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Decreased c-*fos* Responses to Dopamine D_1 Receptor Agonist Stimulation in Mice Deficient for D_3 Receptors*

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Mi-Young Jung‡§ and Claudia Schmauss‡¶

From the ‡Department of Psychiatry/Neuroscience, Columbia University, New York, New York 10032 and the §Graduate Program in Neurobiology, Mount Sinai School of Medicine, New York, New York 10029

The acute administration of dopamine D_1 receptor agonists induces the expression of the immediate early gene c-*fos*. In wild type mice, this induction is completely abolished by pretreatment with the D_1-selective antagonist SCH23390, and pretreatment with the D_2-like receptor antagonist eticlopride reduces the levels of c-*fos* expressed in response to D_1 receptor stimulation. Mice deficient for the dopamine D_3 receptor express levels of D_1 agonist-stimulated c-*fos* immunoreactivity that are lower than c-*fos* levels of their wild type littermates. Moreover, the acute blockade of D_2 receptors in D_3 mutant mice further reduces c-*fos* expression levels. These data indicate that the basal activity of both D_2 and D_3 receptors contributes to D_1 agonist-stimulated c-*fos* responses. The findings therefore indicate that not only D_2 but also D_3 receptors play a role in dopamine-regulated gene expression.

An increase in neuronal activity triggers the transcription of immediate early genes, including the proto-oncogene c-*fos*, which, in turn, stimulates the transcription of AP-1-promotor-containing late response genes that are responsible for adaptive changes in mature neurons (1, 2). It is well established that the concentration of second messengers and differences in the activation threshold for calcium-dependent signaling are important factors controlling cellular responses to stimulatory signals, including the transcriptional activation of Fos genes (3, 4). Two key regulators of c-*fos* transcription are the phosphorylated forms of mitogen-activated protein kinase (MAPK)[1] and CREB (4). CREB is a Ca^{2+}/cAMP-responsive transcription factor that in its phosphorylated form (pCREB; phosphorylated at Ser^{133}) stimulates transcription of the c-*fos* gene by interacting with the CRE transcriptional regulatory element (for review see Ref. 2). The MAPK cascade is thought to participate in the activation of c-*fos* transcription either by phosphorylating CREB at Ser^{133} or by phosphorylating ternary complex factors that, together with serum response factor, bind to the SRE element of the c-*fos* promotor (2). Activation of the MAPK cascade occurs in response to serum or growth factor stimulation (5) as well as in response to increased calcium levels via a Ras-dependent pathway (6).

Induction of c-*fos* expression is an important mechanism in the control of neurotransmitter-regulated gene expression. One extensively studied example is the dopamine-regulated expression of Fos (7). In the striatum, for example, induction of c-*fos* expression has been shown to occur in response to the acute administration of cocaine and amphetamine (8–13). These drugs indirectly activate dopamine receptors, and the Fos responses elicited by them have been linked to the activation of dopamine D_1-like receptors, which couple to stimulatory subsets of heterotrimeric proteins to stimulate cytosolic second messengers (14).

Moreover, blockade of dopamine D_2-like receptors (which couple to inhibitory G proteins) by neuroleptic drugs results in increased c-*fos* expression levels. Anatomically, a close topographic relationship exists between c-*fos* effects and the expression of receptors upon which various typical and atypical neuroleptics act (15–18). Furthermore, if mesotelencephalic dopaminergic neurons are destroyed by 6-hydroxydopamine, neuroleptic drugs no longer increase c-*fos* expression levels, indicating that dopamine exerts a tonic inhibitory effect on basal c-*fos* levels via its action on D_2-like receptors (15).

Pharmacological studies further revealed synergistic c-*fos* responses to combined D_1- and D_2-like receptor stimulation in the striatum of normal and dopamine-depleted rats (19–21). Whereas the topographic overlap between increase in c-*fos* expression and expression of receptors blocked by various neuroleptic drugs suggest a cellular mechanism that regulates basal c-*fos*-expression levels, synergistic c-*fos* responses to combined D_1- and D_2-like receptor stimulation are more likely to be regulated at the level of neuronal circuitry.

The studies that found synergistic effects of D_1- and D_2-like receptor activation on c-*fos* responses (20, 21) used D_2-like agonists that do not discriminate between the D_2 and D_3 receptor subtypes (for review see Ref. 22). Thus, it remains unresolved whether D_2 and D_3 receptors contribute similarly or differently to the modulation of c-*fos* responses. However, the current lack of antagonists specific for each member of the D_2 class of dopamine receptors complicates attempts to elucidate the role of both receptor subtypes in the modulation of c-*fos* expression levels. Therefore, the present study used mutant mice generated by gene targeting via homologous recombination that lack D_2, D_3, and D_2/D_3 receptors (23) to test whether the absence of D_2 and D_3 receptors alters dorsal striatal and extrastriatal c-*fos* responses to D_1 receptor stimulation.

EXPERIMENTAL PROCEDURES

Animals—The generation of D_2, D_3, and D_2/D_3 mutant mice is described elsewhere (23). For studies that compared c-*fos* responses to application of the D_1 agonist SKF82958 across different mutants, homozygous mutants and wild type littermates were derived from crosses of heterozygous D_2 and D_3 single mutants with a hybrid 129/Sv × C57Bl/6 genetic background. Heterozygous D_2/D_3 double mutants were generated via cross-breeding of homozygous D_2 males with homozygous D_3 females and were subsequently cross-bred to generate wild type and the various genetic combinations of D_2/D_3 double mutants. For studies

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¶ To whom correspondence should be addressed: Columbia University, Depts. of Psychiatry and Neuroscience, 1051 Riverside Dr., Unit 42, New York, NY 10032. Tel.: 212-543-6505; Fax: 212-543-6017; E-mail: schmauss@neuron.cpmc.columbia.edu.

