Development/Plasticity/Repair

Sustained Extracellular Signal-Regulated Kinase 1/2 Phosphorylation in Neonate 6-Hydroxydopamine-Lesioned Rats after Repeated D_1-Dopamine Receptor Agonist Administration: Implications for NMDA Receptor Involvement

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Extracellular signal-regulated kinase (ERK) 1/2, a well known regulator of gene expression, is likely to contribute to signaling events underlying enduring neural adaptations. Phosphorylated (phospho)-ERK was examined immunohistochemically after both single and repeated (i.e., sensitizing) doses of the partial D_1-dopamine (DA) receptor agonist SKF-38393 (2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benazepine HCl) to adult rats lesioned as neonates (neonate lesioned) with 6-hydroxydopamine. Remarkably, prolonged phospho-ERK accumulated primarily in layers II–III of medial prefrontal cortex (MPC), where it declined gradually yet remained significantly elevated for at least 36 d after repeated doses of SKF-38393. Sustained ($\geq$7 d) phospho-ERK was observed for shorter periods in various other cortical regions but was not detectable in striatum or nucleus accumbens. At 36 d, an additional injection of SKF-38393 to sensitized rats restored phospho-ERK to maximal levels only in MPC when examined 7 d later. Phosphorylated cAMP response element-binding protein (CREB), examined 7 d after the sensitizing regimen, was observed exclusively in MPC, where it was abundant throughout all layers. Systemic injections of SL327 (α-[amino[(4-aminophenyl)thio]methylene]-2-(trifluoromethyl)benzeneacetonitrile), an inhibitor of the upstream ERK activator mitogen ERK kinase, attenuated both ERK and CREB phosphorylation in layers II–III of MPC. Pretreatment with the D_1 antagonist SCH-23390 ((*R*)-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-OL maleate) inhibited the prolonged increase in MPC phospho-ERK, whereas the 5-HT_2 receptor antagonist ketanserin (3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl]-2,4(1H,3H)-quinazolinedione tartrate) was ineffective. Competitive and noncompetitive NMDA receptor antagonists also blocked sustained ERK phosphorylation. Collectively, the present results demonstrate coupling of D_1 and NMDA receptor function reflected in sustained activation of the ERK signaling pathway in MPC of SKF-38393-sensitized neonate-lesioned rats. Ultimately, long-lasting phosphorylation of ERK and CREB in MPC may play a pivotal role in any permanent adaptive change(s) in these animals.

Key words: **ERK; neonatal 6-hydroxydopamine; D_1; dopamine; NMDA; prefrontal; striatum**

Introduction

Administration of the neurotoxicant 6-hydroxydopamine (6-OHDA), with desmethylimipramine pretreatment, results in the selective destruction of dopamine-containing neurons (Smith et al., 1973). Rats bilaterally lesioned with 6-OHDA as neonates have increased susceptibility for aggression, hyperexcitability, and self-injurious behavior in response to dopamine (DA) receptor agonists (Breese et al., 1984). These behaviors are similar to those observed clinically with Lesch–Nyhan syndrome (LNS), a developmental disorder characterized by reduced brain DA, choreoathetoid movements, and compulsive self-injurious behavior (Lesch and Nyhan, 1964). The susceptibility for self-injurious behavior observed in neonate-lesioned rats is not present in rats lesioned with 6-OHDA as adults, the latter of which mimic Parkinson's disease (Breese et al., 1984; Marsden, 1984). Thus, the neonatal rat brain must retain sufficient plasticity to allow for the development of compensatory mechanisms that attenuate, to some degree, the debilitating effects of DA depletion.

Repeated dosing with the D_1-dopamine receptor agonist SKF-38393 (2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benazepine HCl) to neonate-lesioned rats results in long-lasting behavioral sensitization, which is seen as an agonist-induced increase in locomotor activity for at least 6 months after the sensitization (Breese et al., 1984; Criswell et al., 1989). Treatment with D_1 receptor antagonists blocks the increasing motor responsive-

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ness (Breese et al., 1985a,b; Criswell et al., 1989, 1990), a finding that demonstrates a requirement for D_1 receptor activation in SKF-38393-mediated sensitization of these animals. Studies have shown that the sensitization is not dependent on contextual cues associated with SKF-38393 administration (Criswell et al., 1989), nor is it related to changes in density or binding affinity of D_1 receptors in striatum (Breese et al., 1987).

Central to the notion that SKF-38393-mediated sensitization of neonate-lesioned rats relates to behavioral and neural plasticity is the discovery that pretreatment with the noncompetitive NMDA receptor antagonist MK-801 ((+)-5-methyl-10,11-dihydroxy-5H-dibenzo(a,d)cyclohepten-5,10-imine) abolishes this sensitization (Criswell et al., 1990). It is well established that NMDA receptor activation is critical for development of psychomotor stimulant-induced behavioral sensitization (Karler et al., 1989; Stewart and Druhan, 1993; Wolf and Jeziorski, 1993) and that NMDA receptor antagonists interfere with long-term potentiation, a model of learning and memory formation (Lynch et al., 1983; Davis et al., 1992; for review, see Riedel et al., 2003). These findings have established a critical role for NMDA receptor-dependent mechanisms that can lead to persistent cellular and behavioral adaptive changes. Thus, the finding that MK-801 antagonizes SKF-38393-mediated sensitization in neonate-lesioned rats supports consideration of this model as a type of "neuronal learning" (Criswell et al., 1989).

The discovery of specific neurobiological substrates of adaptive change(s) that accompany the enduring hyper-responsiveness of neonate-lesioned rats to repeated D_1 receptor stimulation might provide insight into developmental and other disorders involving permanent DA-mediated sensitization, such as schizophrenia and psychostimulant abuse. A likely candidate, extracellular signal-regulated kinase (ERK) 1/2, is a cell-signaling molecule thought to be critical for various forms of neuroplasticity. Stimulation of D_1 and NMDA receptors can recruit several second messenger systems to activate ERK (Xia et al., 1996; Sweatt, 2001). In this respect, Gerfen et al. (2002) demonstrated enhanced striatal ERK phosphorylation after acute SKF-38393 administration in unilateral adult 6-OHDA-lesioned rats. Furthermore, immediate-early gene transcription in DA-depleted striatum was ERK dependent, a finding that suggests ERK pathway involvement in adaptive changes occurring in these animals (Gerfen et al., 2002). Neuronal ERK phosphorylation has been linked to a number of other cellular signaling processes (for review, see Adams and Sweatt, 2002). Among these, activation of the transcription factor cAMP response element-binding protein (CREB) has emerged as a major regulatory mechanism for activity-dependent neuroplasticity (Lonze and Ginty, 2002).

The aim of the present study was to investigate whether repeated SKF-38393 administration to neonate-lesioned rats would result in enhanced ERK activation in the primary DA-terminal fields of the forebrain. Immunostaining for phosphorylated (phospho)-ERK revealed distinct temporal patterns in dorsal striatum (striatum), nucleus accumbens (accumbens), and cortex. The medial prefrontal cortex (MPC) was unique in that it demonstrated remarkably sustained phospho-ERK that was accompanied by increased phospho-CREB immunostaining. Moreover, the prolonged MPC phospho-ERK immunostaining was dependent on D_1 and NMDA receptor coactivation. Our data strongly suggest that the sustained ERK phosphorylation observed in MPC reflects a neuroadaptive change that occurs with D_1 agonist-induced sensitization of neonate-lesioned rats.

Materials and Methods

Preparation of neonate 6-OHDA-lesioned rats. Pregnant Sprague Dawley rats obtained from Charles River Laboratories were individually housed, with Wayne Lab Blox laboratory chow and water available *ad libitum*. On day 3 after delivery, male and female rat pups were anesthetized with ether and then administered 100 μg (free base) of 6-OHDA intracisternally (i.c.), 60 min after desipramine (20 mg/kg, i.p.), to protect noradrenergic neurons (Breese et al., 1984). The bilateral lesion causes >90% loss of dopamine innervation into the striatum and disrupts basal ganglia–cortical system circuits (Smith et al., 1973). Some rats received desipramine (20 mg/kg, i.p.) and saline (i.c.) and served as unlesioned (sham-lesioned) controls. Rats treated with 6-OHDA or saline neonatally were weaned at day 30 and testing began at 40–60 d of age. All animal use procedures were in strict accordance with the Institutional Animal Care and Use Committee (2003), and all efforts were made to minimize the number of animals used.

Although several lines of evidence implicate gender differences in the activation of ERK and CREB (Cardona-Gomez et al., 2002; Bi et al., 2003; Wade and Dorsa, 2003), no significant SKF-38393-dependent differences in phospho-ERK or phospho-CREB immunoreactivity were found between male and female rats, regardless of neonatal lesioning (data not shown). Thus, SKF-38393-induced phospho-ERK, ERK, phospho-CREB, or CREB immunoreactivity was examined in the striatum, accumbens, MPC, and other selected cortical regions of both male and female rats.

The following sections describe all adult drug treatments, dosing regimens, and times of killing for animals used in this study. Data in supplemental Figure A (available at www.jneurosci.org) are provided as an additional guide for the experimental paradigm.

Single SKF-38393 treatment to neonate-lesioned adult rats. To assess the acute effects of a single dose of SKF-38393, naive neonate-lesioned and sham-lesioned rats were injected with 3 mg/kg SKF-38393 at 40–60 d of age and killed at 15 min ($n = 4$ per treatment group), 30 min ($n = 4$), 60 min ($n = 4$–5), 120 min ($n = 5$–12), and 360 min ($n = 2$) after agonist treatment. A separate group of adult neonate-lesioned or sham-lesioned rats were administered saline, rather than agonist, and killed at 15, 60, and 120 min to serve as controls ($n = 2$). Neonate-lesioned and sham-lesioned rats were also killed at 3 d ($n = 4$) and 7 d ($n = 4$–6) after this treatment regimen to examine the long-term effects of a single dose of agonist to these animals.

Repeated SKF-38393 treatment to neonate-lesioned adult rats. Neonate-lesioned rats do not show maximal sensitivity to D_1 agonists unless exposed repeatedly to such agonists (Breese et al., 1985b; Criswell et al., 1989). Therefore, beginning at 40–50 d of age, neonate-lesioned rats in this treatment group received repeated treatments with SKF-38393 sufficient to allow the animals to reach a plateau of maximal behavioral supersensitivity (Criswell et al., 1989, 1990). To accomplish this sensitization process, lesioned animals were administered a total of 12 mg/kg SKF-38393, divided into three doses as follows: 6, 3, and 3 mg/kg, each spaced 1 week apart as described previously (Breese et al., 1985a,b; Criswell et al., 1989). A separate group of sham-lesioned rats received the same agonist-dosing regimen. To assess acute effects of repeated SKF-38393 administration, animals were killed at 15 min ($n = 4$–5 per treatment group), 60 min ($n = 4$–5), 120 min ($n = 4$), and 360 min ($n = 2$) after agonist treatment. To serve as controls, groups of neonate-lesioned and sham-lesioned rats were administered three consecutive injections of saline at weekly intervals and killed at 15 min ($n = 2$–4), 60 min ($n = 2$), 120 min (2–3), and 360 min ($n = 2$) after the final saline administration. To examine the chronic effects of repeated SKF-38393 administration, animals were killed at 3 d ($n = 4$–6), 7 d ($n = 10$–15), 14 d ($n = 5$–8), 21 d ($n = 6$–7), or 36 d ($n = 5$–6) after the final agonist or saline treatment. At 36 d, a separate group of previously treated neonate-lesioned and sham-lesioned rats were administered an additional dose of SKF-38393 (3 mg/kg) or saline and killed 7 d later ($n = 5$–6).

