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Region-dependent dynamics of cAMP response element-binding protein phosphorylation in the basal ganglia

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ABSTRACT **The cAMP response element-binding protein (CREB) is an activity-dependent transcription factor that is involved in neural plasticity. The kinetics of CREB phosphorylation have been suggested to be important for gene activation, with sustained phosphorylation being associated with downstream gene expression. If so, the duration of CREB phosphorylation might serve as an indicator for time-sensitive plastic changes in neurons. To screen for regions potentially involved in dopamine-mediated plasticity in the basal ganglia, we used organotypic slice cultures to study the patterns of dopamine- and calcium-mediated CREB phosphorylation in the major subdivisions of the striatum. Different durations of CREB phosphorylation were evoked in the dorsal and ventral striatum by activation of dopamine D1-class receptors. The same D1 stimulus elicited (*i*) transient phosphorylation (≤15 min) in the matrix of the dorsal striatum; (*ii*) sustained phosphorylation (≤2 hr) in limbic-related structures including striosomes, the nucleus accumbens, the fundus striati, and the bed nucleus of the stria terminalis; and (*iii*) prolonged phosphorylation (up to 4 hr or more) in cellular islands in the olfactory tubercle. Elevation of Ca^{2+} influx by stimulation of L-type Ca^{2+} channels, NMDA, or KCl induced strong CREB phosphorylation in the dorsal striatum but not in the olfactory tubercle. These findings differentiate the response of CREB to dopamine and calcium signals in different striatal regions and suggest that dopamine-mediated CREB phosphorylation is persistent in limbic-related regions of the neonatal basal ganglia. The downstream effects activated by persistent CREB phosphorylation may include time-sensitive neuroplasticity modulated by dopamine.**

Dopamine is a classical neurotransmitter that can influence neurologic functions ranging from movement to emotion, memory, and reinforcement. The behavioral aspects of reward and motivation are known to be modulated by the nigrostriatal and mesolimbic pathways. The primary striatal targets of these dopamine-containing systems are different. The nigrostriatal system primarily innervates the caudoputamen. The mesolimbic system innervates a number of forebrain structures including the ventral striatum, which is formed by the nucleus accumbens and olfactory tubercle. Although both the dorsal and ventral striatum are targets of dopamine-containing afferents from the midbrain and share a striatal architecture, they differ from each other in neurochemical organization and in their connectivity with other brain regions (1, 2). The dorsal striatum is engaged primarily in processing information from the cerebral cortex and thalamus and is involved in motor control, aspects of cognitive control, and some forms of learning and memory. The ventral striatum receives inputs from limbic structures including the amygdala, the hippocampus, and the limbic prefrontal cortex, and projects back to limbic structures via the ventral pallidum. These limbic-associated circuits are thought to underlie motivational and viscero-affective aspects of neurologic function served by the ventral striatum. The midbrain dopamine pathways innervating the dorsal and ventral striatum are critically involved in controlling these functions, including, for the ventral striatum, the reinforcing properties of psychostimulant and other drugs (3).

A distinct feature of much reinforcement-based learning is that the modified behaviors develop with time and are highly sensitive to the temporal organization of events (4, 5). The time-dependent nature of the learning suggests that the underlying neural plasticity may share similar time sensitivity at the molecular level. The mesolimbic dopamine–ventral striatum system undergoes a series of cellular and molecular changes in response to psychoactive and addictive drugs (6). However, time-sensitive neural adaptations of this system have not been identified.

In previous work with organotypic striatal slice cultures, we demonstrated that the cAMP response element-binding protein (CREB) may be a key regulator of time-dependent plasticity in the dorsal striatum (7). CREB is an activity-dependent transcription factor that can integrate multiple signals including cAMP and Ca^{2+} (8, 9). We found that the kinetics of CREB phosphorylation are regulated in different ways in the two main compartments of the developing dorsal striatum (striosomes and matrix). In these parts of the striatum, dopamine/cAMP and Ca^{2+} signals lead to transient Ser-133 CREB phosphorylation, but compartment-specific phosphatase control mechanisms lead to sustained (ca. 30 min) duration phosphorylation and subsequent c-*fos* expression targeted to different compartments. These experiments suggested that the duration of CREB phosphorylation may be a key signal used by neurons in the dorsal striatum to regulate CRE-mediated gene expression and long-term effects induced by dopamine.

Because the ventral striatum is so strongly implicated in reinforcement-based learning and memory, we decided to study the kinetics of CREB phosphorylation in this striatal system in response to dopamine and calcium signals. Our results, obtained with organotypic slices, suggest striking differences in the temporal control of CREB phosphorylation induced by dopamine in the dorsal and ventral striatum. Our results also suggest marked differences in the effects of Ca^{2+} signals in the dorsal and ventral striatum. We suggest that the region-specific kinetics of CREB phosphorylation may have important implications for functions carried out by different components of the dorsal and ventral striatum.