[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; P*n*, postnatal day *n*.

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that compared c-*fos* responses to D_1 agonist stimulation between wild type and D_3 single mutants, congenic (8th generation of back-crossing) C57Bl/6 D_3 mutants and their wild type littermates were used.

Male animals at postnatal ages P15, P30, P60, and P70 were used in this study. Their genotypes were verified by Southern blotting as described in Jung *et al.* (23). Animals were housed in a 12-h light/dark cycle colony room at 22 °C with free access to food and water. The following drugs were administered: 1 mg/kg SKF82958, 1 mg/kg SCH23390, and 0.5 mg/kg eticlopride. All drugs were purchased from Research Biochemicals, Inc. (Natick, MA). Drugs were dissolved in saline and administered intraperitoneally between 2 and 5 p.m. Animals were killed at defined time points after systemic drug administration, and their brains were quickly removed. The dorsal striatum (caudoputamen) was immediately dissected from all brains. This dissection did not include the ventral part of the striatum containing the nucleus accumbens. The dorsal striatal tissue and the remaining brain tissue were collected in separate tubes and stored at −80 °C until use. All animal procedures were approved by the Institutional Animal Care and Use Committee.

Expression of c-fos, pMAPK, and pCREB Immunoreactivities—For immunoblot analyses (c-*fos*, pMAPK, and pCREB), proteins were extracted in a buffer containing 1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS (RIPA buffer) supplemented with 2 mM Na_3VO_4, 20 mM NaF, 1 mM EGTA, 1 mM dithiothreitol, 1 μM microcystin, and protease inhibitors. The protein concentration in each lysate was determined using the BCA protein assay kit (Pierce).

c-*fos* immunoreactivity was detected with two different rabbit polyclonal anti-c-*fos* antibodies. One antibody was purchased from Santa Cruz (Santa Cruz, CA; lot number H067) and used at a dilution of 1:1000. The other antibody (dilution, 1:2,500 to 1:5000) was obtained from Oncogene Research (San Diego, CA; lot number D08330). A sheep polyclonal anti-phospho MAPK antibody (Upstate Biotechnology, Inc., Lake Placid, NY) was used at a dilution of 1:1000, and a rabbit polyclonal anti-pCREB antibody (Upstate Biotechnology) was used at a dilution of 1:2,500. A mouse monoclonal anti-TH antibody (Incstar, Stillwater, MN; dilution, 1:10,000) was used to reprobe all immunoblots. Bound antigen was visualized using the appropriate peroxidase-conjugated secondary antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, MD) in conjunction with ECL (Pierce). The ECL signals on autoradiograms were assessed densitometrically using the National Institutes of Health Image Analysis Software. Optical density measurements of standards on the film were made to construct a standard correlation curve. Relative optical densities were determined for optical densities of signals located in equal size sample areas.

RESULTS

The Role of D_2 and D_3 Receptors in Modulating c-fos Responses to D_1 Agonist Stimulation—To test whether mice lacking dopamine D_2, D_3, and D_2/D_3 receptors maintain the wild type specificity between stimulation of D_1 receptors and transcription of the c-*fos* gene, mice were treated with the full D_1 agonist SKF82958 (1 mg/kg intraperitoneal), a ligand known to stimulate c-*fos* expression in rat striatum (21, 24). A first series of experiments performed on wild type animals determined the time course of c-*fos* induction and tested whether D_1- and D_2-like receptor antagonists block or reduce this induction.

As shown in Fig. 1*A*, the earliest time point at which a robust induction of expression of c-*fos* can be detected on Western blots is 60 min after the systemic application of the drug. In general, however, the onset of detectable c-*fos* expression varies between 60 and 90 min, a variability that is most likely accounted for by the route of drug administration (*i.e.* intraperitoneal). c-*fos* immunoreactivity remains detectable up to 120 min after drug administration and then returns to base-line levels (not shown). In addition to the induction of c-*fos* expression, SKF82958 administration also leads to phosphorylation of the MAPK. The kinetics of MAPK phosphorylation is more rapid and increased levels of pMAPK immunoreactivity are detected 20 min after drug administration (Fig. 1*A*). The expression levels of pMAPK return to base line 30–45 min after drug administration (not shown). Interestingly, although the anti-pMAPK antibody recognizes the phosphorylated pMAPK isoforms ERK 1 and ERK 2 (see "Experimental Procedures"), only the 42-kDa protein pERK 2 was detected following SKF treatment.

