor antagonist SCH23390 (18, 19, 26). The NMDA antagonist MK-801 also inhibits the striatal induction of c-*fos* mediated by cocaine (26, 29). We tested whether these compounds can also inhibit the effect of RU24969. The D_1 receptor antagonist SCH23390 alone did not change c-*fos* IR in the CPu but completely abolished the effect of 5 mg/kg RU24969 (Fig. 4). The NMDA antagonist MK-801 by itself induced a strong immunostaining in a reduced number of neurons in the medial CPu. When MK-801 and RU24969 were coadministered, the pattern of c-*fos* IR was similar to that induced by MK-801 alone and the number of positive cells was much lower than in animals which only received RU24969 (Fig. 4).

Because 5-HT_{1B} receptors have been suggested to activate dopamine neurons via a reduction in $GABA_B$-mediated inhibitory postsynaptic potentials (14, 15), we also tested the ability of the $GABA_B$ receptor agonist baclofen to inhibit the effect of RU24969. A 30 mg/kg dose of baclofen by itself had no effect on basal c-*fos* IR in the CPu, but it completely abolished the induction elicited by 5 mg/kg RU24969 (Fig. 4). We then investigated how the same dose of baclofen would affect the previously shown (Fig. 1) striatal induction of c-*fos* IR elicited by 30 mg/kg cocaine. Interestingly, the mice that received both baclofen and cocaine showed no increase in striatal c-*fos* IR and were indistinguishable from those that were administered only saline or baclofen.

The 5-HT_{1B}/$_{1D}$ antagonist GR127935 reduces the effects of cocaine. The results obtained in the knockout mice prompted us to study the effect of 5-HT_{1B} antagonists on the induction of c-*fos* elicited by cocaine. The recently generated compound GR127935 has been identified as a very potent (pK_D = 9.9) and *in vivo* active 5-$HT_{1D\beta}$ receptor antagonist (30). Although the affinity of this compound for 5-$HT_{1D\beta}$ receptors is 1 order of magnitude higher than that for 5-HT_{1D} α (pK_D = 8.9) and 5-HT_{1B} (pK_D = 8.5) receptors in transfected cells (31), it has also been reported to have similar affinities for 5-HT_{1D} and 5-HT_{1B} receptors in guinea pig and