MATERIALS AND METHODS

Organotypic Cultures of Striatum. Vibratome slices of forebrain containing the striatum were taken from the brains

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Abbreviations: CREB, cAMP response element-binding protein; NMDA, *N*-methyl-D-aspartate; PCREB, phospho-CREB.

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of newborn Sprague–Dawley rat pups or, in one set of experiments, postnatal day 2 (P2) pups. The slices were cultured according to a previously described procedure (7, 10). Brain slices were placed on top of 0.4-μm membranes in culture plate inserts (Millicell-CM, Millipore, Bedford, MA) and were then maintained in 1 ml SF21 serum-free medium (11) at 33°C with 5% CO_2/95% air for 3 days before the experiments were carried out.

Drug Application. For the initial time-course study, striatal slices were incubated for 7, 15, or 30 min, or 1, 2, 4, 8, or 24 hr, with the following reagents: the D1-selective agonist, SKF-81297 HCl (100 nM, kindly provided by J. Weinstock of SmithKline Beecham); the D2-selective agonist, R(−)-quinpirole HCl [10 μM; Research Biochemicals (RBI), Natick, MA]; the L-type voltage-sensitive Ca^{2+} channel agonist, S(−)-BAY K 8644 (1 μM; RBI); the adenylate cyclase activator, forskolin (10 μM; RBI); KCl (50 mM; Mallinckrodt); or the *N*-methyl-D-aspartate receptor agonist, NMDA (10 μM, 100 μM; RBI). Washout experiments were carried out with SKF-81297 and forskolin. Fifteen minutes after drug administration, the culture medium was replaced with fresh, prewarmed SF21 medium, and a second wash of fresh medium was carried out 5 min after the first wash. For blocking experiments, slice cultures were pretreated for 30 min with the D1-class antagonist, R(+)-SCH-23390 HCl (1 μM; RBI), before SKF-81297 treatment, or with the L-type voltage-sensitive Ca^{2+} channel antagonists, nifedipine (10 μM; RBI) and nitrendipine (10 μM; RBI), or the Ca^{2+} chelator, EGTA (10 mM; Sigma), before BAY K 8644 treatment. Each blocking agent was also tested alone. All drug treatments were carried out at least in duplicate.

Immunocytochemistry. Slice cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at different times after the drug incubations. The slices were then processed for immunocytochemistry by the avidin–biotin–peroxidase complex (ABC) method (7, 10). The dilutions of primary antibodies were as follows: affinity-purified rabbit polyclonal anti-Ser-133 phosphorylated CREB (1:300–1,000), rabbit polyclonal anti-Ser-133 phosphorylated and nonphosphorylated CREB [1:8,000, both antisera kindly provided by D. D. Ginty and M. E. Greenberg (12); mouse monoclonal anti-MAP-2 (1:2,000, Boehringer Mannheim Biochemica); mouse monoclonal anti-GFAP (1:1,000, Sigma); mouse monoclonal anti-nestin (1:1, antibody kindly provided by R. D. G. McKay, National Institutes of Health)]. Controls for phospho-CREB (PCREB) immunostaining were carried out by omitting the primary antiserum or by replacing it with the preimmune serum. The controls showed no staining (omission of primary antiserum) or nonspecific staining (preimmune serum). Some slices were stained for Nissl substance with cresylecht violet. To estimate the intensity of immunostaining, sections were judged by eye, and these estimates were checked by computer-aided densitometry. Estimates were made by a rating scale of 0–5 as follows: 5, very strong staining; 4, strong staining; 3, moderate staining; 2, moderate to weak staining; 1, weak staining; and 0, background staining.

RESULTS

Contrasting Time Course of CREB Phosphorylation in the Dorsal Striatum and Ventral Striatum Induced by Activation of Dopamine D1-Class Receptors. Stimulation of striatal slice cultures with the D1-selective agonist, SKF-81297, induced time-varying patterns of CREB phosphorylation in the dorsal and ventral striatum. In the caudoputamen (dorsal striatum), the D1 agonist induced strong PCREB immunoreactivity in both striosomes and matrix at 7 min, but by 30 min, PCREB expression in the matrix had declined, leaving a persistent CREB signal in striosomes (Fig. 1*A*). This spatial selectivity confirmed our earlier findings (7). A detailed time-course