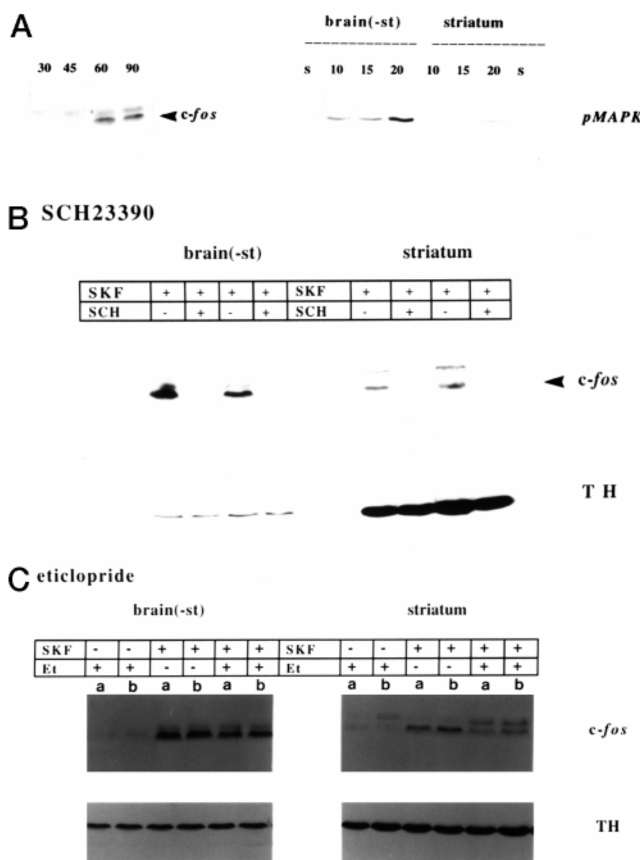

FIG. 1. **Induction of c-*fos* and pMAPK expression following SKF82958 (1 mg/kg) treatment of wild type mice.** *A*, *left*, expression of extrastriatal c-*fos* immunoreactivity 30–90 min after drug administration. The migration of the c-*fos* immunoreactive protein band, detected with a rabbit polyclonal antibody (Santa Cruz), is indicated by an *arrowhead*. *Right*, pMAPK expression in brain and dorsal striatum at 10–20 min time points after drug administration. Time points are indicated on *top* of each *lane*. The anti-pMAPK antibody detects only one phosphorylated MAPK of 42 kDa (ERK 2). *Lanes s*, saline. *B*, c-*fos* induction in brain (without striatum) and striatum 90 min following SKF82958 (*SKF*; 1 mg/kg) administration to mice pretreated with either SCH23390 (*SCH*; 1 mg/kg) or saline. Results of duplicate experiments are shown. *C*, SKF82958-induced expression of c-*fos* immunoreactivity 60 min (*lanes a*) and 90 min (*lanes b*) with or without eticlopride pretreatment (*Et*; 0.5 mg/kg). Total striatal and extrastriatal (*brain(−st)*) protein (100 μg) extracted from the combined tissues of two animals was loaded onto each lane. The blots shown in *B* and *C* were reprobed with an antibody directed against tyrosine hydroxylase (*TH*) to demonstrate that equal amount of protein was loaded onto each lane.

SKF-induced c-*fos* responses are completely abolished when mice are pretreated with the D_1-selective antagonist SCH23390 (1 mg/kg intraperitoneal). The results of duplicate experiments with dorsal striatal and extrastriatal tissues of mice are shown in Fig. 1*B*. Furthermore, as shown in Fig. 1*C*, pretreatment of mice with the D_2-like antagonist eticlopride (0.5 mg/kg intraperitoneal) reduces c-*fos* responses to SKF stimulation, an effect that is most apparent in the dorsal striatum and that is further examined below.

The next series of experiments analyzed the expression of c-*fos* immunoreactivity 60 and 90 min after SKF-82958 (1 mg/kg) application in the dorsal striatum and brain without dorsal striatum tissues of mutant mice lacking D_2, D_3, and D_2/D_3 receptors. These experiments were performed on mice with a hybrid C57Bl/6 × 129Sv genetic background (see "Experimental Procedures"), and the results are shown in Fig. 2. In both

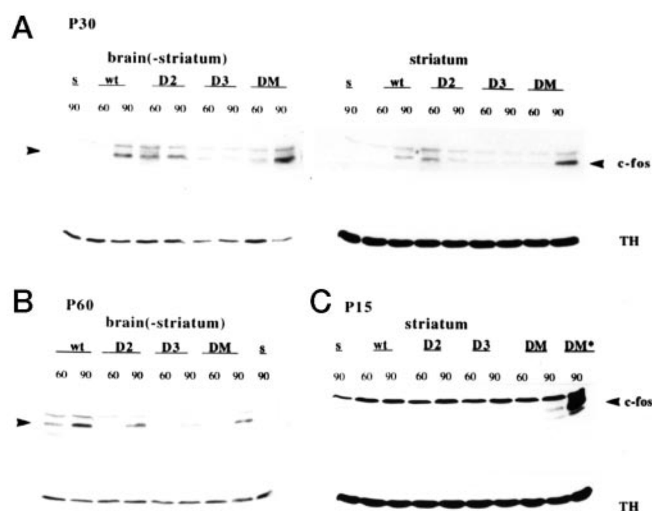

FIG. 2. **Expression of striatal and extrastriatal c-*fos* immunoreactivity following SKF82958 (1 mg/kg) treatment of wild type (*wt*) and mutant mice.** Shown is the expression of c-*fos* immunoreactivity 60 and 90 min after drug administration to P30 (*A*), P60 (*B*), and P15 mice (*C*). c-*fos* immunoreactivity (*arrowhead*) was detected with a rabbit polyclonal antibody (Santa Cruz). *DM*, homozygous D_2/D_3 double mutants. *Lanes s*, saline; *DM**, homozygous D_2/D_3 double mutants at P30. 100 μg of total protein extracted from combined brain tissues of two animals/genetic group was loaded onto each lane. Each blot was reprobed with an anti-TH antibody.

striatal and extrastriatal tissues of mice at P30, the most striking difference in the levels of c-*fos* immunoreactivity is seen in D_3 single mutants, which express lower levels compared with wild type, D_2 single mutants, and D_2/D_3 double mutants. In Fig. 2*A*, other differences include an earlier induction of c-*fos* expression in D_2 single mutants and a slightly stronger induction in D_2/D_3 double mutants, but these differences are no longer apparent in mice at P60. A representative example of c-*fos* levels expressed in extrastriatal tissues of P60 mice is shown in Fig. 2*B*. Similar to results obtained with P30 mice, c-*fos* levels of D_3 single mutants are decreased when compared with wild type, D_2 single mutants, and D_2/D_3 mutants. The optical density of signals measured 60 and 90 min following drug administration is 5.17 for wild type and 1.98 for D_3 single mutants and indicate a 62% decrease in c-*fos* responses in the mutant animals. Optical density measurements of corresponding signals obtained from D_2 single mutants (optical density, 3.51) and D_2/D_3 double mutants (optical density, 3.01) also indicate a reduction of c-*fos* expression. However, this reduction (32% in D_2 single mutants and 42% in the double mutants) is substantially less than the reduction seen in D_3 single mutants. Furthermore, c-*fos* levels expressed in the dorsal striatum of both D_2 and D_2/D_3 mutants are reduced by only 20% (not shown).