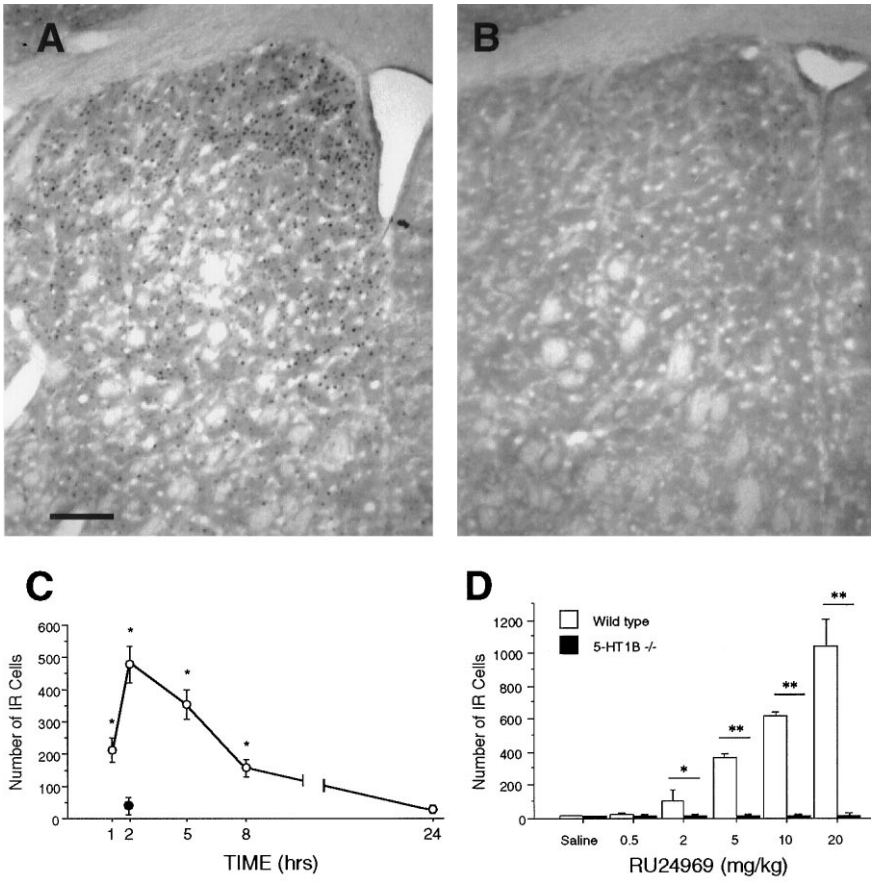

Fig. 2. Induction of c-*fos* IR in the CPu of wild-type but not 5-HT$_{1B}$ knockout mice by RU24969. Effect of a 5 mg/kg intraperitoneal dose of RU24969 on c-*fos* IR in the anterior CPu of (A) wild-type and (B) 5-HT$_{1B}$ knockout mice. *Scale bar*, 200 μm for both photographs. C, Time course of the effect of a 5 mg/kg intraperitoneal dose of RU24969 on c-*fos* IR in the CPu of wild-type mice. The number of c-*fos* IR cells was determined 1, 2, 5, 8, and 24 hr after injection of 5 mg/kg RU24969 (○) and compared with the saline injection (*t* test; ∗, $p < 0.01$). The number of c-*fos* IR cells after saline (●) injection was determined 2 hr after injection. D, Dose-response effect of RU24969 on c-*fos* IR in the CPu of wild-type mice and knockout mice. Analysis of variance revealed a significant effect of genotype ($p < 0.0001$), dose ($p < 0.0001$), and the interaction between dose and genotype ($p < 0.0001$). *Post hoc* Scheffé's comparisons revealed significant differences between genotypes at 2, 5, 10, and 20 mg/kg doses of RU24969. ∗∗, $p < 0.005$; ∗, $p < 0.01$.

rat striatal membranes, respectively (32). We therefore tested the ability of this compound to antagonize the effect of RU24969 on striatal c-*fos* IR, which is, as we previously demonstrated, mediated by 5-HT$_{1B}$ receptors. As shown in Fig. 3, pretreatment with a 10 mg/kg intraperitoneal dose of GR127935 completely abolished the effect of RU24969 on striatal c-*fos* IR, indicating that this compound is an effective antagonist of 5-HT$_{1B}$ receptors *in vivo*. The 10 mg/kg dose was the lowest dose of GR127935 that completely abolished the effect of 5 mg/kg RU24969 on striatal c-*fos* IR. A significant reduction in the effect of RU24969 was also observed with a 3 mg/kg dose of GR127935 (data not shown).

We then analyzed the effect of GR127935 on the increase in striatal c-*fos* IR elicited by cocaine in both wild-type and knockout mice. As shown in Fig. 5, a 10 mg/kg intraperitoneal dose of GR127935 by itself had no significant effect on the level of c-*fos* expression in the CPu of either wild-type or knockout mice. However, when coadministered to wild-type mice with a 30 mg/kg intraperitoneal dose of cocaine, GR127935 produced a significant reduction (42%) in the number of c-*fos* IR cells induced by cocaine alone. Interestingly, the number of c-*fos* IR cells in the CPu of wild-type mice that received both GR127935 and cocaine was similar to the one shown by knockout mice treated with cocaine only. These results therefore suggest that the reduced effect of cocaine observed in the knockout mice is due to the absence of the 5-HT$_{1B}$ receptor.

5-HT$_{1D}$ α-receptors are expressed at the same levels in wild-type and knockout mice and do not contribute significantly to the effect of cocaine. The 5-HT$_{1B}$ receptor is the rodent homologue of the human 5-HT$_{1D}$ β-receptor, whereas the 5-HT$_{1D}$ α-receptor is a close relative present in both humans and rodents (for a review, see Ref. 11). Because the affinity of GR127935 for 5-HT$_{1D\alpha}$ receptors has been reported to be similar or higher than that for 5-HT$_{1B}$ receptors, GR127935 must be interacting with 5-HT$_{1D\alpha}$ receptors as well. A precise interpretation of the effect of GR127935 on cocaine-induced c-*fos* IR requires an evaluation of the distribution and level of expression of 5-HT$_{1D\alpha}$ receptors in both wild-type and knockout mice. We performed autoradiographic studies with S-CM-G-^{125}I-TNH$_2$ in wild-type and knockout mice. This radioligand has been shown to label 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors exclusively (24). In wild-type mice, S-CM-G-^{125}I-TNH$_2$ sites were found in substantia nigra, globus pallidus, superior colliculus, central gray, entopeduncular nucleus, raphe, and subiculum (Fig. 6A and not shown). In knockout mice, the level of specific S-CM-G-^{125}I-TNH$_2$ binding was much lower than that in wild-type mice