Immediately after the final dose of SKF-38393 or saline to neonate-lesioned or sham-lesioned rats, behavioral activity was assessed to assure maximal responsiveness of neonate-lesioned rats to the agonist. Rats were placed in a clear 17 × 17 inch computer-monitored activity cham-

ber (Med Associates, St. Albans, VT), and horizontal, vertical, and stereotypical activity was recorded in 5 min bins over a 3 hr testing period. ANOVA *F* test of model fit for motor activity between treatment groups yielded $F_{(3,206)} = 54.90$, $p < 0.0001$ for horizontal activity, $F_{(3,206)} = 17.68$, $p < 0.0001$ for vertical activity, and $F_{(3,206)} = 11.94$, $p < 0.0001$ for stereotypical activity. As determined by *post hoc* analysis using Fisher's PLSD test (mean ± SEM), neonate-lesioned rats receiving multiple SKF-38393 treatments demonstrated 120,486.24 ± 10,641.87 total horizontal counts, 2348.51 ± 263.30 total vertical counts, and 15,167.80 ± 535.27 total stereotypical counts compared with 20,681.32 ± 2357.34 total horizontal counts ($p < 0.0001$), 799.09 ± 77.84 total vertical counts ($p < 0.0001$), and 11,646.37 ± 476.32 total stereotypical counts ($p < 0.0001$) observed for neonate-lesioned rats injected with saline, 20,389.82 ± 2751.24 total horizontal counts ($p < 0.0001$), 1040.34 ± 118.37 total vertical counts ($p < 0.0001$), and 12,371.19 ± 772.15 total stereotypical counts ($p = 0.0026$) observed for sham-lesioned rats dosed repeatedly with SKF-38393, and 20,579.15 ± 1648.53 total horizontal counts ($p < 0.0001$), 965.13 ± 71.12 total vertical counts ($p < 0.0001$), and 10,864.39 ± 579.04 total stereotypical counts ($p < 0.0001$) observed for sham-lesioned rats injected with saline. The novelty of the testing environment unlikely affected our results, because no significant differences were found in motor activity between the SKF-38393-sensitized neonate-lesioned animals and a separate group of neonate-lesioned animals (not used in this study) that were placed in activity chambers after each of four weekly treatments with SKF-38393 (supplemental Table A, available at www.jneurosci.org) ($p = 0.4229$, horizontal activity; $p = 0.3034$, vertical activity; $p = 0.5824$, stereotypical activity indicating no difference with Fisher's PLSD test). This finding is consistent with previous data accumulated in neonate-lesioned rats showing that behavioral sensitization results in comparable levels of activity in response to SKF-38393 when the rats are repeatedly dosed in the same or a different environment from that in which the rats are finally tested (Criswell et al., 1989).

Pretreatment with the MEK inhibitor SL327 before repeated SKF-38393 administration to neonate-lesioned rats. For this experimental series, SL327 (α-[amino[(4-aminophenyl)thio]methylene]-2-(trifluoromethyl) benzeneacetonitrile) (100 mg/kg), a selective inhibitor of the upstream ERK activator MEK, was administered to neonate-lesioned rats 30 min before each of three weekly doses of SKF-38393 (3 mg/kg), with the fourth weekly treatment consisting of only dimethylsulfoxide (DMSO) vehicle followed by SKF-38393 ($n = 5$). Control treatment groups included (1) four weekly treatments of DMSO followed by SKF-38393 ($n = 4$) and (2) four weekly treatments of DMSO followed by saline ($n = 4$). All rats thus received DMSO before the fourth and final dose of SKF or saline and were killed 7 d later.

Pretreatment with the D_1 antagonist SCH-23390 or the 5-HT_2 receptor antagonist ketanserin before repeated SKF-38393 administration. Groups of neonate-lesioned and sham-lesioned rats each received four doses of SCH-23390 ((*R*)-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-OL maleate; 0.3 mg/kg), ketanserin (3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl]-2,4(1H,3H)-quinazolinedione tartrate; 2 mg/kg), or saline 15 min before each of four weekly doses of SKF-38393 or saline. The primary treatment groups were as follows: (1) SCH-23390 followed by SKF-38393 ($n = 6–10$) and (2) ketanserin followed by SKF-38393 ($n = 4$). Control treatment groups included (1) saline followed by SKF-38393 ($n = 2–10$), (2) SCH-23390 followed by saline ($n = 2–10$), and (3) saline followed by saline ($n = 4–5$). Rats were killed 7 d after the final drug treatment.

Pretreatment with NMDA receptor antagonist MK-801 or CGS-19755 before repeated SKF-38393 administration. For the noncompetitive NMDA receptor antagonist MK-801, male rats were dosed with 0.3 mg/kg, whereas females received 0.14 mg/kg because female rats have much greater responsiveness to the motor effects of MK-801 (Fleischmann et al., 1991; Blanchard et al., 1992; Honack and Loscher, 1993; Haggerty and Brown, 1996; Frantz and Van Hartesveldt, 1999). MK-801, the competitive NMDA receptor antagonist CGS-19755 (*cis*-4-(phosphonomethyl)-2-piperidinecarboxylic acid; 10 mg/kg), or saline was administered 15 min before four weekly doses of SKF-38393 (3 mg/kg) or saline. The two primary treatments were (1) MK-801 followed by SKF-38393 ($n = 13$ per treatment group) and (2) CGS-19755 followed by SKF-38393 ($n = 6–18$ per treatment group). Control treatments included (1) saline followed by SKF-38393 ($n = 5$), (2) MK-801 followed by saline ($n = 6–8$), (3) CGS-19755 followed by saline ($n = 6–15$), and (4) saline followed by saline ($n = 7–10$). Rats were killed 7 d after the final drug treatment.

Drugs. 6-OHDA hydrobromide (ICN Biochemicals, Irvine, CA) was dissolved in saline containing 0.5% ascorbic acid and administered intracisternally. Desipramine hydrochloride (Sigma, St. Louis, MO), SKF-38393 (Sigma), SCH-23390 (Schering Corporation, Bloomfield, NJ), ketanserin (Sigma), MK-801 (a gift from Merck, Rahway, NJ), and CGS-19755 (a gift from CIBA-GEIGY Corporation, Summit, NJ) were dissolved in saline and administered intraperitoneally. The MEK inhibitor SL327 (kindly provided by DuPont Pharmaceuticals Company, Boston, MA) was dissolved in 100% DMSO and administered intraperitoneally (2 ml/kg) (Atkins et al., 1998; Selcher et al., 1999; Yamagata et al., 2002).

Immunohistochemistry: tissue preparation and immunostaining. Rats were deeply anesthetized with an overdose of sodium pentobarbital (100 mg/kg), perfused transcardially for 4 min with PBS (150 mM NaCl, 100 mM sodium phosphate, pH 7.4) followed by 7 min of 4% phosphate–buffered paraformaldehyde (100 mM sodium phosphate), and their brains were collected and postfixed for 24 hr at 4°C. Forty-micrometer-thick sections were then cut with a vibrating microtome for the immunohistochemistry. Standard avidin–biotin–horseradish peroxidase methods were used as described previously (Knapp et al., 1998, 2001). After rinsing in fresh PBS three times (10 min each), free-floating tissue sections were blocked in 10% normal goat serum and 0.2% Triton X-100 in PBS for 1 hr. Affinity-purified polyclonal tyrosine hydroxylase (TH) (1:4000; Calbiochem, La Jolla, CA), phospho-ERK [phospho-p44/42 MAP kinase (thr202/tyr204); 1:500; Cell Signaling Technology, Beverly, MA], phospho-CREB, and CREB (both 1:500; Cell Signaling Technology) were used to detect protein expression. All sections were incubated in 3% normal serum, 0.2% Triton X-100, and antisera for 48–72 hr at 4°C with agitation. An antibody-blocking peptide to phospho-ERK containing phosphorylated amino acid residues threonine 202 and tyrosine 204 (Santa Cruz Biotechnology, Santa Cruz, CA) was used to verify specificity of the antibody. A 10-fold higher concentration of the blocking peptide was incubated with phospho-ERK primary antibody at room temperature for 30 min and then incubated with selected brain tissue as described above. Tissue sections were further processed using Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA) per the manufacturer's instructions with immunochemical detection using nickel-cobalt intensification of the diaminobenzidine reaction product. For analysis, great care was taken to match sections through the same region of brain at the same level. All visible positive nuclei or cell bodies within a 10 × 10 eyepiece reticule field were counted and expressed as number of cells per square millimeter of tissue for each brain site. For a single brain site, counts were averaged from three sections from each animal.

Western blot analysis: tissue preparation and immunoblotting. For Western blotting, rats were killed by decapitation, their brains were rapidly removed from the skulls, and the MPC and striatum were dissected on ice and stored at −80°C until use. Tissues were homogenized by sonication in solubilization buffer (10 mM Tris-Cl, 50 mM NaCl, 1% Triton X-100, 30 mM sodium pyrophosphate, 50 mM NaF, 5 nM $ZnCl_2$, 100 μM Na_3VO_4, 1 mM DTT, 5 nM okadaic acid, 2.5 μg aprotinin, 2.5 μg pepstatin, and 2.5 μg leupeptin). Insoluble material was removed by centrifugation (13,000 rpm for 20 min at 4°C), and protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL). Samples were mixed with Novex 2× Tris-glycine SDS sample buffer (San Diego, CA) containing 5% 2-mercaptoethanol and heated to 90°C for 3 min. Aliquots of 20 μg of protein per lane were separated on 8–16% gradient Tris-glycine gels (Novex) under reducing conditions using the Novex Xcell II minicell apparatus. Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). Membranes were incubated in PBS with 0.05% Tween 20 (PBS-T), containing 1% milk powder for 2 hr at room temperature to block nonspecific binding. Blots were probed with an antibody corresponding to the inactive form of ERK (p44/42 MAPK; 1:1000; Cell Signaling Technology) or to phospho-ERK (1:1000; Cell Signaling Technology), followed by goat anti-rabbit IgG conjugated with horseradish peroxidase

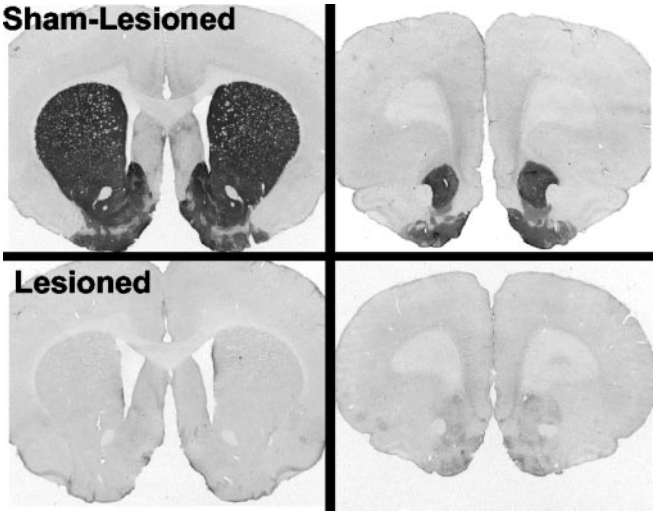

Figure 1. Immunohistochemistry for tyrosine hydroxylase (1:5000; Calbiochem) in coronal sections representing the striatum, accumbens, and MPC in sham-lesioned and neonate 6-OHDA-lesioned (Lesioned) adult rats.