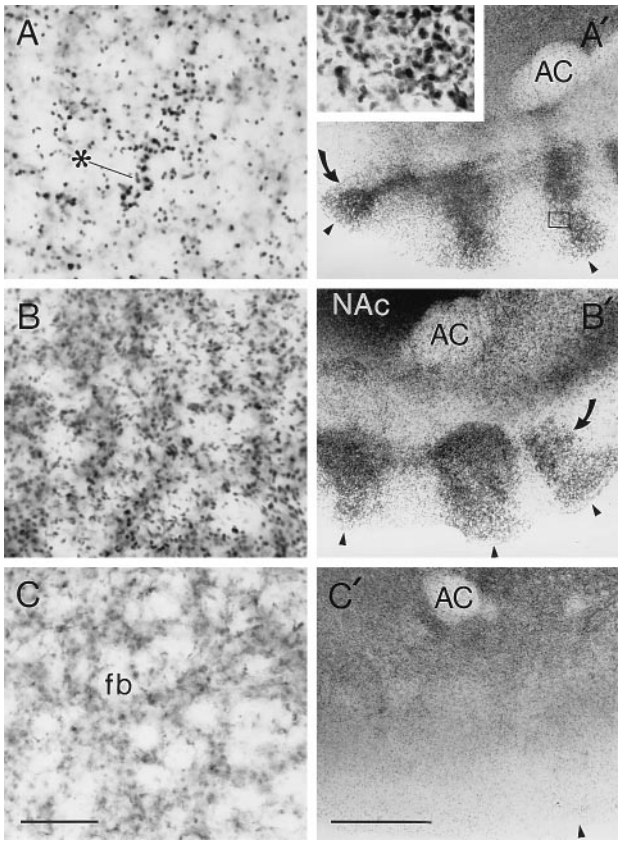

FIG. 1. Patterns of CREB phosphorylation in the dorsal and ventral striatum induced by D1-class dopamine receptor agonist stimulation (*A* and *A′*), stimulation of cAMP (*B* and *B′*), and of L-type Ca^{2+} channel activation (*C* and *C′*). Photomicrographs of PCREB immunostaining are shown for the caudoputamen (*A–C*) and the olfactory tubercle (*A′–C′*) of slice cultures treated for 30 min with different agonists. SKF-81297 induces CREB phosphorylation in striosomal patches of PCREB-positive nuclei (*A*, see asterisk), whereas forskolin induces expression of PCREB in many evenly distributed PCREB-positive nuclei in the caudoputamen (*B*). Box in *A′* shows the region enlarged in the *Inset*. Both SKF-81297 (*A′*) and forskolin (*B′*) induce expression of strongly PCREB-positive nuclei in a horizontal band and ventral islands (curved arrows) in the olfactory tubercle. The L-type calcium channel agonist BAY K 8644 induces PCREB expression in the matrix of the caudoputamen (*C*), but it induces only low levels of PCREB expression in the olfactory tubercle (*C′*). The arrowheads in *A′–C′* indicate the bottom edges of the sections. AC, anterior commissure; NAc, nucleus accumbens; fb, fiber bundles. [Bar in *C* (for *A–C*) = 100 μm; bar in *C′* (for *A′–C′*) = 0.5 mm.]

study showed that the amount of sustained CREB phosphorylation detectable immunohistochemically varied systematically with time. The high levels of PCREB immunoreactivity in striosomes decreased steadily with time to moderate levels at 1–2 hr and to very low levels at 4 hr. No PCREB was detectable at the 8- or 24-hr time points (Fig. 4*A*).

Dopamine D1-class receptor stimulation with SKF-81297 also induced PCREB expression in the ventral striatum and associated regions, including the shell and core regions of the nucleus accumbens, the fundus striati, and bed nucleus of the stria terminalis (Fig. 2). The time course of PCREB expression in these regions was similar to the time course of PCREB in striosomes of the caudoputamen. Fig. 2 shows the pattern at 30 min.

Strikingly, activation of D1-class dopamine receptors induced intense PCREB immunoreactivity in the olfactory tubercle subdivision of the ventral striatum (Figs. 1*A′* and 2), and this CREB phosphorylation persisted for hours (Fig. 3).

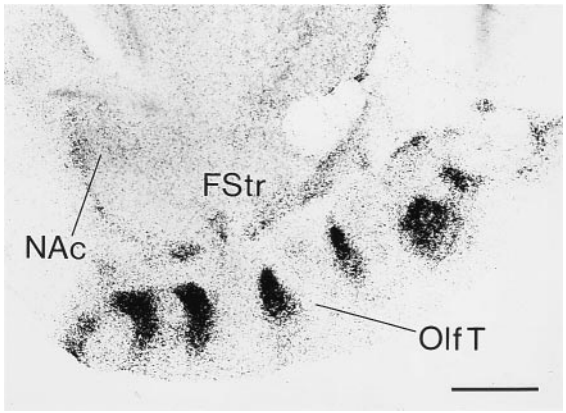

FIG. 2. Photomicrograph illustrating the typical pattern of PCREB immunostaining in the ventral striatum and the olfactory tubercle. Application of the D1-class dopamine receptor agonist SKF-81297 for 30 min induces strong PCREB immunoreactivity in nuclei in the nucleus accumbens (NAc), fundus striati (FStr), and olfactory tubercle (OlfT). Regularly spaced islands of densely packed PCREB-positive nuclei are present in the olfactory tubercle. (Bar = 0.5 mm.)