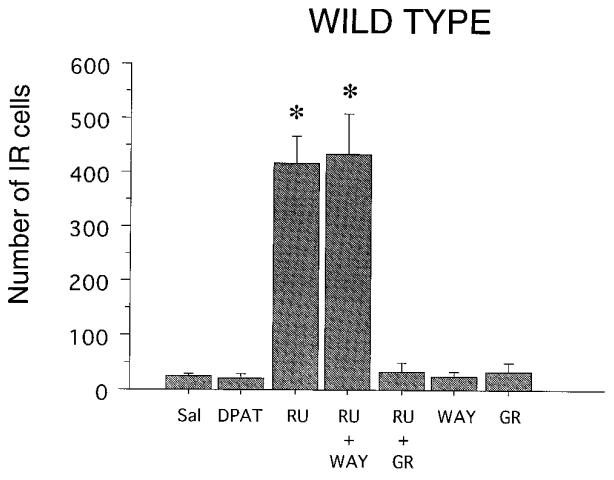

Fig. 3. The effect of RU24969 on c-*fos* IR in the CPu is blocked by the 5-$HT_{1B/1D}$ antagonist GR127935 but not by the 5-HT_{1A} antagonist WAY-100635. Each animal received two intraperitoneal injections at a 30-min interval. Control animals received two saline injections (*Sal*). The 5-HT_{1A} agonist 8-OH-DPAT (*DPAT*, 3 mg/kg) and the 5-$HT_{1A/1B}$ agonist RU24969 (*RU*, 5 mg/kg) were administered as second injections preceded by an injection of either saline or antagonist. The 5-HT_{1A} antagonist WAY-100635 (*WAY*, 1 mg/kg) and the 5-$HT_{1B/1D}$ antagonist GR127935 (*GR*, 10 mg/kg) were administered as first injections followed by an injection of either saline or RU24969. RU24969 and RU24969 + WAY-100635 were the only treatments that differed significantly from saline (*t* test; *, $p < 0.01$).

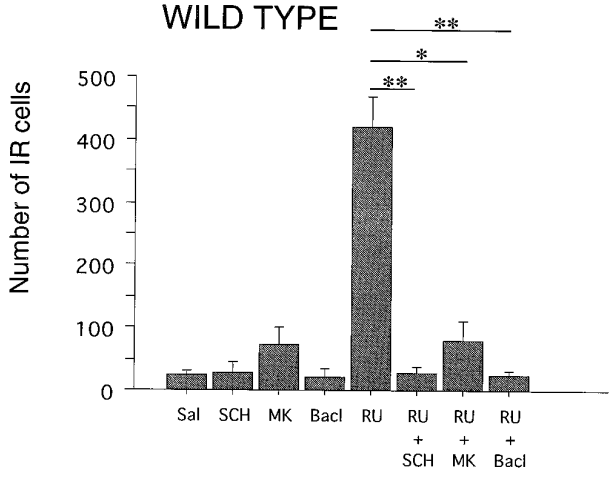

Fig. 4. Pharmacology of the effect of RU24969 on c-*fos* IR in the CPu. Each animal received two intraperitoneal injections at a 30-min interval. Control animals received two saline injections (*Sal*). The dopamine D_1 antagonist SCH23390 (*SCH*, 0.5 mg/kg), the NMDA antagonist MK-801 (*MK*, 1 mg/kg), and the $GABA_B$ agonist baclofen (*Bacl*, 30 mg/kg) were administered as first injections followed by an injection of either saline or RU24969 (*RU*, 5 mg/kg). Unpaired *t* test comparison revealed that RU24969 + SCH23390, RU24969 + MK-801, and RU24969 + baclofen differ significantly from RU24969. **, $p < 0.005$; *, $p < 0.01$.