(Chemicon, Temecula, CA) at a 1:20,000 dilution in blocking solution for 60 min. Membranes were then washed three times with PBS-T. Bands were detected using enhanced chemiluminescence (Pierce) apposed to x-ray film under nonsaturating conditions and analyzed by densitometric measurements using NIH Image 1.57 (public domain software developed by the National Institutes of Health and available at http://rsb.info.nih.gov/nih-image). Data are representative of four animals per treatment group and normalized on the basis of estimates obtained in the samples from sham-lesioned, saline-treated controls.

Quantification and statistical analysis. Details of brain region identification and cell counting strategy have been reported previously in our laboratory (Knapp et al., 1998). Average counts for a specific brain region for each animal were grouped by treatment and averaged for each time point to obtain the mean counts per square millimeter ± SEM for that brain site. Average cell counts for each defined brain region were compared within and between treatment groups using ANOVA. Statistical comparisons between control groups versus lesioned groups administered either SKF-38393 or saline were followed by *post hoc* tests. A more conservative significance level was set at $p = 0.01$ for all time-course experiments to correct for multiple comparisons, whereas the significance level for all other experiments was set at the traditional 0.05 level. The Fisher's PLSD test was performed when comparing combinations of means.

Results

Assessment of dopamine-containing neuronal destruction in neonate-lesioned adult rats

Previous studies (Smith et al., 1973; Breese et al., 1985a,b) documented that DA content within the striatum, accumbens, and cortex is drastically reduced in adult rats lesioned as neonates with 6-OHDA. Such loss of DA-containing terminals in the striatum of neonate-lesioned rats is illustrated in Figure 1 by the >90% reduction in TH immunoreactivity compared with the TH level in a sham-lesioned control animal. Because TH is the rate-limiting enzyme in the biosynthesis of dopamine (Nagatsu et al., 1964), TH immunoreactivity was determined for all lesioned and control animals in this investigation to establish that the neonate 6-OHDA lesioning induced an adequate loss of DA-containing neurons.

Acute effects of single and repeated D_1 agonist SKF-38393 administration on phospho-ERK immunoreactivity in neonate-lesioned rats

Phospho-ERK was evaluated in the primary forebrain DA-terminal regions (i.e., striatum, accumbens, and MPC) of

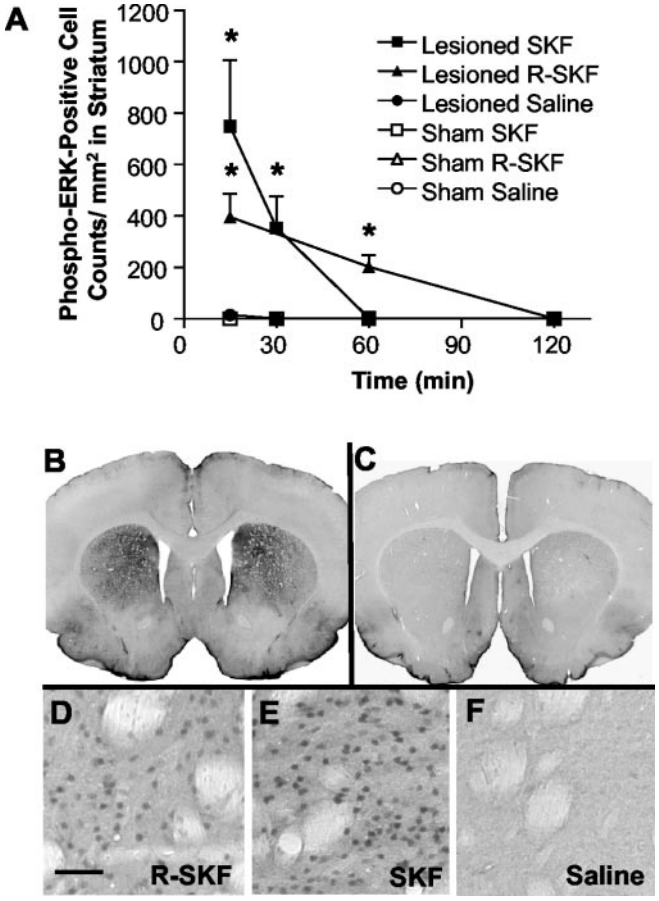

Figure 2. Administration of the partial D_1 agonist SKF-38393 to neonate 6-OHDA-lesioned rats transiently activates ERK in striatum. *A*, Time-dependent ERK phosphorylation in striatum (0–120 min). Treatment groups are represented by (■) Lesioned SKF = single dose of SKF-38393 to neonate-lesioned rats; (▲) Lesioned R-SKF = repeated doses of SKF-38393 to neonate-lesioned rats; (●) Lesioned Saline = saline treatment to neonate-lesioned rats; (□) Sham SKF = single dose of SKF-38393 to sham-lesioned rats; (△) Sham R-SKF = repeated doses of SKF-38393 to sham-lesioned rats; (○) Sham Saline = saline treatment to sham-lesioned rats. Phospho-ERK-positive cell counts did not significantly differ between rats receiving single or multiple injections of saline; thus these data were collapsed for each time point examined. Symbols remain consistent throughout this paper to represent each treatment group. ANOVA *F* test of model fit: $F_{(19,74)} = 9.890$; $p < 0.0001$. *B*, *C*, At 15 min after SKF-38393 treatment to neonate-lesioned rats, abundant phospho-ERK immunoreactivity is observed in striatum (*B*) but is not present in striatum of neonate-lesioned rats administered saline treatments (*C*). *D*, Representative low-magnification (100×) image of phospho-ERK-expressing cells at 15 min in neonate-lesioned rats administered repeated doses of SKF-38393. *E*, Neonate-lesioned rats administered a single dose of SKF-38393. *F*, Neonate-lesioned rats injected with saline.

neonate-lesioned rats at various time points after D_1 agonist administration. The initial 15 min time point was chosen on the basis of Gerfen et al. (2002), who demonstrated maximum phospho-ERK immunoreactivity in striatum of unilateral adult-lesioned rats 15 min after a single SKF-38393 administration. In the present study, distinctive patterns of time-dependent ERK phosphorylation were observed across these brain regions in response to SKF-38393.

Striatum and nucleus accumbens

In the striatum, both single and repeated SKF-38393 treatments to neonate-lesioned rats produced acute, transient increases in phospho-ERK that peaked at 15 min after drug administration (Fig. 2*A*,*D*,*E*). Although slightly more phospho-ERK-positive cells were observed at 15 min in naive neonate-lesioned rats com-

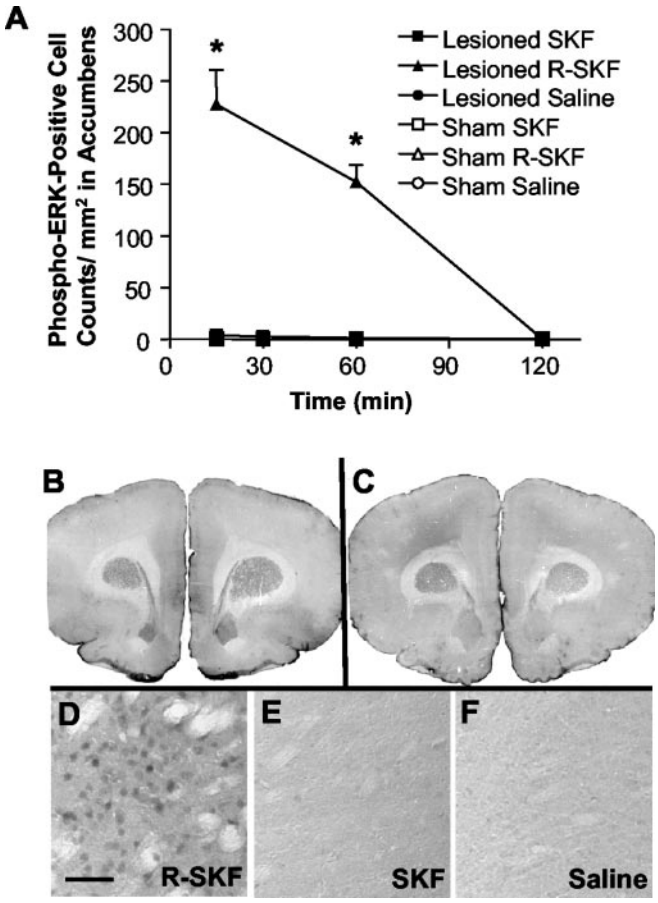

Figure 3. Repeated administration of the partial D_1 agonist SKF-38393 to neonate 6-OHDA-lesioned rats transiently increases phospho-ERK in accumbens. *A*, Time-dependent phospho-ERK immunoreactivity in accumbens (0–120 min). ANOVA *F* test of model fit: $F_{(19,74)} = 27.335$; $p < 0.0001$. *B*, *C*, At 15 min, phospho-ERK immunostaining is observed in accumbens of neonate-lesioned rats administered repeated doses of SKF-38393 (*B*) but is not present in accumbens of neonate-lesioned rats administered only a single dose of SKF-38393 (*C*). *D*, Representative low-magnification (100×) image of phospho-ERK-expressing cells at 15 min in neonate-lesioned animals administered repeated doses of SKF-38393. *E*, Neonate-lesioned rats administered a single dose of SKF-38393. *F*, Neonate-lesioned rats injected with saline. *$p <$ 0.01 with Fisher's PLSD test. Scale bar, 250 μm.

pared with rats sensitized with the agonist, the difference was not significant ($p = 0.2096$). ERK activation was absent in the striatum by 60 min in neonate-lesioned rats administered a single dose of SKF-38393; however, phospho-ERK immunoreactivity remained significantly elevated at this time point in rats administered repeated doses of SKF-38393 ($p < 0.0001$). This prolongation of ERK phosphorylation appeared to occur most prominently in the dorsomedial and dorsolateral quadrants of the striatum, with lighter staining occurring ventrally (Fig. 2*B*). Phospho-ERK was no longer detected at 120 min in striatum of SKF-38393-treated neonate-lesioned rats. No phospho-ERK immunoreactivity was detected in striatum of neonate-lesioned rats treated with saline or in sham-lesioned rats treated with single or repeated doses of SKF-38393 or saline at any of the time points examined (Fig. 2*A*,*C*,*F*).