Even at the shortest incubation time, most of the induction was in vivid, regularly spaced islands of tissue containing densely packed, strongly PCREB-positive nuclei. These were partly connected to a dorsal PCREB-positive band but protruded ventrally as distinct islands (Figs. 1*A′* and 2). CREB phosphorylation in these islands remained at nearly full intensity for 2 hr after SKF-81297 treatment and only then declined gradually (Fig. 4*A*). Even 8 hr after the D1 agonist stimulation, moderate to weak levels of PCREB immunoreactivity were still present in the islands (Fig. 3*A′*). By 24 hr, the PCREB had disappeared.

To characterize the PCREB-positive islands in the olfactory tubercle, we immunostained slices for microtubule-associated protein-2 (MAP-2), a neuronal marker, for glial fibrillary acidic protein (GFAP, an astrocyte marker), and for nestin (a marker for neuronal and glial precursors). There were MAP-2-positive neurons, GFAP-positive astrocytes, and nestin-positive cells throughout the slice cultures, including in the olfactory tubercle, but none of them appeared as tightly packed cell clusters similar to the PCREB-rich ventral islands. Nor did we see Nissl-stained cells in comparable clusters (data not shown). Because of the presence of cells in the slice cultures expressing MAP-2, GFAP, and nestin, the cultured slices were likely still undergoing development *in vitro*. Despite the known late development of the olfactory tubercle (13), we found no particular marker indicating that the PCREB-positive cell clusters might correspond to clusters of immature neurons or glia. However, to test whether the finding of prolonged CREB phosphorylation was specific to the cultures taken at P0, we prepared other cultures from postnatal day 2 rats by the same methods. We found a similar intense induction of PCREB in islands of the olfactory tubercle at 30 min as we found in the cultures from postnatal day 0 rat pups (data not shown). We tentatively identified the islands as developing islands of Calleja, but were not able to make the identification definitive even in the P2 plus 3-day slices.

The changes in intensity of PCREB immunostaining over time did not appear attributable to alterations in the total quantity of CREB protein. Immunostaining of SKF-81297-treated slices with the CREB antiserum, which detects both unphosphorylated and phosphorylated CREB proteins, did not show significant changes with time (data not shown). This also applied to the striatal slice cultures treated with forskolin and BAY K 8644 (see below).

The PCREB induction in the cellular islands of the olfactory tubercle, like that elsewhere in the ventral and dorsal striatum,

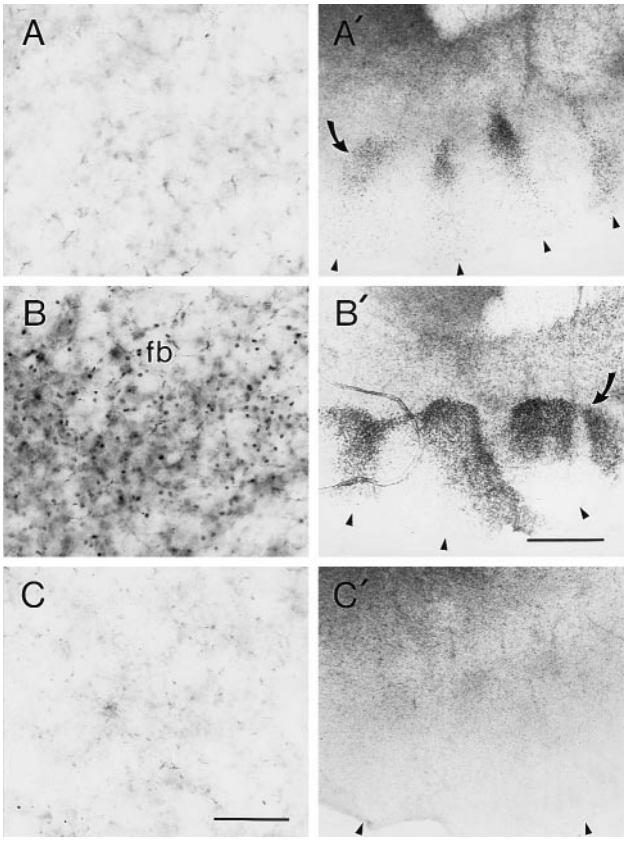

FIG. 3. Photomicrographs of PCREB immunostaining in the caudoputamen (*A–C*) and in the olfactory tubercle (*A′–C′*) of slice cultures treated for 8 hr with the dopamine D1-class receptor agonist (*A* and *A′*), forskolin (*B* and *B′*), or the L-type Ca^{2+} channel agonist, BAY K 8644 (*C* and *C′*). Eight hours after SKF-81297 stimulation of D1-class dopamine receptors, PCREB immunoreactivity is reduced to background levels in the caudoputamen (*A*), but persistent, weak PCREB immunostaining remains in the islands of the olfactory tubercle (curved arrow in *A′*). With 8-hr forskolin treatment, only weak PCREB immunoreactivity is found in the caudoputamen (*B*), but moderate levels of PCREB immunostaining are still present in the olfactory tubercle islands (curved arrow in *B′*). Only background levels of PCREB immunoreactivity are present in the caudoputamen (*C*) and olfactory tubercle (*C′*) 8 hr after BAY K 8644 stimulation. The arrowheads in *A′–C′* mark the ventral edges of the tissue. fb, fiber bundles. [Bar in *C* (for *A–C*) = 100 μm; bar in *B′* (for *A′–C′*) = 0.5 mm.]