(~10–15%) and was localized in the same brain regions, with the exception of the subiculum, in which no binding was detected (Fig. 6B and not shown). The 5-HT_{1B}-specific ligand CP-93129 (10^{-7} M) displaced 90% of the S-CM-G-^{125}I-TNH_2 binding in wild-type brains, whereas it had no effect in knockout mice (Fig. 6, C and D). The remaining S-CM-G-^{125}I-TNH_2 binding found in wild-type mice in the presence of CP-93129 was very similar in intensity and distribution to that found in knockout mice (Fig. 6, compare C with B and D). These remaining sites were displaced by two "5-HT_{1D}-preferring" ligands, sumatriptan (10^{-7} M) and 4[2-[4-[3-(trifluoromethyl)phenyl]-1-piperazinil]ethyl]benzeneamine (10^{-7} M) in both wild-type and knockout mice, therefore suggesting that these sites correspond to 5-$HT_{1D\alpha}$ receptors. The distribution and level of expression of 5-$HT_{1D\alpha}$ receptors in these mice are in close agreement with those from a previous report in rats (33). These observations therefore rule out major compensatory changes in the knockout mice concerning the level and pattern of expression of 5-$HT_{1D\alpha}$ receptors. This, together with the fact that the increase in c-*fos* IR induced by cocaine in the CPu of knockout mice was not affected by coadministration of GR127935 (Fig. 5), suggests that 5-$HT_{1D\alpha}$ receptors do not play a major role in the induction of c-*fos* elicited by cocaine and that the reduction induced in wild-type mice by GR127935 is mediated through blockade of 5-HT_{1B} receptors exclusively.

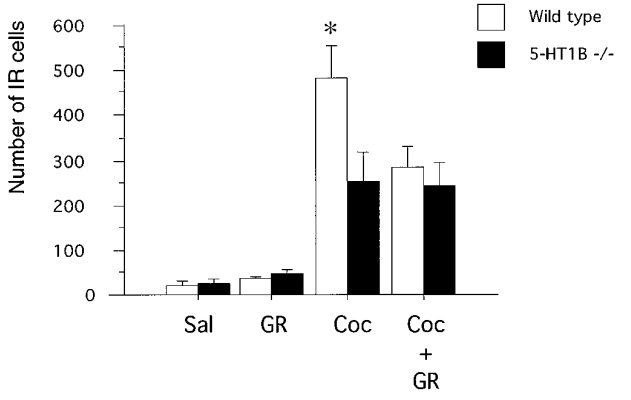

Fig. 5. Effect of GR127935 on cocaine-elicited c-*fos* IR in the CPu of wild-type and knockout mice. Each animal received two intraperitoneal injections at a 30-min interval. Control animals received two saline injections (*Sal*). GR127935 (*GR*, 10 mg/kg) was administered as first injection. Cocaine was administered as second injection (*Coc*, 30 mg/kg) preceded by either saline or GR127935. Analysis of variance followed by *post hoc* Scheffé's comparison revealed that pretreatment with GR127935 significantly decreased the number of c-*fos* IR cells induced by cocaine in wild-type mice. *, $p < 0.01$.

Discussion

Synergism of genetic and pharmacological approaches

The 5-HT_{1B} knockout mice are indistinguishable from their wild-type littermates in terms of development, body weight, fertility, open field behavior, and anxiety measures (21, 23). To test for neurochemical compensatory changes, we compared the levels of several neurotransmitters and receptors in different brain regions of wild-type and mutant mice. No significant differences in the levels of 5-HT, dopamine, or their metabolites were found in any of the studied regions.[2] 5-$HT_{1D\alpha}$ receptors, although expressed at much lower levels than 5-HT_{1B} receptors, have a similar anatomical distribution and the same coupling to G proteins as 5-HT_{1B} receptors (11, 12). They are, therefore, good candidates to undergo changes in their level of expression to compensate for the absence of 5-HT_{1B} receptors in the knockout mice. We have shown with autoradiography that the level and pattern of expression of 5-$HT_{1D\alpha}$ receptors are not altered in the knockout mice. Furthermore, the levels of 5-HT_{1A}, 5-HT_{2A}, and

[2] N. H. Chen and R. Hen, unpublished observations.