When SKF82958 was administered to mice at P15, only a marginal (and barely detectable) c-*fos* response was detected in all animals, regardless of their genotype. A representative example of c-*fos* responses in the striatum of these mice is shown in Fig. 2*C*. Thus, a robust c-*fos* expression in response to D_1 agonist stimulation occurs only at most advanced postnatal ages. In fact, the differences in c-*fos* responses of P30 and P60 mice suggest that the maturity of c-*fos* responses correlates with the maturity of the animal.

Despite of the differences in c-*fos* responses seen in D_2 and D_2/D_3 mutants at P30 and P60, c-*fos* levels of D_3 single mutants are consistently lower at both postnatal ages. To test whether possible differences in the genetic background could have accounted for the lower c-*fos* responses to SKF82958 in brains of

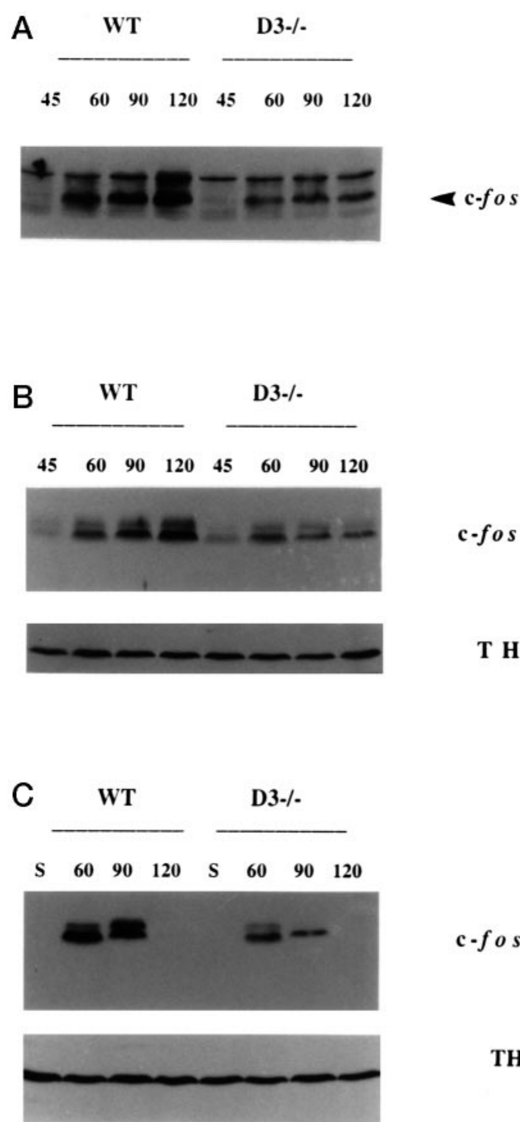

FIG. 3. **Expression of striatal and extrastriatal c-*fos* immunoreactivity following SKF82958 treatment in congenic wild type and D_3 mutants.** *A*, extrastriatal expression of c-*fos* immunoreactivity 45, 60, 90, and 120 min following SKF82598 treatment of P30 mice. c-*fos* immunoreactivity (*arrowhead*) was detected with a rabbit polyclonal antibody (Santa Cruz). *B*, protein samples loaded onto the gel shown in *A* were probed with a rabbit polyclonal anti-c-*fos* antibody that exclusively recognizes c-*fos* immunoreactivities of 55 and 57 kDa (Oncogene Research). *C*, striatal expression of c-*fos* immunoreactivity 60, 90, and 120 min following SKF82598 treatment of P30 mice. On all gels, 100 μg of protein extracted from brain tissue of one animal/time point was loaded. *Lanes S*, saline. The blots shown in *B* and *C* were reprobed with an anti-TH antibody.

D_3 single mutants, additional experiments were performed with congenic (C57Bl/6) D_3 mutants and their wild type littermates. As shown in Fig. 3, also congenic D_3 mutants showed a substantial reduction in the levels of c-*fos* expressed following SKF treatment. Fig. 3*A* shows a representative example of a Western blot of proteins extracted from brain (without striatum) tissue that was probed with the same antibody used to probe the blots shown in Fig. 2 (a rabbit polyclonal anti-c-*fos* antibody; Santa Cruz). Identical results were obtained when the blot was probed with another polyclonal anti-c-*fos* antibody (Oncogene Research) (Fig. 3*B*). This antibody, like the first antibody, recognizes an epitope located within peptide sequences that constitute the amino terminus of the c-*fos* protein. Unlike the first antibody, however, the second antibody does