was specific to the activation of dopamine D1-class receptors, because it could be blocked by pretreatments with the dopamine D1-class receptor antagonist, SCH-23390 (1 μM) (data not shown). Furthermore, activation of dopamine D2-class receptors with the D2-selective agonist, quinpirole (10 μM), did not induce detectable PCREB expression anywhere in the striatum, dorsally or ventrally (data not shown). The vehicle for SKF-81297 (and those for forskolin and BAY K 8644, see below) induced only weak, transient PCREB expression that was not detectable 30 min after the treatments (data not shown).

Extended Time Course of CREB Phosphorylation Induced by Activation of Adenylate Cyclase. Dopamine D1-class receptors are coupled to adenylate cyclase. We therefore next studied the time course of CREB phosphorylation induced by activation of adenylate cyclase with forskolin (10 μM). In the caudoputamen, forskolin induced intense and sustained CREB phosphorylation that did not differ perceptibly in striosomes and matrix (Fig. 1*B*) (7). The PCREB immunostaining was very intense from the earliest time point (7 min) and remained so for 30–60 min. Two hours after forskolin

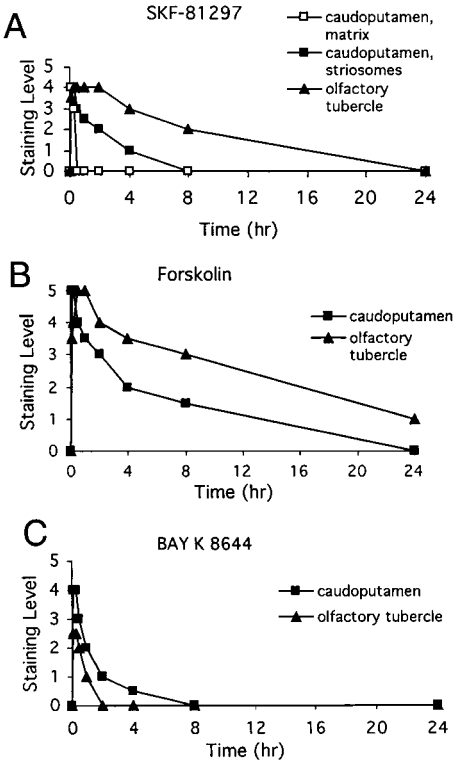

FIG. 4. Time courses of CREB phosphorylation activated by the D1-class dopamine receptor agonist, SKF-81297 (*A*), the cAMP stimulator, forskolin (*B*), and the L-type calcium channel agonist, BAY K 8644 (*C*). D1 agonist stimulation induces longer phosphorylation in striosomes than in the matrix of the caudoputamen, and the duration of CREB phosphorylation induced by SKF-81297 and forskolin is longer in the olfactory tubercle than in either compartment of the dorsal striatum. By contrast, in BAY K 8644-treated slice cultures, CREB phosphorylation is shorter in the olfactory tubercle than in the caudoputamen.

stimulation, strong to moderate levels of PCREB immunoreactivity remained in the caudoputamen. Diminishing PCREB immunoreactivity appeared at 4–8 hr (Figs. 3*B* and 4*B*). PCREB immunoreactivity was not detectable in the caudoputamen at 24 hr. The rate of decline of PCREB was similar in both striosomes and matrix. No PCREB cell clusters were observed at any time point studied.

Forskolin induced PCREB expression in both the shell and the core regions of the nucleus accumbens, and in the fundus striati and the bed nucleus of the stria terminalis. Strong PCREB immunoreactivity also was present in the endopiriform nucleus. The time course of PCREB expression in these regions followed that in the caudoputamen.

Like SKF-81297, forskolin induced more intense immunostaining for PCREB in the olfactory tubercle than in other ventral striatal regions or the caudoputamen, and the phosphorylation was more persistent (Fig. 4*B*). There was very strong PCREB immunoreactivity in the horizontal band and islands of the olfactory tubercle, and the number of PCREB-positive nuclei in the zones between the islands also was greater, although not dramatically so, with forskolin stimulation (Fig. 1*B′*). The intensity of PCREB immunoreactivity in the islands only very gradually decreased with time (Fig. 4*B*). The PCREB immunostaining was at the highest scoring level for 1 hr and was maintained at high levels for 4 hr. Dense PCREB immunoreactivity was still present in the ventral islands at 8 hr (Fig. 3*B′*), and weak PCREB immunoreactivity was detectable even 24 hr after forskolin stimulation. The levels of PCREB expression in slices treated with forskolin were higher than those in slices treated with SKF-81297 in both the caudoputamen and the olfactory tubercle at matched time points (Figs. 1, 3, and 4).