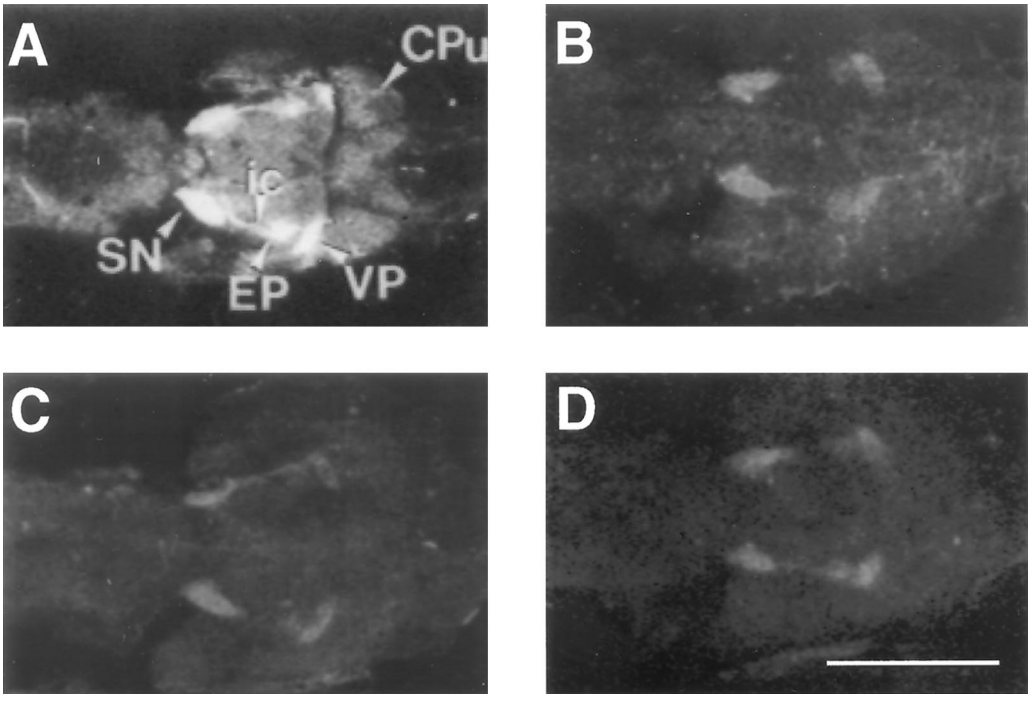

Fig. 6. Comparison of the levels of 5-HT$_{1D\alpha}$ receptors in wild-type and knockout mice. Autoradiograms showing the distribution of sites labeled by 0.3 nM S-CM-G-^{125}I-TNH$_2$ in horizontal brain sections of (A and C) wild-type and (B and D) 5-HT$_{1B}$ knockout mice. A and B, Distribution pattern with the radioligand alone. C and D, Labeling remaining in the presence of 100 nM of CP 93129. *VP*, ventral pallidum; *EP*, entopeduncular nucleus; *SN*, substantia nigra; *ic*, internal capsule. *Scale bar*, 0.5 cm.

5-HT$_{2C}$ receptors and of the dopamine transporter are not altered in the knockout mice.[3]

It seems that the knockout mice do not experience any major compensatory changes in the serotonergic or dopaminergic systems. We cannot, however, rule out the possibility that the knockout mice have undergone changes in other neurotransmitter systems that would influence their response to cocaine. We decided therefore to compare the results obtained with the knockout mice with results obtained with the 5-HT$_{1B/1D}$ antagonist GR127935. This compound has been shown to effectively antagonize 5-HT$_{1D\beta}$ receptors *in vivo* (16, 30, 32). Although the affinity of GR127935 for 5-HT$_{1B\beta}$ receptors is 1 order of magnitude lower than for 5-HT$_{1D\beta}$ receptors, we have shown in the current study that GR127935 is able to antagonize 5-HT$_{1B}$ receptors *in vivo*. GR127935 also has a high affinity for 5-HT$_{1D\alpha}$ receptors. However, because GR127935 does not affect the induction of c-*fos* elicited by cocaine in knockout mice, 5-HT$_{1D\alpha}$ receptors do not seem to play a role in this phenomenon. Therefore, by combining studies with the antagonist and the specificity of the mutation in the knockout mice, we have been able to demonstrate that 5-HT$_{1B}$ receptors play a role in mediating the cellular effects of cocaine.

Mechanism of 5-HT$_{1B}$ receptor modulation of cocaine effects. The 5-HT$_{1B}$ mRNA is found in the CPu, NAc, Purkinje cells, CA1 pyramidal neurons, and raphe neurons (12), whereas 5-HT$_{1B}$ binding sites are localized in the projection zones of neurons expressing the 5-HT$_{1B}$ mRNA (i.e., the substantia nigra, VTA, globus pallidus, deep cerebellar nuclei, and subiculum) (12, 33). On the basis of this observation and lesion experiments (13, 34), it was postulated that 5-HT$_{1B}$ receptors are localized on axon terminals (33, 34). In keeping with this localization, 5-HT$_{1B}$ receptors have been shown to inhibit neurotransmitter release from nerve terminals. Activation of 5-HT$_{1B}$ autoreceptors on serotonergic terminals results in inhibition of 5-HT release (13). Similarly, in the substantia nigra and VTA, 5-HT$_{1B}$ agonists inhibit GABA release (14, 15).