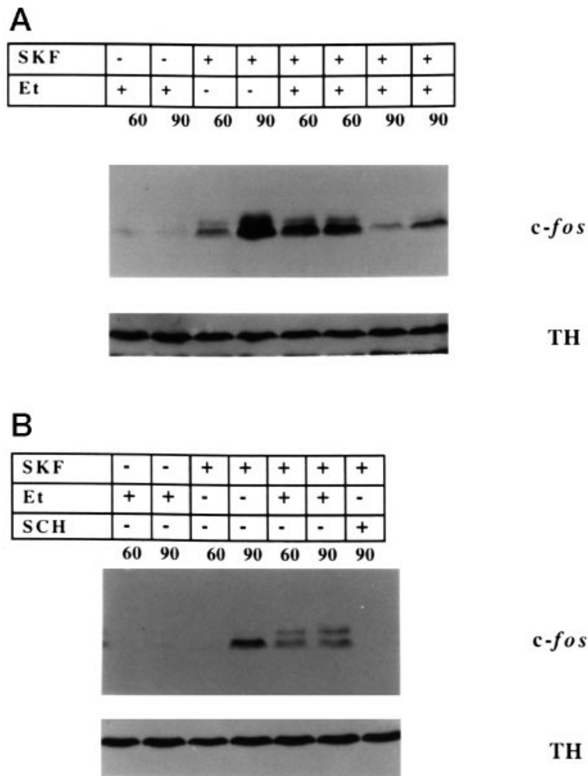

FIG. 4. **Expression of c-*fos* immunoreactivity following treatment of congenic D_3 mutants with eticlopride and SKF82958.** Shown is expression of extrastriatal (brain (without striatum)) (*A*) and striatal c-*fos* immunoreactivity (*B*) 60 and 90 min after SKF administration to mice that were pretreated either with eticlopride (Et; 0.5 mg/kg) or saline. The last lane on the blot shown in *B* demonstrates that SCH23390 (*SCH*; 1 mg/kg) pretreatment completely abolishes the SKF-induced c-*fos* induction. A rabbit polyclonal anti-c-*fos* antibody (Oncogene Research) was used to probe both blots. Total protein (100 μg) extracted from brain tissue of one animal/time point was loaded onto each lane.

not recognize other nonspecific protein bands (like the one migrating above the c-*fos* band in Fig. 2). In fact, it recognizes exclusively one major c-*fos* protein of 55 kDa and a second protein of 57 kDa that is known to result from posttranslational modifications of the 55-kDa c-*fos* protein (25). A densitometric analysis of signals shown in Fig. 3*B* revealed an optical density of 2.95 for the entire field containing c-*fos* signals of wild type animals 45, 60, 90, and 120 min after SKF treatment. The optical density for an equal size field containing corresponding signals of D_3 mutants is only 1.68, indicating a 57% reduction in c-*fos* expression levels. Thus, the results obtained with congenic D_3 mutants are very similar to the results obtained with hybrid (129Sv × C57Bl/6) D_3 mutant mice (Fig. 2*B*). Moreover, Fig. 3*C* illustrates that, similar to the results shown in Fig. 2*A*, congenic D_3 mutants also express lower levels of dorsal striatal c-*fos* immunoreactivity in response to D_1 agonist stimulation. Optical density measurements of these signals (wild type, 4.84; D_3 mutants, 1.42) revealed a 70% reduction of c-*fos* levels in D_3 mutants.

The next experiments tested whether the D_2-like antagonist eticlopride would further affect the D_1 agonist-stimulated c-*fos* response of D_3 mutants. The results are summarized in Fig. 4. As expected, in both extrastriatal and striatal tissue, administration of eticlopride (0.5 mg/kg) alone does not lead to detectable c-*fos* expression levels. It should be noted that in the experiment shown in Fig. 4, the c-*fos* responses of mice treated with SKF alone are low at 60 min and robust at 90 min after drug administration, whereas c-*fos* responses of mice treated with eticlopride and SKF are clearly detectable at both time points. As outlined above, the onset of detectable c-*fos* expression following the systemic administration of SKF varies between 60 and 90 min. This variability in the kinetics of c-*fos* induction necessitates that c-*fos* levels are measured at 60 and 90 min after drug administration so that the sums of optical densities of both time points can be compared. In Fig. 4*A*, results of duplicate experiments on extrastriatal tissues of mice treated with SKF and eticlopride and mice treated with SKF alone are compared. The optical density of the 60- and 90-min signals obtained from SKF-treated animals is 7.74. By comparison, the mean optical density of the 60- and 90-min c-*fos* signals of animals treated with SKF and eticlopride is 4.43. Thus, a 43% reduction of c-*fos* expression levels is found in mice pretreated with eticlopride. Similar results are obtained for the dorsal striatum (Fig. 4*B*). The optical density of c-*fos* signals following SKF treatment alone (2.51) is 20% higher than the corresponding optical density of c-*fos* signals following SKF and eticlopride treatment (2.01). It is further noted that in contrast to mice treated with SKF alone, striatal tissues of mice treated with SKF and eticlopride express similar levels of the 55- and 57-kDa c-*fos* proteins (see Figs. 1*C* and 4*B*). The reason for this expression pattern is presently unclear. Finally, as shown in Fig. 4*B*, the D_1-selective antagonist SCH23390 completely abolishes the c-*fos* response to SKF82958 administration in D_3 single mutants (see also Fig. 1*B*).