In accumbens, a single dose of SKF-38393 to drug-naive neonate-lesioned animals or controls failed to produce significant change in phospho-ERK at any time point (Fig. 3*A*,*C*,*E*,*F*). Neonate-lesioned rats administered repeated doses of SKF-38393, however, exhibited significant phospho-ERK immunoreactivity at acute time points before 120 min (Fig. 3*A*,*B*,*D*) compared with neonate-lesioned animals administered saline ($p < 0.0001$, 15 min; $p < 0.0001$, 60 min). This enhanced ERK phosphorylation was observed specifically in the rostral pole of the accumbens and was not present in the caudal shell or core of neonate-lesioned rats receiving repeated doses. Although the time course of accumbens phospho-ERK immunoreactivity was similar to that of striatum, total phospho-ERK-positive cell counts in the accumbens were ~20–40% of striatum, and the intensity of phospho-ERK immunostaining in the accumbens was much lower. It is unlikely that these effects were artifacts of tissue processing, because representative sections of the striatum and accumbens were always stained concurrently.

Medial prefrontal cortex

A baseline of ~18–25 phospho-ERK-positive cells per square millimeter was observed consistently in MPC and did not significantly differ among the sham or saline-treated lesioned groups (Fig. 4*A*). Neonate-lesioned rats that received a single dose of SKF-38393 demonstrated a transient (0–60 min) increase in ERK phosphorylation over baseline in MPC, which was similar in profile and duration to the striatum of these animals (compare Figs. 2*A*, 4*A*). Phospho-ERK immunoreactivity in this treatment group was significantly elevated over control groups only at 15 min after agonist administration ($p < 0.0001$ for Lesioned SKF vs control groups).

Among the dopamine-rich regions, MPC displayed a unique, highly prolonged increase in phospho-ERK in neonate-lesioned rats previously administered repeated doses of SKF-38393. In these animals, phospho-ERK-immunoreactive cell counts were increased four- to sixfold, primarily within layers II–III of the MPC. These phospho-ERK-positive counts remained elevated over the course of the examination (up to 360 min) (Fig. 4*A–H*) ($p = 0.0085$ at 15 min, $p = 0.0015$ at 60 min, $p < 0.0001$ at 120 min for Lesioned R-SKF vs Lesioned SKF groups; $p < 0.01$ at 15, 60, and 120 min, and $p < 0.02$ at 360 min for Lesioned R-SKF vs other control groups). The conspicuous persistence of ERK hyperphosphorylation in MPC, but not in striatum or accumbens, suggests that different processes of neuroadaptation occur among these brain regions and, furthermore, that the adaptive changes occurring in the MPC are more enduring.

Sustained duration of ERK phosphorylation in MPC of neonate-lesioned rats after repeated SKF-38393 administration

Because the persistent increase in ERK phosphorylation was observed for up to 360 min in MPC of neonate-lesioned rats sensitized with SKF-38393, an additional evaluation of the time course of the sustained ERK response in this region was performed. As shown in Table 1, control groups did not significantly differ from each other across time points, with phospho-ERK-positive cell counts remaining at the baseline level of ~18–25 cells per square millimeter. Neonate-lesioned rats sensitized with repeated doses of SKF-38393 demonstrated a sustained increase in phospho-ERK immunoreactivity in MPC that was significantly elevated over neonate-lesioned rats injected with saline (as well as sham-lesioned control groups) across all extended time points ($p < 0.0001$ at 3, 7, and 14 d; $p = 0.0109$ at 21 d; $p = 0.0025$ at 36 d comparing R-Saline and R-SKF groups). Prominent phospho-ERK in MPC of these animals persisted for >7 d after the final agonist treatment (Table 1). Beyond 7 d, sensitized phospho-ERK immunoreactivity began to diminish, yet remained significantly elevated from that observed in saline-treated

neonate-lesioned and sham-lesioned rats for up to 36 d ($p < 0.01$ for all comparisons).

As shown in Table 1, an additional dose of SKF-38393 administered at 36 d to previously sensitized neonate-lesioned rats resulted in significantly elevated levels of phospho-ERK immunoreactivity 7 d later (i.e., at 42 d) compared with levels observed at 36 d ($p = 0.0001$). Phospho-ERK-positive cell counts in these animals were nearly identical to those found in neonate-lesioned rats killed 3 and 7 d after the initial SKF-38393 dosing regimen ($p \geq 0.1110$, indicating no significant difference). In neonate-lesioned rats injected repeatedly with saline, an additional dose of saline at 36 d did not result in elevated phospho-ERK immunoreactivity at 7 d after injection (data not shown), nor did a single dose of SKF-38393 to neonate-lesioned or sham-lesioned rats (Table 1). Sham-lesioned rats that received saline treatment at 36 d also did not exhibit a significant increase in phospho-ERK immunoreactivity at 7 d after injection (data not shown).

To ascertain that the observed immunohistochemical staining was phospho-ERK and not another phosphoprotein or nonspecific reaction, immunoblotting was performed with protein isolated from MPC of lesioned rats administered repeated doses of SKF-38393 or injected with saline (data not shown). Blots probed with the same concentration of phospho-ERK antibody used for immunohistochemistry revealed the typical doublet of bands near 42 and 44 kDa related to phospho-ERK1 and phospho-ERK2, respectively. No other bands were visualized, a finding that provides strong evidence that the elevated immunohistochemical staining observed in MPC of rats dosed repeatedly with SKF-38393 was specific for phospho-ERK.

A plausible explanation for our findings with sustained phospho-ERK would be that total ERK protein levels were elevated chronically in the neonate-lesioned animals after repeated exposure to SKF-38393. Therefore, we examined whether sensitization of neonate-lesioned rats to SKF-38393 would produce long-lasting changes in total ERK protein in MPC. Because immunohistochemistry for unphosphorylated ERK failed to produce a consistent signal, total ERK protein was analyzed by SDS-PAGE and immunoblotting of equally loaded total proteins from MPC and striatum (blot not shown). No significant differences were found for optical density measurements (mean ± SEM) of total ERK levels among neonate-lesioned rats repeatedly dosed with SKF-38393 (MPC, 101.86 ± 5.42; striatum, 91.0 ± 5.59), sham-lesioned rats repeatedly dosed with SKF-38393 (MPC, 95.60 ± 4.75; striatum, 91.56 ± 1.96), saline-treated neonate-lesioned rats (MPC, 105.30 ± 3.30; striatum, 94.00 ± 1.12), or saline-treated sham-lesioned rats (MPC and striatum standardized to 100%) at day 7 after the final injection of agonist (ANOVA F test of model fit: $F_{(3,12)} = 1.040$, $p = 0.4102$ for MPC; $F_{(3,12)} = 1.865$, $p = 0.1893$ for striatum). Because total ERK levels were not changed by the repeated D_1 agonist treatments in neonate-lesioned rats, the sustained increase in phospho-ERK immunoreactivity in MPC could not be attributed to a sustained increase in total ERK protein.

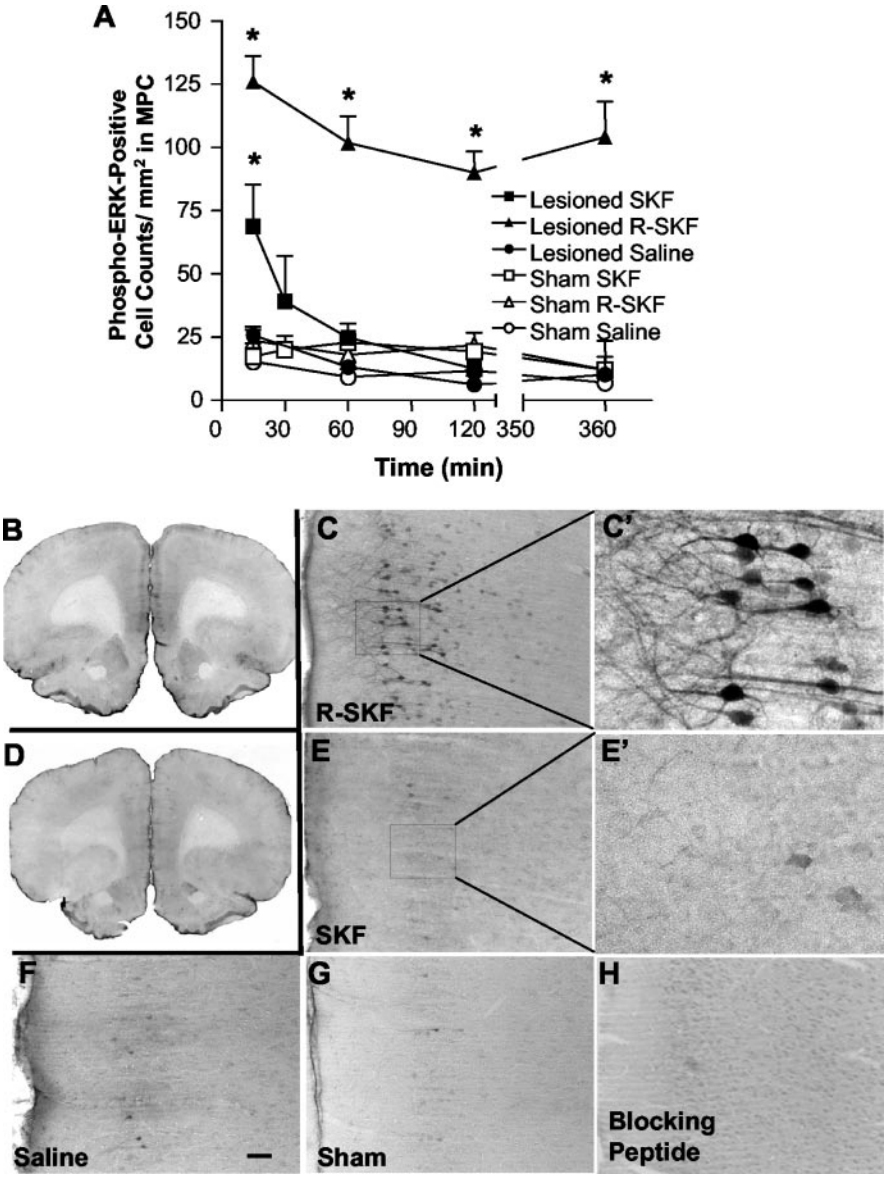

Figure 4. Repeated administration of the partial D_1 agonist SKF-38393 to neonate-lesioned rats produces a sustained increase in ERK phosphorylation in MPC. *A*, Time-dependent phospho-ERK immunoreactivity (0–360 min). ANOVA *F* test of model fit: $F_{(23,79)} = 12.943$; $p < 0.0001$. *B*, Neonate-lesioned rats administered repeated doses of SKF-38393 demonstrated robust phospho-ERK immunostaining at 120 min. *C*, *C'*, Representative low-magnification (100×) (*C*) and high-magnification (400×) (*C'*) images of phospho-ERK-expressing cells (120 min) in neonate-lesioned rats administered repeated doses of SKF-38393. *D*, Increased phospho-ERK immunostaining was not present at 120 min in MPC of neonate-lesioned rats administered a single dose of SKF-38393. *E*, *E'*, Representative low-magnification (100×) (*E*) and high-magnification (400×) (*E'*) images of phospho-ERK-expressing cells (120 min) in neonate-lesioned rats administered a single dose of SKF-38393. *F*, Neonate-lesioned rats administered repeated saline injections. *G*, Sham-lesioned rats administered repeated doses of SKF-38393. *H*, Corresponding blocking peptide inhibited sustained phospho-ERK immunoreactivity in neonate-lesioned rats repeatedly dosed with SKF-38393. *$p <$ 0.01 with Fisher's PLSD test. Scale bar, 250 μm.