The striatum, in which inactivation of 5-HT$_{1B}$ receptors results in a markedly reduced effect of cocaine, belongs to a group of highly interconnected structures referred to as the basal ganglia. In these circuits, 5-HT$_{1B}$ receptors are found predominantly on axon terminals of striatal GABAergic neurons projecting to the substantia nigra, VTA, and globus pallidus (Fig. 7). 5-HT$_{1B}$ autoreceptors are also found on the axon terminals of raphe neurons that project throughout the brain and might also modulate the activity of the basal ganglia circuits.

The fact that the D$_1$ receptor antagonist SCH23390 blocks the striatal induction of c-*fos* elicited by RU24969 demonstrates that dopaminergic transmission is essential for this phenomenon and suggests an involvement of those 5-HT$_{1B}$ receptors that modulate the activity of dopaminergic neurons. Activation of 5-HT$_{1B}$ receptors in substantia nigra and VTA results in disinhibition of dopaminergic neurons (14, 15) and in facilitation of dopamine release in the striatum (9, 16). 5-HT$_{1B}$ receptors located in substantia nigra and VTA therefore seem to play a crucial role in mediating the striatal induction of c-*fos* elicited by RU24969. However, 5-HT$_{1B}$ receptors located in the globus pallidus that might be modulating the activity of the direct and indirect loops of striatothalamocortical projections (Fig. 7) might also significantly contribute to the effect of RU24969.

The activation of dopaminergic neurons by 5-HT$_{1B}$ ago-

[3] L. Segu and R. Hen, unpublished observations.

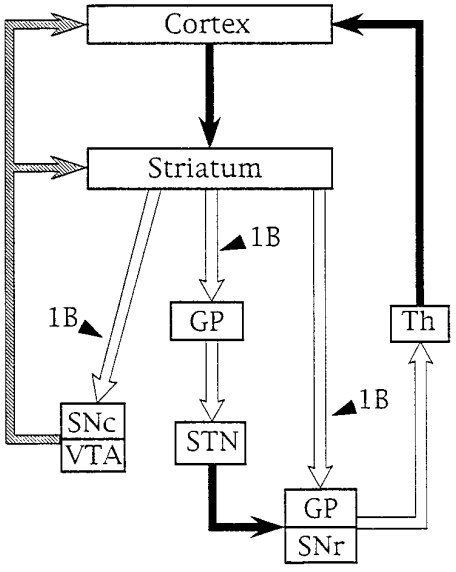

Fig. 7. 5-HT_{1B} receptors in the basal ganglia. *Black arrows*, excitatory pathways (glutamatergic). *White arrows*, inhibitory pathways (GABAergic). *Slashed arrows*, nigrostriatal and mesocorticolimbic dopaminergic pathways. *Arrowheads*, 5-HT_{1B} receptors localized on GABAergic terminals. Abbreviations: *SNc*, substantia nigra pars compacta; *SNr*, substantia nigra pars reticulata; *GP*, globus pallidus; *STN*, subthalamic nucleus; *Th*, thalamus.

nists observed in substantia nigra and VTA slice preparations is mediated by a reduction in GABA release and $GABA_B$-mediated inhibitory postsynaptic potentials (14, 15). These observations prompted us to test the effect of the $GABA_B$ receptor agonist baclofen on the RU24969-mediated induction of c-*fos* . The fact that baclofen completely blocks the effect of RU24969 is consistent with an involvement of 5-HT_{1B} receptors located in the substantia nigra and VTA. However, alteration in GABA transmission by baclofen at different points of the basal ganglia circuits (Fig. 7) might also account for its effect. Interestingly, we found that baclofen also blocks the striatal induction of c-*fos* elicited by cocaine, which is in good agreement with the previously reported ability of intra-VTA injections of baclofen to prevent the motor stimulant action of cocaine (35). The NMDA receptor antagonist MK-801 also blocks the effect of both RU24969 (Fig. 4) and cocaine (26, 29). Concerning the localization of the relevant NMDA receptors, it has recently been shown that D_1 receptor-mediated induction of c-*fos* in cultured striatal neurons requires functional activation of NMDA receptors (36). These observations point toward an involvement of glutamatergic corticostriatal projections, although they do not rule out a contribution of glutamate receptors at other levels in the basal ganglia circuitry (Fig. 7).