Agonist Washout and Persistent Phosphorylation of CREB. To test whether the persistent phosphorylation of CREB by SKF-81297 and forskolin required the continuous presence of agonist in the culture medium, we carried out experiments in which we decreased the concentration of applied agonists. The culture medium containing the agonist was discarded and was replaced with fresh medium 15 min after agonist treatments, and this initial wash was followed by a second replacement with fresh medium 5 min later. At a 50% efficiency of replacement, this should have reduced the agonist concentrations to a maximum of 25% of the original values. At 80% efficiency, the values would be 4% of the original concentrations. The cultures were then processed for PCREB immunostaining at different times after drug treatments.

The washout treatments did not change the distributions of PCREB-positive nuclei found in the caudoputamen and olfactory tubercle after the SKF-81297 and forskolin treatments, but did result in a more rapid reduction of detectable PCREB both in the caudoputamen and in the olfactory tubercle (Fig. 5). The duration of detectable CREB phosphorylation activated by SKF-81297 was reduced from 4 hr to 2 hr in the striosomes of the caudoputamen and limbic ventral striatal regions other than the islands, and from 8 hr to 4 hr in the olfactory tubercle (Fig. 5*A*). The washout procedure reduced the duration of CREB phosphorylation activated by forskolin from 8 hr to 4 hr in the caudoputamen, and from 24 hr to 8 hr in the olfactory tubercle (Fig. 5*B*). There were not sharp decreases of PCREB immunostaining immediately after the rinses of culture medium, i.e., at the 30-min time point after drug stimulation. Instead, the phosphorylation detected diminished gradually with time.

Time Course of CREB Phosphorylation Induced by Activation of L-Type Voltage-Sensitive Ca^{2+} Channels. We confirmed that treatment of the cultures with BAY K 8644 (1 μM), which increases the channel open time of L-type Ca^{2+} channels, results in sustained phosphorylation of CREB in many matrix cells of the caudoputamen in slices incubated for 30 min

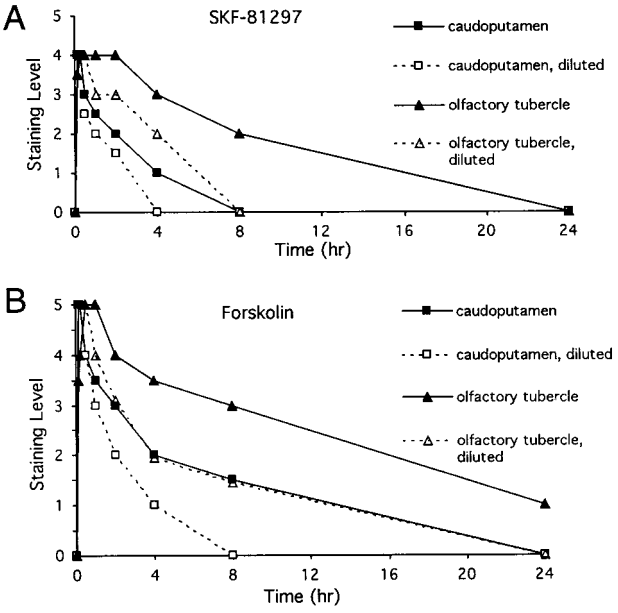

FIG. 5. Effects of agonist washout on the time courses of CREB phosphorylation activated by SKF-881297 (*A*) and by forskolin (*B*). Reducing concentrations of SKF-81297 and forskolin by replacing the agonist solutions with fresh medium (2×5 min) shifts the time-course curves of PCREB expression to the left for both the caudoputamen and the olfactory tubercle. Note that the differences in time course between the two regions are maintained.

(Fig. 1*C*) (7). Low levels of activation occurred in limbic striatal regions such as the nucleus accumbens. As shown in Fig. 4*C*, with increasing time, the sustained CREB phosphorylation in the matrix and elsewhere decreased with a fast kinetics of dephosphorylation. Two hours after BAY K 8644 stimulation, only weak PCREB immunoreactivity remained in the caudoputamen, and by 4 hr, PCREB immunostaining was nearly absent (Fig. 4*C*).

In contrast to the robust PCREB expression induced in the caudoputamen by BAY K 8644, the same treatment resulted in at most weak to moderate expression of PCREB immunoreactivity in the olfactory tubercle at 7–30 min (Fig. 1*C′*). PCREB expression was even lower at 1 hr, and PCREB was undetectable by 2 hr after BAY K 8644 stimulation (Figs. 3*C′* and 4*C*). Pharmacological experiments suggested that this weak PCREB induction by BAY K 8644 in the olfactory tubercle was specific to the activation of L-type Ca^{2+} channels. Pretreating slice cultures with nifedipine (10 μM) and nitrendipine (10 μM) blocked the induction of PCREB immunoreactivity by BAY K 8644 (data not shown; see also ref. 7).