Table 1. Chronic time course of ERK phosphorylation in MPC after repeated doses of SKF-38393 or saline to neonate- and sham-lesioned rats (mean ± SEM)

Time of killing[a]	Lesioned R-Saline	Lesioned R-SKF	Sham R-Saline	Sham R-SKF
ERK phosphorylation at extended time points after SKF-38393 treatment				
3 d	18.42 ± 7.29	94.38 ± 4.63*†	30.08 ± 4.77	27.50 ± 5.38
7 d	19.90 ± 5.95	114.40 ± 13.37*†	18.15 ± 5.60	16.80 ± 2.70
14 d	19.30 ± 5.95	53.59 ± 4.30*‡	19.17 ± 3.06	
21 d	22.78 ± 3.10	42.43 ± 7.24*‡	25.42 ± 2.64	
36 d	17.10 ± 3.70	37.25 ± 2.55*	15.00 ± 4.00	
ERK phosphorylation after reinstatement dose of SKF-38393 at 36 d				
7 d after additional treatment[b]	28.08 ± 4.88	96.10 ± 2.36*†	18.50 ± 10.26	

ANOVA *F* test of model fit: $F_{(19,117)} = 16.057$; $p < 0.0001$. Cells were counted at 100× magnification.

[a]Interval between repeated SKF-38393 or saline treatment and killing.

[b]Rats were killed at day 42, 7 d after an additional dose of SKF-38393 at 36 d.

*Counts are significantly different from all other counts (across all treatment groups and time points) at the $p = 0.01$ significance level with Fisher's PLSD test.

†,‡Counts within a treatment group (column) significantly differ from each other at the $p = 0.01$ significance level with Fisher's PLSD test.

Table 2. Chronic time course of ERK phosphorylation in various cortical regions after repeated doses of SKF-38393 to neonate-lesioned rats (mean ± SEM)

	Time of killing[a]			
Brain region	7 d	14–21 d[b]	36 d	7 d after additional dose at 36 d (42 d)[c]
VLOC	53.21 ± 14.86*	29.53 ± 7.49	16.50 ± 7.27	39.83 ± 17.32
CgC	50.58 ± 10.28*	19.91 ± 4.18	7.90 ± 3.30	28.00 ± 9.88
MC	106.08 ± 20.05*	49.44 ± 9.60	15.10 ± 3.80	47.07 ± 18.06
SSC	113.96 ± 23.44*	37.19 ± 7.99	25.50 ± 8.86	62.07 ± 23.23
PirC	135.5 ± 28.75*	53.84 ± 7.82	28.90 ± 9.82	64.07 ± 15.91

ANOVA *F* test of model fit: VLOC, $F_{(7,65)} = 2.863$, $p = 0.0115$; CgC, $F_{(7,65)} = 5.551$, $p < 0.0001$; MC, $F_{(7,65)} = 5.681$, $p < 0.0001$; SSC, $F_{(7,65)} = 6.960$, $p < 0.0001$; PirC, $F_{(7,65)} = 6.090$, $p < 0.0001$. Cells were counted at 100× magnification for each region.

[a]Interval between repeated SKF-38393 or saline treatment and killing.

[b]Values at 14–21 d did not significantly differ within treatment groups for each cortical region examined and thus collapsed.

[c]Rats were killed at day 42, 7 d after an additional dose of SKF-38393 at 36 d.

*Counts within a given cortical region are significantly different from all other counts in that region at the $p = 0.01$ significance level, except for Lesioned R-SKF 7 d versus Lesioned-R-SKF 42 d in VLOC.

Sustained duration of ERK phosphorylation in other cortical regions of neonate-lesioned rats after repeated SKF-38393 administration

Because of the extraordinarily sustained phospho-ERK response observed in MPC, we examined other cortical areas of neonate-lesioned rats dosed repeatedly with agonist to determine whether ERK phosphorylation was prolonged in these regions. (Fig. 5, Table 2). Phospho-ERK immunostaining was elevated in ventrolateral orbital cortex (VLOC) and in layer II–III cells of cingulate (CgC), motor (MC), somatosensory (SSC) and piriform (PirC) cortices when examined at day 7 after the final agonist treatment. As shown in Table 2, levels of phospho-ERK immunoreactivity for each cortical region were significantly higher at day 7 than levels observed at days 14–21 and 36 after the agonist treatment ($p < 0.01$ for all comparisons). Levels at day 7 after agonist treatment were also significantly higher than levels observed at all time points in saline-treated neonate-lesioned rats and sham-lesioned controls for each cortical region examined (Fig. 5) ($p < 0.01$ for all comparisons). Although apparent phospho-ERK-positive cell counts were still elevated (although not statistically significant) at 14–21 d after repeated agonist treatment, phospho-ERK in these cortical regions was clearly reduced by day 36. An additional dose of SKF-38393 at day 36 restored some phospho-ERK immunoreactivity in these cortical regions when examined 7 d later, but these levels were not significantly different from levels observed at day 36 before the additional dose (Table 2). Likewise, these levels were not different from those observed in saline-treated neonate-lesioned rats killed at day 36 or in saline-treated neonate- or sham-lesioned rat groups administered a single dose of SKF-38393 at day 36 and killed 7 d after injection (data not shown). In contrast to cortex, neither the striatum nor the accumbens of SKF-38393-sensitized rats exhibited phospho-ERK immunoreactivity at 7 d after the final sensitizing dose of agonist (Fig. 5).

Together, these data demonstrate that sustained phospho-ERK immunoreactivity occurs in multiple areas of the cortex, but not in the striatum or accumbens, of D_1 agonist-sensitized neonate-lesioned rats. These changes are most remarkable in MPC, where ERK phosphorylation lingers longest and remains most sensitive to full reinstatement with an additional dose of agonist. That the ERK pathway could respond to repeated D_1 agonist stimulation in such a prolonged and regionally selective manner suggests that specific neuroadaptive changes accompany D_1 sensitization in the neonate-lesioned rat. With respect to the MPC, neuroadaptive changes in this region are thought to play a role in diseases related to dopaminergic dysfunction such as schizophrenia, attention-deficit–hyperactivity disorder, and drug abuse (Laruelle, 2000; Vanderschuren and Kalivas, 2000; Steketee, 2003; Sullivan and Brake, 2003). Because behavioral alterations in the neonate-lesioned rat have been suggested to model each of these diseases (Schwarzkopf et al. 1992; Stevens et al., 1996; Fahlke and Hansen 1999; Moy and Breese 2002; Davids et al., 2003), we chose to focus most of the remainder of our study on exploring this phenomenon in the MPC.

Sustained CREB phosphorylation in MPC associated with prolonged phospho-ERK in neonate-lesioned rats after repeated SKF-38393 administration

Phosphorylation of ERK can activate the transcription factor CREB to affect long-term gene expression (Lonze and Ginty, 2002). Thus, phosphorylated CREB potentially represents a functional product of the sustained ERK response after repeated dosing with SKF-38393 to neonate-lesioned rats. When sections from the same animals were immunostained concurrently, we

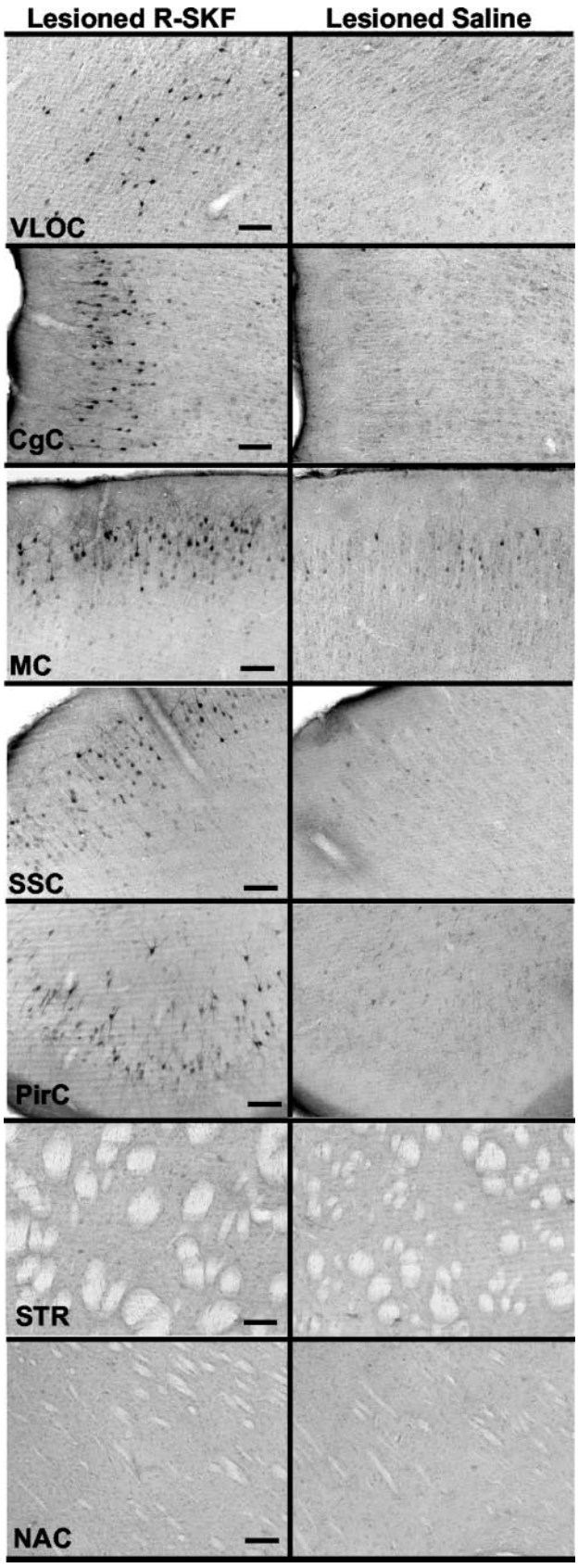

Figure 5. Photomicrographs depicting ERK phosphorylation in various cortical regions, striatum, and accumbens of neonate-lesioned rats at day 7 after repeated SKF-38393 administration (R-SKF) or saline treatment. VLOC, Ventrolateral orbital cortex, orbitofrontal cortex; CgC, Cg1, and Cg2, cingulate cortex; MC, M1, and M2, motor cortex; SSC, S1, somatosensory cortex; PirC, piriform cortex; STR, striatum; NAC, accumbens. Scale bar, 100 μm.