In summary, the results shown above indicate that D_3 single mutants express lower levels of c-*fos* in response to SKF treatment alone and that the D_2-like antagonist eticlopride further reduces this response. These results are consistent with the results obtained with wild type mice (Fig. 1*C*), and they suggest that the basal activity of both D_2 and D_3 receptors is required for the expression of maximum levels of c-*fos* responses to D_1 agonist stimulation. It is therefore very surprising that compared with D_3 single mutants, the c-*fos* responses of D_2/D_3 double mutants are reduced to a substantially lesser extent. In fact, given that c-*fos* responses are also reduced in D_2 single mutants (albeit to a much lesser extent compared with D_3 single mutants), one would expect that D_2/D_3 double mutants express the lowest levels of c-*fos* in response to D_1 agonist stimulation. Results of two additional experiments, however, suggest that mice lacking D_2 and D_2/D_3 receptors develop compensatory mechanisms that enable them to express higher levels of c-*fos* in response to D_1 receptor stimulation. First, as shown in Fig. 5*A*, it is obvious at a glance that double-mutant mice that are homozygous for the D_3 mutation and heterozygous for the D_2 mutation (D_3−/−;D_2+/−; labeled *D2*+/− in Fig. 5*A*) express drastically reduced levels of c-*fos* compared with mice that are either heterozygous for the D_3 mutation and homozygous for the D_2 mutation (D_3+/−;D_2−/−; labeled *D3*+/− in Fig. 5*A*) or homozygous for both mutations (D_3−/−; D_2−/−; labeled *MD*−/− in Fig. 5*A*). These results suggest that on a D_3 mutant genetic background, c-*fos* levels are reduced substantially when only one intact D_2-encoded allele is lacking. Second, as shown in Fig. 5*B*, c-*fos* responses of homozygous D_2 single mutants are unaffected by eticlopride, an antagonist with high affinity for both D_2 and D_3 receptors (22). This result suggests that adaptive mechanisms involving systems other then the dopaminergic system also operate in brains of D_2 single mutants.

Expression of pCREB and pMAPK in Response to D_1 Agonist Stimulation—It has previously been shown that differences in MAPK activity and CREB phosphorylation are critical in regulating c-*fos* expression (4). The following experiments therefore tested whether the expression of these two key transcrip-

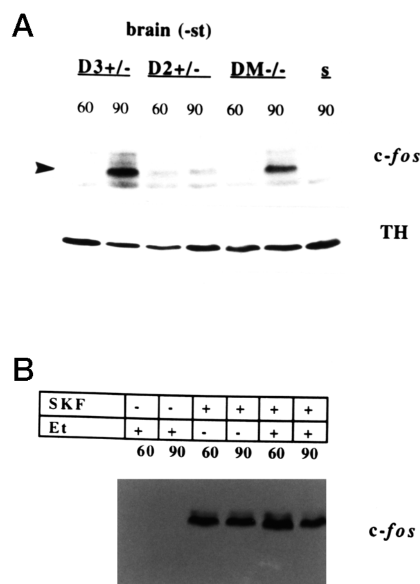

FIG. 5. **SKF82958-induced expression of extrastriatal c-*fos* immunoreactivity in D_2 and D_2/D_3 mutants.** *A*, c-*fos* expression in three different genetic combinations of D_2/D_3 double mutants. *D3+/−*, D_3+/−;D_2−/−; *D2+/−*, D_3−/−;D_2+/−; *DM−/−*, D_3−/−;D_2−/−. Total protein (100 μg) extracted from brain tissues of two P70 animals/genetic group was loaded onto each lane. c-*fos* immunoreactivity (*arrowhead*) was detected with a rabbit polyclonal anti-c-*fos* antibody (Santa Cruz), and the blot was reprobed with an anti-TH antibody. *B*, SKF-induced expression of c-*fos* immunoreactivity in D_2 single mutants pretreated with eticlopride (*Et*; 0.5 mg/kg) or saline. A rabbit polyclonal anti-c-*fos* antibody (Oncogene Research) was used to probe the blot which contains 100 μg of total protein/lane. Time points after drug administration (60 and 90 min) are indicated on *top* of each *lane*.

tional regulators is affected by the lack of D_3 and/or D_2 receptors.

Immunoblotting experiments were performed with pCREB- and pMAPK-specific antibodies to test whether the levels of pCREB and pMAPK differ between wild type and the various mutants that received SKF82958 treatment (1 mg/kg). These experiments were done with C57Bl/6 × 129Sv hybrid mice. As shown in Fig. 6, SKF administration led to the expression of similar levels of pCREB and pMAPK in wild type, D_2 and D_3 single mutants, and D_2/D_3 double mutants. The slightly lower level of pCREB immunoreactivity shown in Fig. 6 for D_3 mutants could not be verified with additional pCREB immunoprecipitation experiments (not shown). Thus, the results indicate that differences in the expression levels of pCREB and pMAPK do not account for the differences seen in the c-*fos* responses described above.

DISCUSSION

D_1 Agonist-stimulated c-fos Responses in Wild Type Mice and D_3 Mutant Mice—The present study revealed a blunted c-*fos* response to D_1 agonist stimulation in mice lacking the dopamine D_3 receptor. In both wild type and D_3 mutants, c-*fos* responses to stimulation with the full D_1 agonist SKF82958 could be completely abolished by pretreatment with the D_1-selective antagonist SCH23390. Furthermore, in both wild type and D_3 mutant mice, pretreatment with the D_2-like antagonist eticlopride reduced the magnitude of c-*fos* responses to D_1 agonist stimulation. These results indicate that although the induction of c-*fos* responses is dependent on D_1 receptor stimulation (26), maximum c-*fos* responses require a steady-state activity of *both* D_2 and D_3 receptors. The reduced c-*fos* response to D_1 agonist stimulation in D_3 mutants and the further reduction of c-*fos* expression levels following blockade of D_2 receptors in these mutants indicate cooperativity between D_1, D_2, and D_3

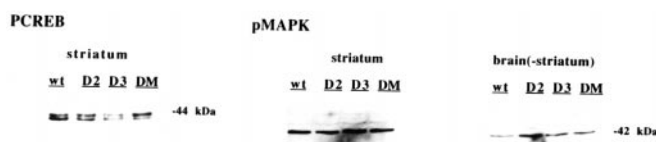

FIG. 6. **Expression of pCREB and pMAPK immunoreactivities in striatal and extrastriatal brain tissue of wild type (*wt*) and mutant mice treated with SKF82958.** *Left*, striatal pCREB levels (*PCREB*) were determined 5 min following drug administration. 60 μg of total protein extracted from brains of three animals/genetic group was loaded onto each lane. The blot was probed with an anti-pCREB antibody. pCREB levels remained unchanged up to 2 h after drug administration (not shown). *Right*, the expression of pMAPK (ERK 2; 42 kDa) was determined 20 min after drug administration using an anti-pMAPK antibody. *D2*, homozygous D_2 mutants; *D3*, homozygous D_3 mutants; *DM*, homozygous D_2/D_3 double mutants.

receptors in the modulation of c-*fos* responses to D_1 receptor stimulation.