Differential Phosphorylation of CREB in the Caudoputamen and in the Olfactory Tubercle by KCl-Induced Depolarization and by NMDA Stimulation. Because the stimulation of L-type Ca^{2+} channels produced surprisingly little activation of CREB in the olfactory tubercle, we treated other striatal slice cultures with KCl to induce depolarization or with NMDA to activate Ca^{2+} flux through glutamate NMDA channels. Remarkably, although treatments of the slice cultures with KCl (50 mM, for 30 min) induced PCREB in many nuclei throughout the caudoputamen and nucleus accumbens/fundus striati (Fig. 6*A*; see also ref. 7), the KCl treatment induced little PCREB expression in the olfactory tubercle (Fig. 6*A′*). Treatment of striatal cultures with NMDA (10 μM, 30 min), also resulted in high levels of PCREB immunoreactivity in the caudoputamen (Fig. 6*B* and ref. 7), but induced at most low levels of PCREB expression in the olfactory tubercle (Fig. 6*B′*). Only high, probably damaging concentrations of NMDA (100 μM) induced significant PCREB immunoreactivity in the ventral islands of the olfactory tubercle (data not shown). Depolarization with KCl and activation of NMDA receptors presumably increase intracellular Ca^{2+} concentrations, as does BAY K 8644. The uniformly low stimulation of CREB phosphorylation in the olfactory tubercle by these treatments suggests that elevation of intracellular Ca^{2+} levels did not facilitate CREB phosphorylation in this part of ventral striatum.

To explore this possibility further, we pretreated striatal cultures with EGTA (10 mM), a Ca^{2+} chelator, followed by treatment with BAY K 8644 (1 μM). The EGTA pretreatment abolished the PCREB expression induced by BAY K 8644 in the caudoputamen (Fig. 6*C*), but, unexpectedly, it strongly enhanced PCREB immunoreactivity in the olfactory tubercle, especially in the islands (Fig. 6*C′*). Thus, a decrease of Ca^{2+} concentration facilitated the phosphorylation of CREB by BAY K 8644 in the olfactory tubercle, while blocking CREB phosphorylation in the dorsal striatum. Treatment of cultures with EGTA alone did not induce phosphorylation of CREB.

DISCUSSION

Our experiments demonstrate that the kinetics of CREB phosphorylation induced by dopamine D1-class receptors and cAMP are markedly different in the dorsal striatum and the olfactory tubercle and other subdivisions of the ventral striatum. Our results further suggest striking contrasts in the effects of Ca^{2+} signaling in stimulating CREB phosphorylation in these striatal regions. Ca^{2+} activates CREB phosphorylation in the caudoputamen, but either poorly activates or inhibits CREB phosphorylation in the olfactory tubercle.

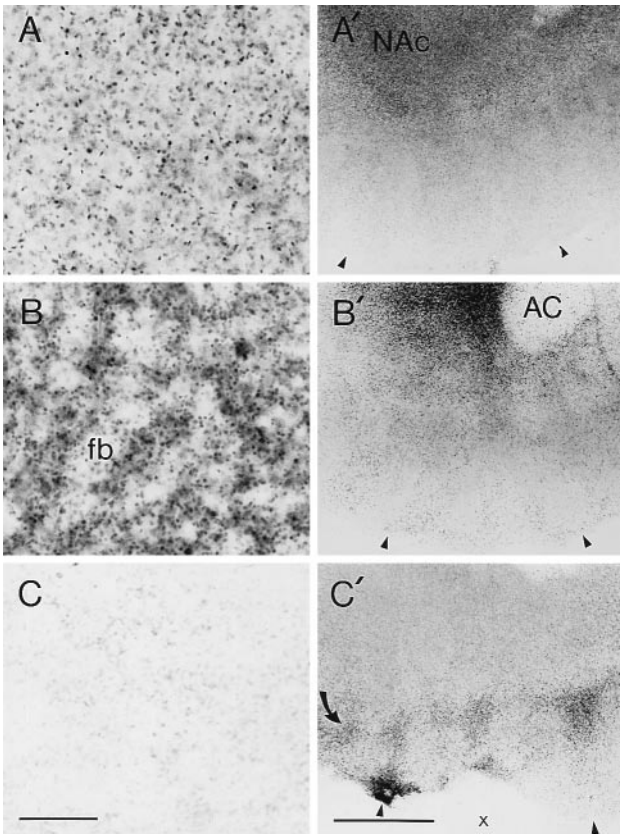

FIG. 6. Contrasting effects of treatments inducing calcium flux on CREB phosphorylation in the caudoputamen (*A–C*) and in the olfactory tubercle (*A′–C′*). Both KCl (*A*) and NMDA (*B*) induce high levels of PCREB expression through the caudoputamen. In sharp contrast, KCl induces little PCREB expression (*A′*) and NMDA induces only weak PCREB expression (*B′*) in the olfactory tubercle. Pretreatment with EGTA before BAY K 8644 stimulation decreases the PCREB immunostaining to background levels in the caudoputamen (*C*), but significantly enhances PCREB immunoreactivity in the islands of the olfactory tubercle (curved arrow in *C′*). All drug treatments were for 30 min. The arrowheads in *A′–C′* point to ventral edges of the tissue; × indicates a region of tissue damage. NAc, nucleus accumbens; AC, anterior commissure; fb, fiber bundles. [Bar in *C* (for *A–C*) = 100 μm; bar in *C′* (for *A′–C′*) = 0.5 mm.]