observed a robust increase in phospho-CREB immunoreactivity accompanying phospho-ERK in MPC at day 7 after repeated SKF-38393 administration (Fig. 6*A*). To our surprise, however, increased phospho-CREB immunoreactivity was observed not only in layers II–III, but throughout all MPC layers (Fig. 6*B*,*F*). When quantified across all MPC layers, neonate-lesioned animals repeatedly dosed with SKF-38393 demonstrated approximately a four- to fivefold increase in phospho-CREB-positive cell counts (mean ± SEM; 795.07 ± 137.81) compared with neonate-lesioned rats injected repeatedly with saline (146.50 ± 40.28; $p < 0.0001$) and sham-lesioned rats treated repeatedly with SKF-38393 (127.13 ± 12.21; $p = 0.0002$) or saline (226.42 ± 78.00; $p < 0.0003$). Cell counts specifically in layers II–III in the agonist-sensitized rats exhibited nearly a ninefold increase in phospho-CREB-positive cell counts compared with control animals (Fig. 6*A*) ($p < 0.0001$ for Lesioned R-SKF vs Lesioned R-Saline, Sham R-SKF, or Sham R-Saline). The elevation in number of phospho-CREB-positive cells was not reflective of increased total CREB protein expression (mean values ± SEM were 343.75 ± 8.68 for Lesioned R-SKF, 319.67 ± 8.56 for Lesioned R-Saline, and 329.38 ± 16.55 for Sham R-Saline; ANOVA *F* test of model fit: $F_{(2,13)} = 1.475$; $p = 0.265$). Notably, only scattered phospho-CREB-positive cells were noted throughout the striatum and accumbens at day 7, regardless of drug treatment (Fig. 6*H*,*I*). Sustained CREB phosphorylation was not observed in any other cortical regions examined (ANOVA *F* test of model fit: $F_{(3,19)} = 1.181$, $p = 0.345$ for VLOC; $F_{(3,19)} = 0.575$, $p = 0.639$ for CgC; $F_{(3,19)} = 1.086$, $p = 0.380$ for MC; $F_{(3,19)} = 1.051$, $p = 0.394$ for SSC; and $F_{(3,19)} = 0.506$, $p = 0.683$ for PirC). Thus phospho-CREB immunolabeling, unlike phospho-ERK, was restricted to MPC. Furthermore, the multilamellar expression of phospho-CREB did not mirror the predominant appearance of phospho-ERK in layers II–III, suggesting that some, although perhaps not all, of the phospho-CREB immunolabeling was caused by mechanisms other than activation of ERK.

Effect of MEK-inhibitor (SL327) pretreatment on sustained ERK and CREB phosphorylation in MPC of neonate-lesioned rats after repeated administration of SKF-38393

The involvement of MEK in sustained ERK phosphorylation was demonstrated using systemic injections of SL327, a MEK inhibitor that crosses the blood–brain barrier (Atkins et al., 1998; Selcher et al., 1999; Yamagata et al., 2002). Phospho-ERK immunoreactivity was suppressed in neonate-lesioned rats pretreated with SL327 before each SKF-38393 administration, compared with those pretreated with vehicle (Fig. 7*A–C*) ($p < 0.0001$). The number of phospho-ERK-positive cells in SL327-pretreated rats did not differ significantly from rats treated with vehicle alone. These data provide further evidence that the elevated immunostaining of MPC cells observed in SKF-38393-sensitized neonate-lesioned rats is indeed represented by MEK-dependent ERK phosphorylation.

We also examined the effects of SL327 pretreatment on phospho-CREB immunolabeling in MPC. Phospho-CREB immunoreactivity was reduced to some extent throughout all layers of MPC by pretreatment with SL327 (Fig. 7*D*,*F*) ($p = 0.0238$ for SL R-SKF vs R-SKF). The most dramatic suppression of phospho-CREB immunoreactivity by the MEK inhibitor, however, was produced in layers II–III (Fig. 7*D–F*) ($p < 0.0001$ for SL R-SKF vs R-SKF), in which cell counts did not significantly differ from rats that were not given the agonist-sensitizing regimen. These findings are consistent with direct involvement of both MEK and ERK activation in sustained CREB phosphorylation in

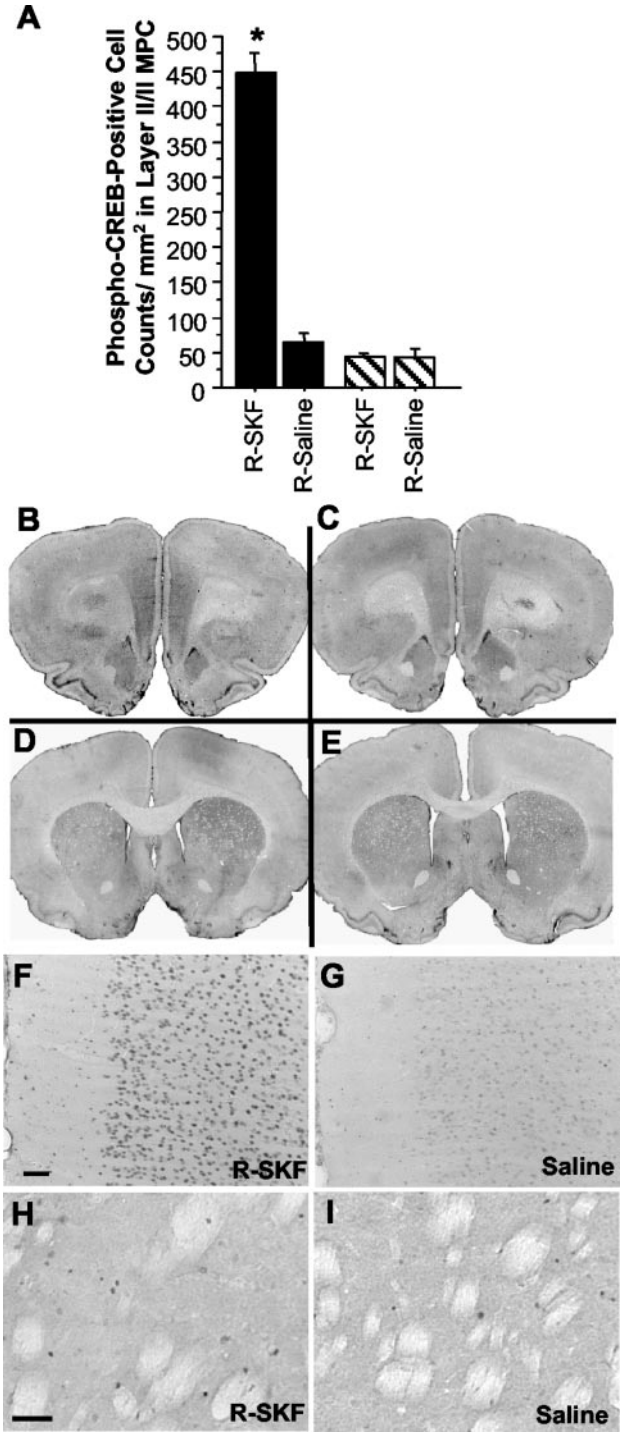

Figure 6. Repeated administration of SKF-38393 to neonate-lesioned rats produces long-lasting CREB phosphorylation in MPC. *A*, Graphic representation of layers II–III phospho-CREB-positive cell counts in MPC at day 7 after repeated SKF-38393 or saline treatment to neonate-lesioned (solid bars) or sham-lesioned (striped bars) rats. *$p < 0.05$ with Fisher's PLSD test. R-SKF, Rats administered repeated doses of SKF-38393; R-Saline, rats injected repeatedly with saline. ANOVA *F* test of model fit: $F_{(3,28)} = 82.148$; $p < 0.0001$. *B*, *C*, Robust phospho-CREB immunostaining is observed only in MPC of neonate-lesioned rats at day 7 after repeated SKF-38393 administration (*B*) but not in MPC of neonate-lesioned rats treated with saline rather than the agonist (*C*). *D*, *E*, Only scattered phospho-CREB-positive cells were noted in striatum and accumbens of neonate-lesioned rats administered repeated SKF-38393 administration (*D*) and neonate-lesioned rats injected with saline (*E*). *F*, *G*, Representative low-magnification (100×) images of phospho-CREB-immunoreactive cells in MPC of neonate-lesioned rats dosed repeatedly with SKF-38393 (*F*) or saline (*G*). Scale bar, 250 μm. *H*, *I*, Representative high-magnification (200×) images of phospho-CREB immunoreactive cells in striatum of neonate-lesioned rats dosed repeatedly with SKF-38393 (H) or saline (*I*). Scale bar, 50 μm.