Differences in c-*fos* responses between wild type and D_3 mutants were found in mice with a hybrid C57Bl/6 × 129Sv genetic background as well as in congenic C57Bl/6 mice. Thus, it is unlikely that differences in the genetic background contributed to the reduced c-*fos* expression found in SKF-treated D_3 mutants. Furthermore, the observed differences in c-*fos* responses to D_1 agonist stimulation cannot be explained by differences in D_1 receptor expression levels in D_3 mutants. Previous studies (27, 28), as well as our own unpublished results, have shown that the expression of D_1 receptor ligand-binding sites is unaltered in these mutants.

It is also unlikely that the D_1-selective agonist used in the present study exerts its effects on c-*fos* induction not only via D_1 but also via D_2 receptors. SKF82958 has recently been reported to act as a D_2-like autoreceptor agonist to inhibit the basal firing rate of midbrain dopaminergic units (29). However, as shown in the present study, c-*fos* responses elicited by SKF82598 are completely abolished by pretreatment with the D_1-selective antagonist SCH23390. Moreover, if c-*fos* responses were modulated by an agonist action of this drug at D_2-like autoreceptors, the autoreceptor effect would be inhibitory and, in contrast to our results, pretreatment with eticlopride should have increased c-*fos* responses. In addition, studies on mutant mice have now shown clearly that autoreceptor functions are mediated by D_2 but not D_3 receptors (30, 31). c-*fos* responses of D_3 single mutants (in which D_2 receptors remain expressed), however, are also reduced by eticlopride. Altogether, these results are inconsistent with a possible agonist action of SKF82958 at D_2 autoreceptors to modulate c-*fos* responses.

The present study demonstrates a similar role for D_2 and D_3 receptors in the potentiation of c-*fos* responses to D_1 agonist stimulation. Our results resemble results of previous pharmacological studies that found synergistic effects of concurrent D_1- and D_2-like receptor stimulation on c-*fos* responses in normal and 6-hydroxydopamine-lesioned animals (20, 21). The D_2-like agonists quinpirole and quinelorane used in these studies, however, have high affinities for both D_2 and D_3 receptors (22). Thus, it remained unclear whether both D_2 and D_3 receptors participate in this synergism. The present study, however, employed D_3 mutant mice that were treated either with a D_1 agonist alone or a combination of the D_1 agonist and a D_2-like receptor antagonist. The results revealed not only a cooperative involvement of both D_2 and D_3 receptors in the modulation of D_1 agonist-stimulated c-*fos* responses, they also illustrate that the basal activity of D_2 and D_3 receptors (*i.e.* activity in the absence of exogenous agonist) is sufficient to mediate this synergistic effect.

D_1 Agonist-stimulated c-fos Responses in D_2 and D_2/D_3 Mutants—Studies on D_3 mutant mice revealed a role for both D_2

and D_3 receptors in enhancing c-*fos* responses to D_1 receptor stimulation. However, the minimal reduction of c-*fos* expression levels observed in D_2/D_3 mutants does not reflect the magnitude of the combined contribution of D_2 and D_3 receptors to the modulation of D_1 agonist-stimulated c-*fos* responses. In fact, c-*fos* levels of double mutants are substantially higher than those seen in D_3 single mutants. It is therefore possible that D_2/D_3 double mutants have developed compensatory mechanisms that enable them to maintain relatively high (but still subnormal) c-*fos* responses to D_1 agonist stimulation, processes that, at least to some extent, are also likely to operate in brains of D_2 single mutants. Three observations support this possibility: First, $D_2+/-;D_3-/-$ double mutants express substantially lower levels of c-*fos* in response to D_1 agonist stimulation compared with homozygous double mutants ($D_2-/-$; $D_3-/-$). This result is consistent with the cooperative role of D_2 receptors in the induction of c-*fos* responses (*i.e.* the reduced expression of D_2 receptors leads to reduced cooperativity) and suggests further that the much lesser reduction of c-*fos* responses of homozygous D_2 single mutants results from compensatory adaptations that developed in mice with a homozygous and not a heterozygous D_2 mutant genotype. Second, in contrast to wild type and D_3 single mutants, c-*fos* responses to SKF treatment of homozygous D_2 mutants are not affected by pretreatment with the D_2-like antagonist eticlopride. Because eticlopride has a high affinity for both D_2 and D_3 receptors (22), it should have blocked the effect mediated by D_3 receptors. The inability of eticlopride to alter c-*fos* responses of D_2 mutants suggests that compensatory mechanisms involve systems other than the dopaminergic receptor system. Such systems could, for example, involve the adenosine A_{2A} receptor, which has been shown to modulate c-*fos* responses to D_1 receptor stimulation (21). Third, as shown in Fig. 2*A*, c-*fos* responses of D_2/D_3 double mutants at P30 but not at P60 are higher than corresponding wild type responses. This may indicate that, at P30, the "fine tuning" of developing compensatory mechanisms is not yet completed.