In summary, because pharmacological manipulation of the dopamine D_1, NMDA, and $GABA_B$ receptors blocks the effect of RU24969 and cocaine on striatal c-*fos* expression, the two drugs seem to share similar effector systems. The mechanism through which 5-HT_{1B} receptors modulate cocaine actions might be as follows. Cocaine blocks the 5-HT transporter localized on the terminals of tonically active serotonergic neurons that innervate the substantia nigra and VTA, thereby increasing the synaptic concentration of 5-HT. In fact, cocaine has been shown to increase 5-HT levels in the VTA (37). The increased 5-HT levels result in increased activation of 5-HT_{1B} receptors localized on GABAergic afferents to ventral midbrain dopaminergic neurons. This in turn results in decreased GABA release and subsequent disinhibition of dopaminergic neurons.

Physiological relevance of the modulation by 5-HT_{1B} receptors of cocaine effects. The work we present is the first demonstration that cocaine is an indirect agonist of 5-HT_{1B} receptors *in vivo*. We also show that the activation of 5-HT_{1B} receptors by cocaine is relevant to the level of induction of c-*fos* in different brain structures and, most markedly, in the striatum. Interestingly, 5-HT denervation has been shown to result in a reduced induction of c-*fos* by cocaine in this brain structure (38). Because c-*fos* is both a marker of neuronal activation and an inducible transcription factor postulated to mediate plastic changes in the central nervous system (17, 20), the agonist action of cocaine at 5-HT_{1B} receptors that we report here might contribute to both the short and long term effects of cocaine.

The CPu and cingulate cortex, the brain regions in which 5-HT_{1B} knockout mice showed a reduced effect of cocaine on c-*fos* induction, are projection sites of the dopamine nigrostriatal and mesocorticolimbic pathways, respectively. The nigrostriatal dopamine pathway has been postulated to modulate sensorimotor coordination and initiation of movement (39), whereas the mesocorticolimbic pathway has been implicated in the positive-reinforcing (rewarding) and locomotor-stimulating effects of cocaine and other drugs of abuse (2, 40). The indirect agonist action of cocaine at 5-HT_{1B} receptors might therefore play a role in some of these responses to cocaine. Interestingly, we have found that the 5-$HT_{1B/1D}$ antagonist GR127935 also blocks the hyperlocomotion elicited by cocaine (41), suggesting an involvement of 5-HT_{1B} receptors in cocaine-induced locomotion. Due to the association between the locomotor-stimulating and -reinforcing properties of many drugs of abuse (40), the 5-HT_{1B} receptor modulation of cocaine effects might be also relevant to the reinforcing and addictive properties of cocaine. Pretreatment with the 5-HT_{1B} agonist CGS-12066B dose-dependently reduces self-administration of the dopamine uptake inhibitor GBR-12909 (42). Furthermore, RU24969 substitutes for cocaine in drug-discrimination studies (43) and is able to shift the dose-effect function for cocaine self-administration to the left,[4] suggesting that it can enhance the reinforcing properties of cocaine. To further address the contribution of 5-HT_{1B} receptors to the locomotor and reinforcing effects of cocaine, we are currently analyzing cocaine-induced hyperlocomotion and cocaine self-administration in the 5-HT_{1B} knockout mice.

Finally, because the mesocorticolimbic pathway seems to be involved in the reinforcing properties of most drugs of abuse (2, 40), the modulatory role of 5-HT_{1B} receptors might not be restricted to cocaine. In fact, a possible role of 5-HT_{1B} receptors in alcohol abuse is suggested by our recent observations that 5-HT_{1B} knockout mice display reduced sensitivity to alcohol and elevated alcohol consumption (22). Interestingly, the 5-HT_{1B} receptor gene maps to a genetic locus that influences in inbred mouse strains various responses to drugs of abuse, such as alcohol-induced ataxia and cocaine-induced locomotion (44). Alterations in the 5-HT_{1B} gene might therefore account for differences in susceptibility to drugs of abuse.

[4] L. H. Parsons, personal communication.

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