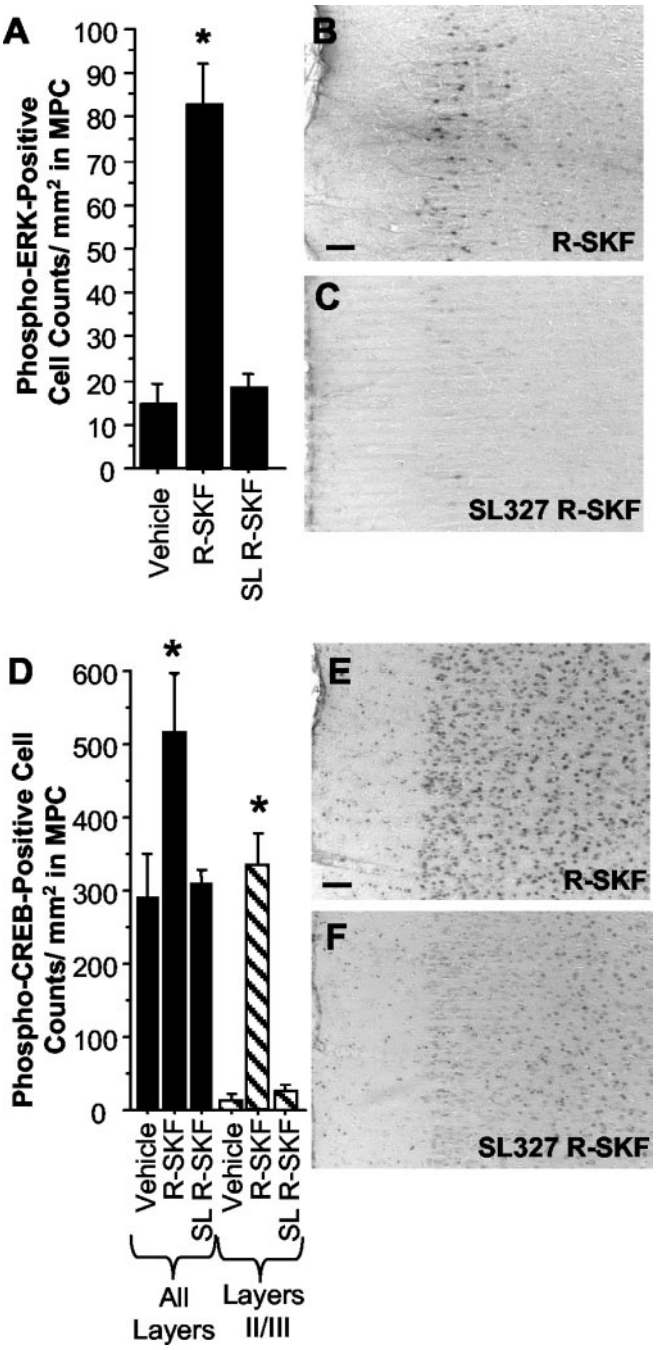

Figure 7. Sustained ERK and CREB phosphorylation is blocked by pretreatment with MEK inhibitor SL327 before each weekly dose of SKF-38393. *A*, Graphic representation of phospho-ERK-positive cell counts in MPC at day 7 after drug treatment. Vehicle, Neonate-lesioned rats pretreated with vehicle (DMSO) before weekly saline injections; R-SKF, neonate-lesioned rats administered vehicle before SKF-38393 treatment; SL R-SKF, neonate-lesioned rats pretreated with SL327 before SKF-38393 treatment. ANOVA *F* test of model fit: $F_{(2,10)} = 40.604$; $p < 0.0001$. *B*, *C*, Representative low-magnification (100×) images of phospho-ERK-immunoreactive cells in R-SKF (*B*) and SL R-SKF (*C*) treatment groups. *D*, Graphic representation of phospho-CREB-positive cell counts across all layers (solid bars) and only layers II–III (striped bars) of MPC after pretreatment with SL327 before each weekly dose of SKF-38393. ANOVA *F* test of model fit: $F_{(2,10)} = 35.82$; $p < 0.0001$. *E*, *F*, Representative low-magnification (100×) image of phospho-CREB immunoreactive cells in R-SKF (*E*) and SL R-SKF (*F*) treatment groups. Note that SL327 pretreatment appeared to reduce phospho-CREB-positive cell counts primarily in layers II–III of MPC, where sustained phospho-ERK immunoreactivity is most prominent. *$p < 0.05$ with Fisher's PLSD test. Scale bar, 250 μm.

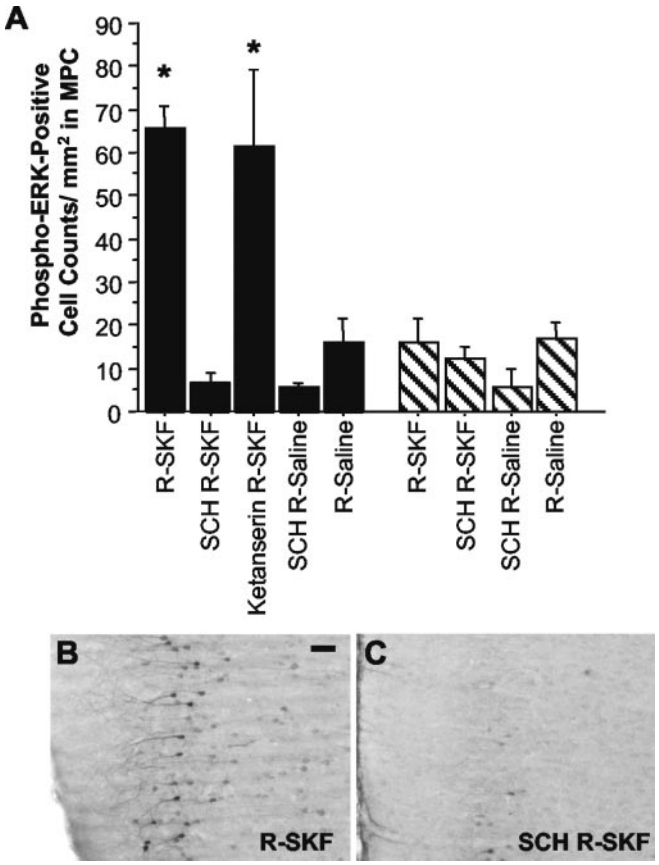

Figure 8. Sustained ERK phosphorylation is blocked by pretreatment with D_1 antagonist SCH-23390 but not with 5-HT_2 antagonist ketanserin. *A*, Graphic representation of phospho-ERK-positive cell counts in MPC of neonate-lesioned (solid bars) and sham-lesioned (striped bars) treatment groups at day 7 after drug treatment. R-SKF, Rats administered saline before SKF-38393; SCH R-SKF, rats pretreated with SCH-23390 before SKF-38393; Ketanserin R-SKF, rats pretreated with ketanserin before SKF-38393; SCH R-Saline, rats pretreated with SCH-23390 before saline injection; R-Saline, rats pretreated with saline (vehicle) before saline injection. ANOVA *F* test of model fit: $F_{(8,44)} = 22.434$; $p < 0.0001$. *B*, *C*, Representative low magnification (100×) images of phospho-ERK immunoreactive cells in R-SKF (*B*) and SCH R-SKF (*C*) neonate-lesioned rat groups. *$p < 0.05$ with Fisher's PLSD test. Scale bar, 250 μm.

layers II–III, but perhaps indirect, if any, involvement in CREB phosphorylation in deeper layers.

Inhibition of sustained SKF-38393-induced ERK phosphorylation in MPC of neonate-lesioned rats after pretreatment with the D_1 antagonist SCH-23390

To determine whether the sustained phospho-ERK observed in MPC of neonate-lesioned rats sensitized with SKF-38393 was dependent on D_1 receptors, rats were pretreated with the D_1 antagonist SCH-23390. As shown in Figure 8, *A* and *C*, SCH-23390 pretreatment to neonate-lesioned rats abrogated phospho-ERK immunoreactivity at this brain site ($p < 0.0001$ compared with Lesioned R-SKF group). The number of phospho-ERK-positive cells for this treatment group did not differ significantly from neonate-lesioned rats administered repeated saline injections or from sham-lesioned control groups. Moreover, repeated SCH-23390 treatment on its own did not enhance phospho-ERK immunoreactivity in either neonate-lesioned or sham-lesioned rats.

Rats were also pretreated with the 5-HT_2 receptor antagonist ketanserin to control for the antagonist binding properties of SCH-23390 at 5-HT_2 receptors (Fig. 8*A*) (Bischoff et al., 1988; McQuade et al., 1988). Ketanserin was ineffective at reducing the persistently increased levels of phospho-ERK ($p = 0.578$ compared with Lesioned R-SKF group), indicating that the prolonged phospho-ERK observed in MPC of SKF-38393-sensitized neonate-lesioned rats is dependent on activation of D_1 receptors.

NMDA receptor blockade prevents the sustained ERK phosphorylation in MPC induced by repeated doses of SKF-38393 to neonate-lesioned rats

Previous work in our laboratory has demonstrated that MK-801 inhibits SKF-38393-induced behavioral sensitization of neonate-lesioned rats, a finding that implicates NMDA receptor function in adaptive processes underlying repeated SKF-38393 treatment to these animals (Criswell et al., 1990). In the present study, administration of MK-801 or CGS-19755 to neonate-lesioned and sham-lesioned animals before repeated injections of saline had no effect on basal phospho-ERK immunoreactivity (Fig. 9*E*). On the other hand, pretreatment with MK-801 or CGS-19755 before repeated doses of SKF-38393 to neonate-lesioned rats resulted in marked inhibition of sustained phospho-ERK immunoreactivity in MPC (Fig. 9*A*,*C*) ($p < 0.0001$ for Lesioned MK-801 R-SKF group vs Lesioned R-SKF group, and for Lesioned CGS R-SKF group vs Lesioned R-SKF group). Consequently, these data point to an involvement of NMDA receptors in the sustained phospho-ERK response in MPC of neonate-lesioned animals.

Discussion

This study demonstrates the remarkably protracted course of ERK phosphorylation in MPC and other cortical regions of neonate 6-OHDA-lesioned rats behaviorally sensitized to the effects of a D_1 agonist in adulthood. Our observations suggest that prolonged MPC phospho-ERK is a neurobiological substrate of long-lasting adaptive change in these animals, as indicated by several key findings. First, MPC is unique among cortical and striatal regions, in that sustained ERK phosphorylation observed primarily in layers II–III declined gradually, yet remained significantly above control levels for at least 36 d. Second, maximal levels of phospho-ERK were restored fully in MPC, but not significantly in other cortical areas, on day 42 after an additional dose of agonist on day 36. Furthermore, an analogous increase in phospho-CREB in layers II–III of MPC, but not other cortical regions, at 7 d after the initial sensitizing regimen was MEK dependent. This finding suggests that a functional effect of sustained MPC ERK phosphorylation is activation of CREB-dependent gene transcription. Finally, the development of sustained phospho-ERK in MPC requires both NMDA and D_1 receptor stimulation, a pattern of dependence resembling that identified in several cellular and behavioral neuroadaptive paradigms that have been described in cortical and striatal systems (for review, see Kelley and Berridge, 2002). These findings, together with the proposed contribution of the MPC in the progressive and enduring behavioral effects of drugs of abuse (Castner and Goldman-Rakic, 2003; Steketee 2003), schizophrenia (Laruelle, 2000; Tzschentke, 2001), and learning and memory (Castner et al., 2000; Elzinga and Bremner, 2002), suggest that the D_1 agonist-sensitized neonate-lesioned rat presents an excellent model for the study of dopamine-dependent neuroadaptations and their functional consequences.