Adaptive changes are known to occur during the development of mice generated with constitutive knockout techniques, and they can be a serious complication for the interpretation of mutant phenotypes. It is perhaps not surprising that D_2/D_3 and possibly also D_2 mutants (but not D_3 mutants) develop adaptive mechanisms to compensate for the loss of the receptor(s). D_2 receptors are far more abundant than D_3 receptors (14), and their absence is likely to have more serious consequences for the normal functioning of the brain. Indeed, a comparison of the motor phenotypes of all three mutants revealed that mice lacking D_2 receptors are more severely impaired than mice lacking D_3 receptors and that D_2/D_3 double mutants are most severely impaired (23). Furthermore, other adaptations have already been described for D_2 single mutants that include, for example, alterations in the expression of glutamic acid decarboxylase mRNA, whose encoded protein is involved in the synthesis of the neurotransmitter γ-amino butyric acid (32). Regardless of the nature of adaptations that result in relatively high c-*fos* responses in mice lacking D_2 receptors, our results suggest that these adaptations obscure the interpretation of the magnitude of effect mediated by D_2 receptors in the complex interaction between D_1, D_2, and D_3 receptors to modulate c-*fos* responses.

What Are the Mechanisms Mediating the Cooperative Interactions between D_1 and D_2-like Receptors in the Modulation of c-fos Responses?—Synergistic or cooperative effects of D_1- and D_2-like receptor stimulation have not only been observed for the induction of c-*fos* expression, they have also been observed in electrophysiological and behavioral experiments. For example, the co-administration of D_1- and D_2-like agonists results in a synergistic inhibition of spontaneous and glutamate-evoked firing (33). Furthermore, in dopamine-depleted animals, unconditional behavioral responses to psychostimulants (increased locomotor activity, sniffing, licking, and biting) have been shown to require stimulation of both D_1 and D_2 receptors (34). However, it is not yet established whether the apparent cooperative interaction between D_1, D_2, and D_3 receptors result from interactions at the cellular level or whether they involve intercellular pathways. For the striatum, for example, anatomic studies suggest a predominant expression of D_1 receptors in neurons of the striatonigral pathway, whereas D_2 receptors are expressed in neurons of the striatopallidal pathway (35). This different cellular distribution would imply that an *intercellular* interaction between different pathways underlies the D_1/D_2 receptor synergism, pathways that may modulate the tonic inhibitory influence of enkephalin and/or γ-amino butyric acid released from local axon collaterals of D_1-containing striatonigral neurons and/or alter the activity of striatal interneurons. However, recent single-cell reverse transcription polymerase chain reaction experiments detected an extensive colocalization of D_1- and D_2-like receptor-encoded mRNA in medium spiny neurons of the striatonigral pathway (36). Furthermore, in contrast to previous reports (37, 38), results of our studies on the expression of D_3 receptor protein (detected with D_3-specific monoclonal antibodies) and D_3-encoded mRNA revealed a significant amount of D_3 receptor expression in the dorsal striatum (23, 39). In addition, imunocytochemical studies on rodent brain tissues identified a high number of D_3-immunoreactive neurons that possibly represent medium spiny cells and many (but not all) large interneurons.[2] Results of single-cell polymerase chain reaction studies further revealed that ~50% of the substance P/D_1 receptor-expressing neurons also expressed D_3 and D_4 receptor mRNA, and results of electrophysiological studies suggest that ~50% of all medium-spiny projection neurons co-express functional D_1- and D_2-like receptors (36). These findings give weight to the possibility that a cellular mechanism underlies the modulatory effects of D_2 and D_3 receptors. However, it should be noted that the effects of D_2 and D_3 receptor expression on c-*fos* responses to D_1 agonist stimulation are not restricted to the dorsal striatum as previously suggested (20). We found that it also operates in extrastriatal brain regions. Because the extrastriatal tissues that we analyzed comprised the entire brain (without dorsal striatum), we can only suggest that the c-*fos* immunoreactivity detected therein is derived mainly from anatomic regions representing the mesolimbic/mesocortical dopaminergic projection areas that express D_1, D_2, and D_3 receptors. For these anatomic structures, however, evidence for co-localization of all three receptors is still lacking.

Despite the present uncertainty regarding the contribution of cellular or intercellular mechanisms, determining the point at which D_1, D_2, and D_3 receptor-mediated signals converge to regulate the transcriptional activity of the c-*fos* gene will be an important next step toward understanding the mechanism of dopaminergic regulation of c-*fos* expression. Our finding that the expression of two key regulators of c-*fos* transcription, pCREB and pMAPK, is not significantly altered in D_1 agonist-treated mice that lack either D_2 or D_3 receptors suggests that the activity of other components of the signaling/transcriptional activator cascade is modulated by D_2 and D_3 receptors and that these components are most likely operating downstream of CREB and MAPK activation.

[2] E. A. Nimchinsky, P. R. Hof, W. G. M. Janssen, C. Schmauss, and J. H. Morrison, unpublished observation.

Implications of the Findings—Although D_3 receptors were identified by molecular cloning almost a decade ago, it has proven difficult to elucidate the function of these receptors with conventional pharmacological studies (22). Recently, however, a behavioral study on D_3 mutant mice showed that one role of D_3 receptors is to diminish the normal cooperative effects of D_1 and D_2 receptor stimulation in the regulation of motor activity and responses to the rewarding properties of psychostimulants (28). The present study identified another role, namely a cooperative participation of D_3 receptors in the regulation of c-*fos* responses to D_1 agonist stimulation. The finding that D_3 receptors play a role in dopamine-regulated gene expression adds an important new aspect to our understanding of D_3 receptor expression and function.

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