Three different kinetics of dopamine-mediated CREB phosphorylation were characterized in the present study: transient CREB phosphorylation in the striatal matrix; sustained CREB phosphorylation in striosomes and in the nucleus accumbens, fundus striati, and bed nucleus of the stria terminalis; and prolonged CREB phosphorylation in cellular islands of the olfactory tubercle. These region-dependent dynamics of CREB phosphorylation raises the intriguing possibility that different levels of CREB activation by dopamine might serve to accommodate different neural information processing in these different brain regions.

We did not assess transcription in this study. Available *in vitro* evidence, however, suggests that prolonged, but not transient, CREB phosphorylation is associated with subsequent activation of CRE-containing genes in neurons (7, 14). The striatal matrix, in which we observed transient CREB phosphorylation, participates in fast sensorimotor control. These functions may make a minimal demand for changes in gene expression unless the actions are extensively repeated. By contrast, striosomes, the nucleus accumbens, fundus striati, bed nucleus of the stria terminalis, and the olfactory tubercle are limbic-related structures that may be engaged significantly in slower neuroplasticity-related changes associated with reinforcement-based memory. These limbic-related regions may

sustain high levels of CREB phosphorylation in response to dopamine to ensure that signal changes can be inscribed at the genetic level.

Some differences in the dopamine-mediated transcriptional response of these striatal regions have been seen in *in vivo* experiments. For example, when the addictive drug, cocaine, is repeatedly given to rats and then is withdrawn, a subsequent challenge with this drug induces enhanced cFos and JunB expression in striosomes and some ventral striatal regions relative to the pattern seen in acutely treated rats (15). Dopaminergic treatments also have been shown to regulate CRE-containing neuropeptide genes in the striatum, including prodynorphin (16–18). We know of no functional corollary, however, for the exceptionally prolonged CREB phosphorylation in the patchy zones of the olfactory tubercle. These may correspond to the late-developing islands of Calleja, noted for their expression of D3 dopamine receptors (2, 19). Conceivably, the prolonged CREB activation reflects a developmental stage.

The prolonged CREB phosphorylation activated by D1 agonists and forskolin was sensitive to the presence of agonists in the culture medium. Nevertheless, even after radical reduction of agonist concentration, persistent CREB phosphorylation still occurred, and the differences among the kinetics of phosphorylation and dephosphorylation in the dorsal and ventral striatal sites were maintained. Thus, the D1 receptor/cAMP-mediated PCREB signaling pathway apparently was not desensitized by the prolonged agonist exposure, but it seems likely that the slow decay of CREB phosphorylation found after the washout rinses reflected an active phosphorylation rather than a gradual depletion of agonist activity.

The different durations of CREB phosphorylation activated in different striatal regions by a single agonist suggests that region-specific signaling molecules may participate in the signal transduction pathways activated by the agonist. The phosphorylation of CREB is under rigid control by protein phosphatases (7, 14, 20, 21). Three isoforms of protein phosphatase-1 have been reported to be expressed in the caudoputamen and the olfactory tubercle (22). Differential regulation of these protein phosphatases and other uncharacterized phosphatases may be one of the mechanisms underlying different kinetics of CREB phosphorylation in these brain regions.

In contrast to SKF-81297 and forskolin, BAY K 8644, KCl depolarization and glutamate NMDA receptor agonists induced low levels of CREB phosphorylation in the olfactory tubercle but strong PCREB expression in the caudoputamen and moderate PCREB expression in the nucleus accumbens and fundus striati. Because these agents all can increase intracellular Ca^{2+} levels, our results suggest that Ca^{2+} signaling is not effective in activating CREB phosphorylation on Ser-133 in the olfactory tubercle. Remarkably, when Ca^{2+} levels were reduced in the slices by pretreating with EGTA and the slices were then stimulated with BAY K 8644, PCREB expression was significantly enhanced in the olfactory tubercle despite the elimination of PCREB expression in the caudoputamen. This highlights the different signaling in the dorsal and ventral striatum.

The phosphorylation of CREB on Ser-133 can be activated not only by neurotransmitters, but also by growth factors. Neural growth factors including BDNF, NT3, NT4, and bFGF are capable of inducing PCREB expression in cortical cell cultures, and the PCREB expression remained strong at 4 hr after stimulation (23). After D1 agonist stimulation, we found CREB phosphorylation in the caudoputamen and most ventral striatal regions to be at low levels by 4 hr, but it still remained at high levels in the olfactory tubercle. The long duration of CREB phosphorylation in the olfactory tubercle induced by D1-class dopamine receptors and cAMP has a kinetics comparable to that induced by growth factors. It is possible that these stimuli have in common the initiation of long-term effects in neurons.

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