Several studies have demonstrated that activation of D_1 receptors, via a protein kinase A (PKA)-dependent mechanism, can phosphorylate ERK to produce adaptive changes in brain (Vossler et al., 1997; Yao et al., 1998; York et al., 1998; Valjent et al., 2000). In unilateral adult 6-OHDA-lesioned rats, Gerfen et al. (2002) demonstrated transient ERK activation in striatum after

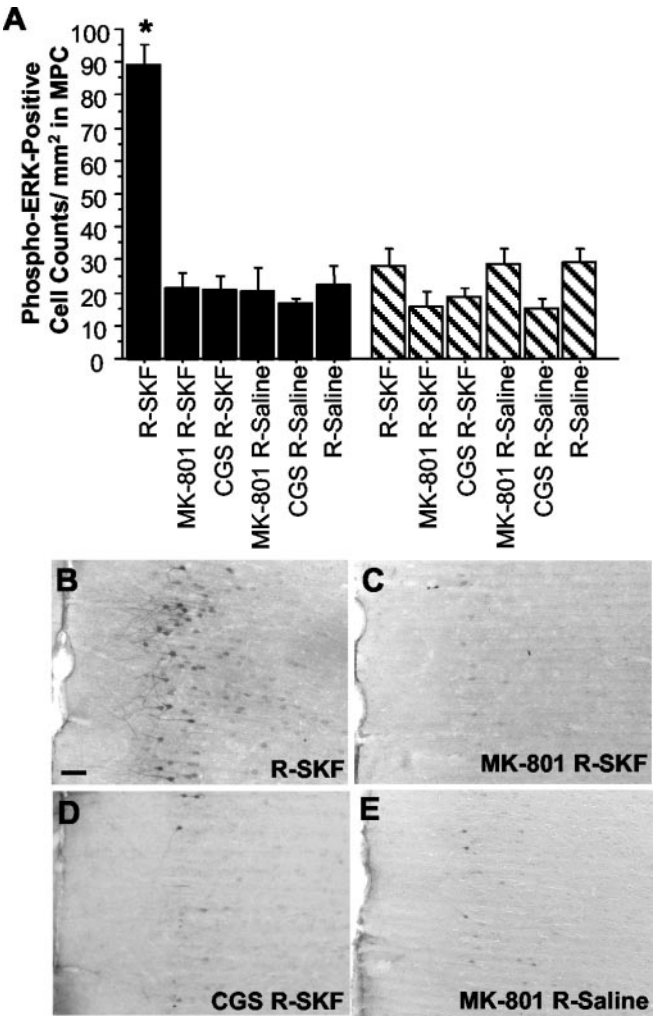

Figure 9. Sustained ERK phosphorylation is blocked by pretreatment with NMDA receptor antagonists MK-801 and CGS-19755 before each repeated weekly dose of SKF-38393. *A*, Graphic representation of phospho-ERK-positive cell counts in neonate-lesioned (solid bars) and sham-lesioned (striped bars) treatment groups at day 7 after drug treatment. R-SKF, Rats administered saline before SKF-38393; MK-801 R-SKF, rats pretreated with MK-801 before SKF-38393; CGS R-SKF, rats pretreated with CGS-19755 before SKF-38393; MK-801 R-Saline, rats pretreated with MK-801 before saline injection; CGS R-Saline, rats pretreated with CGS-19755 before saline injection; R-Saline, rats pretreated with saline (vehicle) before saline injection. ANOVA *F* test of model fit: $F_{(11,100)} = 10.206$; $p < 0.0001$. *B–E*, Representative low-magnification (100×) images of phospho-ERK immunoreactive cells in R-SKF (*B*), in MK-801 R-SKF (*C*), in CGS R-SKF (*D*), and in MK-801 R-Saline (*E*) neonate-lesioned rat groups. *$p < 0.05$ with Fisher's PLSD test. Scale bar, 250 μm.

acute D_1 agonist administration, an effect attributed to sensitized D_1 receptor-dependent responses. Consistent with this finding, the present study demonstrates a transient phosphorylation (<60 min) of ERK induced by a single dose of SKF-38393 in drug-naive neonate-lesioned rat striatum and MPC. Notably, the pattern and duration of ERK phosphorylation in striatum of naive neonate-lesioned rats administered a single dose of SKF-38393 were similar to that published by Gerfen et al. (2002) in unilateral adult 6-OHDA-lesioned rats. In agreement with the transient ERK phosphorylation, previous work has shown that a single dose of SKF-38393 can induce Fos expression in striatum of neonate-lesioned rats (Johnson et al., 1992). Together with the findings of Sgambato et al. (1998) and Gerfen et al (2002), it appears that transient ERK activation can drive immediate-early gene induction in striatum. It remains a question whether transient phospho-ERK observed in the striatum and MPC of neonate-lesioned rats after a single dose of SKF-38393 is sufficient to drive long-term gene regulation.

Several lines of evidence suggest that prolonged ERK activation (>60 min) is necessary to allow sufficient time for translocation of ERK to the nucleus, long-term gene transcription, and subsequent enhancement of enduring plasticity-associated changes in brain (for review, see Marshall, 1995). Repeated D_1 agonist administration prolonged striatal phospho-ERK immunoreactivity nearly twofold. Conversely, in accumbens, ERK activation was absent with a single dose of agonist and was observed only after repeated treatment with SKF-38393. These findings, together with the prolonged presence of ERK phosphorylation in the MPC and various other regions of cortex (VLOC, CgC, MC, SSC, and PirC), point to differing mechanisms of adaptation among these regions. Furthermore, the reinstatement of ERK phosphorylation that occurs only in MPC supports a unique mechanism of adaptation in this region that can trigger rapidly the previous level of ERK activation on reexposure to the agonist.

A well characterized nuclear target for ERK action is the transcription factor CREB (Bourtchuladze et al., 1994; Yin et al., 1994), which is thought to play a central role in long-term plastic changes in brain by controlling the transcriptional expression of several genes (for review, see Curtis and Finkbeiner, 1999). In the present study, immunostaining for phospho-CREB was measured initially as a functional endpoint for sustained ERK activation in MPC. Surprisingly, although phospho-CREB emerged in parallel with ERK phosphorylation in layers II–III, the population of immunolabeled cells was more dense and widespread than those labeled with anti-phospho-ERK. In fact, phospho-CREB immunoreactivity appeared throughout all layers of MPC. At this time, we can only speculate as to the reasons for this seeming incongruence. One obvious possibility is that mechanisms independent of ERK drive CREB phosphorylation. Although phospho-CREB in layers II–III was sensitive to the MEK (and thus ERK) activation state, labeling in the deeper layers of MPC was less responsive to MEK inhibition. Because CREB is a common substrate of multiple kinase pathways in cell model systems (for review, see Herdegen and Leah, 1998; Curtis and Finkbeiner, 1999), other pathways, such as the PKA, CaM kinase, and stress-activated protein kinase cascades, might be responsible for the phospho-CREB effect. Another possibility could be related to functional integration of phospho-ERK-expressing neurons with nonexpressing cells, whereupon ERK-dependent activity in a small population of MPC layer II–III neurons could influence activation, signaling, and appearance of phospho-CREB in cortical and subcortical cells beyond simply those that contain phospho-ERK. The mild reduction in phospho-CREB immunoreactivity observed throughout all MPC layers after MEK inhibition would seem to support a mechanism involving intercellular integration. Nevertheless, additional studies are necessary to resolve these complex issues.

Several lines of evidence suggest that sustained ERK activation may be indicative of neuronal insult, possibly leading to cell death (Stanciu et al., 2000; Kulich and Chu, 2001). Prolonged ERK activation in neurodegenerative processes has been linked to decreased CREB activation (Lee et al., 2002; Trentani et al., 2002). In the present study, sustained ERK phosphorylation in MPC was accompanied by increased, rather than decreased, CREB phosphorylation. On the opposite end of the spectrum, prolonged ERK activation accompanied by an increase in CREB has been linked to a continuum of processes that include cell survival and plasticity (Shen et al., 2001; Sweatt, 2001; Dawson and Ginty,

2002). Thus, it appears that the precise kinetics of ERK and CREB phosphorylation can ultimately determine the fate of a cell within a given region. Although neurodegenerative processes cannot be ruled out, we speculate that the sustained increases in ERK and CREB phosphorylation observed herein are likely to be indicative of cell survival and plasticity in MPC.

The sustained increase in phospho-ERK was dependent on D_1 receptor function, because SCH-23390 antagonist pretreatment blocked the prolonged response to repeated administration of the D_1-selective partial agonist SKF-38393. It is unlikely that SCH-23390 inhibited prolonged ERK phosphorylation through its interaction with 5-HT_2 binding sites (Bischoff et al., 1988; McQuade et al., 1988), because systemic injections of the nonselective 5-HT_2 antagonist ketanserin, before repeated doses of SKF-38393, had no effect on the sustained phospho-ERK response in MPC. In addition, the results with ketanserin further support the notion that long-lasting ERK phosphorylation does not drive expression of locomotor responsiveness to D_1 agonists in SKF-38393-sensitized animals. The spontaneous hyperactive behavior of neonate-lesioned animals has been linked to serotonergic mechanisms (Bishop et al., 2004), and ketanserin blocks the locomotor sensitization observed in these animals (our unpublished data), without affecting the sustained activation of ERK in MPC.

Studies have demonstrated that pharmacological stimulation of NMDA receptors leads to activation of ERK in cortical neurons (Bading and Greenberg, 1991; Xia et al., 1996; Arvanov et al., 1997; Vanhoutte et al., 1999; Wang and O'Donnell, 2001). In this investigation, both the competitive NMDA antagonist MK-801 and the noncompetitive antagonist CGS-19755 eliminated sustained ERK phosphorylation in MPC, a finding suggestive of NMDA dependence for the ERK phosphorylation. Thus, MPC of neonate-lesioned rats sensitized with repeated doses of SKF-38393 might undergo persistent biochemical adaptations similar to other biological substrates of neuroplasticity such as long-term potentiation (Impey et al., 1998) and memory processing (Adams and Sweatt, 2002). In addition, accumulated evidence demonstrates an interaction of D_1 and NMDA receptor functions (Konradi et al.; 1996, Pei et al., 2004) (for review, see Adriani et al., 1998; Salter, 2003) and dopamine modulation of glutamate neurotransmission within the prefrontal cortex (Goldman-Rakic and Selemon, 1997; Gonzalez-Islas et al., 2003; Otani et al., 2003; Sesack et al., 2003). The present findings further illustrate the presence of a dopamine–glutamate interaction in the MPC, whereby repeated D_1 agonist administration maintains NMDA receptor-mediated responses in cells as reflected in ERK phosphorylation.

The neuromolecular mechanisms underlying sustained ERK hyperphosphorylation remain to be determined. Recent studies have established a critical role for protein phosphatases in coordinating neurotransmitter signaling (for review, see Greengard, 2001). Because phosphatases are presumed to rapidly deactivate phosphorylated proteins in brain, the measured increase in cells exhibiting phospho-ERK after SKF-38393 treatment could be related to altered phosphatase activity. Mitogen-activated protein kinase phosphatases (MKPs) 1–3 can directly control nuclear accumulation and persistent activation of ERK (for review, see Pouyssegur et al., 2002). Interestingly, MKP1 and MKP3 expression in frontal cortex and other brain regions is differentially altered by acute and chronic methamphetamine administration to rats (Takaki et al., 2001). Thus, a reduction in MKP1–3 or a related phosphatase might be a means by which ERK phosphorylation could be sustained for an extended period. The answer to this puzzle will have to be resolved in future experiments that examine sustained ERK phosphorylation after SKF-38393-mediated sensitization of neonate-lesioned animals.

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