Desipramine Reduces Stress-Activated Dynorphin Expression and CREB Phosphorylation in NAc Tissue

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ABSTRACT

The nucleus accumbens (NAc) is a critical brain area for reward and motivated behavior. Accumulating evidence suggests that altered function of the transcription factor cAMP response element binding protein (CREB) within the NAc is involved in depressive behavior. In rats, stress activates CREB within the NAc, and elevation of CREB expression in this region produces depressive-like behaviors that are accompanied by activation of CREB-regulated target genes. The depressive-like behaviors seem to be due, at least in part, to CREB-mediated increases in dynorphin function, because they are mimicked by κ-opioid receptor (KOR) agonists and attenuated by KOR antagonists. We hypothesized that if CREB-mediated dynorphin expression in the NAc contributes to depressive behavior, then antidepressants might reduce dynorphin function in this region. Here, we demonstrate that desipramine (DMI), a norepinephrine reuptake inhibitor that has been used for decades to treat clinical depression, blocks swim stress-induced activation of prodynorphin (encodes dynorphin) in the NAc. In primary cultures of NAc neurons originating within the ventral tegmental area and projecting to the nucleus accumbens (NAc), although the NAc is also a substrate for natural rewards, including food, sex, and social interaction (Wise, 2004). In rodents, manipulations within the NAc produce behaviors that may model aspects of clinical depression, including anhedonia, dysphoria, and behavioral despair (Harris and Aston-Jones, 1994; Pliakas et al., 2001; Wise, 2004). Although the NAc has not been a major focus of depression research, it innervates—and is innervated by—regions often studied in depressed humans, including the hippocampus, frontal cortex, and amygdala (Nestler and Carlezon, 2006). In addition, norepinephrine (NE) and serotonin inputs modulate the NAc (Pasquier et al., 1977).

Neuroadaptations within the NAc contribute to the development of depressive-like behaviors. Stress elevates activity of the transcription factor cAMP response element binding protein (CREB) within the NAc (Pliakas et al., 2001). Elevated CREB function within the NAc increases depressive-like behavior in the forced swim test (FST) (Pliakas et al.,...
effects of DMI and forced swimming on prodynorphin gene expression within the NAc shell. The standard FST is a 2-day procedure in which rats swim under conditions in which escape is not possible. On the first day, rats are forced to swim for 15 min. At first the rats attempt to escape from the water, but eventually they adopt a posture of immobility in which they make only the movements necessary to keep their heads above water. When the rats are retested 24 h later, the latency to immobility is decreased. Treatment with standard antidepressant drugs within the 24 h between the first and second exposures to forced swimming can block facilitated immobility, an effect associated with antidepressant efficacy in humans (Detke et al., 1995). In this study, we sacrificed the rats on day 2, at the time when they would have experienced their second exposure to forced swimming. There were four treatment groups (6–13 rats per group): sham (no swim), sham plus DMI, forced swim (FS), and FS plus DMI. On the first day of the experiment, rats were forced to swim for 15 min as described above. Sham-treated rats were placed on a platform set at a height of 45 cm inside a dry Plexiglas cylinder for 15 min, to mimic as closely as possible the FST without actually exposing the rats to forced swimming. After the first forced swimming or sham session, rats were towel-dried and placed in a warm environment for 30 min. All rats were then injected IP with either saline or DMI (10 mg/kg) at 1, 19, and 23 h after the forced swimming session. At 24 h after the first swimming session, the rats were sacrificed by decapitation. The brains were rapidly removed and frozen in −80°C isopentane. Frozen brains were sectioned on a sliding microtome until the NAc shell was exposed (bregma −2.20). Bilateral 1-mm³ punches of tissue were extracted from the NAc shell and placed in Eppendorf tubes on dry ice. Q-PCR methods were similar to those described previously (MacDonald et al., 2005). RNA was extracted from approximately 20 to 30 mg of tissue using the RNeasy kit (Promega, Madison, WI). RNA quality was assessed by analytical gel, and 3.5 μg of total RNA was used for cDNA synthesis with the SuperScript First-Strand Synthesis System for real-time quantitative PCR (Invitrogen, Carlsbad, CA) and oligonucleotide deoxythymidine primer. Primers specific for the prodynorphin and β-actin genes (Genbank accession numbers NM_019374 and NM_031144) were designed with Primer3 software (www-

Materials and Methods

Rats. Thirty-three male Sprague-Dawley rats (325–375 g; Charles River Laboratories, Inc., Wilmington, MA) were used for analysis of prodynorphin mRNA expression. These rats were housed in groups of 4 in a climate-controlled vivarium and maintained on a 12-h light (7:00 AM–7:00 PM)/dark cycle with free access to food and water except during testing. In addition, 16 timed-pregnant Sprague-Dawley rats were used to obtain embryonic day 18 (E18) tissue for the primary cell culture studies. These rats were used on the day of delivery. Experiments were conducted in accordance with the 1996 Guide for the Care and Use of Laboratory Animals (National Institutes of Health), as well as McLean Hospital policies.

Drugs. For in vivo studies in rats, desipramine hydrochloride (DMI; Sigma, St. Louis, MO) was dissolved in distilled water vehicle for 24 h after treatment. Elevated CREB reduces the motivational impact of drugs and natural rewards, a sign of anhedonia (Carlezon et al., 1998). The depressive-like behavioral effects that accompany elevated NAc CREB function seem related to altered transcription of dynorphin (Carlezon et al., 1998); an endogenous peptide that acts at opioid receptors (KORs) (Chavkin et al., 1982). Disruption of CREB function within the NAc produces antidepressant-like effects (Pliaskas et al., 2001) accompanied by decreases in dynorphin expression (Carlezon et al., 1998). Likewise, KOR antagonists attenuate the behavioral effects of elevated CREB expression within the NAc and have antidepressant-like effects (Pliaskas et al., 2001; Newton et al., 2002; Mague et al., 2003; McLaughlin et al., 2003). These findings are consistent with observations that KOR agonists produce depressive signs in humans (Pfeiffer et al., 1986) and rats (Shippenberg and Herz, 1987; Mague et al., 2003; Todtenkopf et al., 2004). Thus, there are strong links between CREB-mediated regulation of dynorphin within the NAc and depressive behavior.

The present studies were designed to test the hypothesis that if CREB function in the NAc contributes to depressive behavior, then desipramine (A NE reuptake inhibitor used for decades to treat clinical depression; Frazer, 1997) might affect CREB-regulated gene expression within this brain region. We first examined the effects of DMI on stress-induced alterations in prodynorphin mRNA expression within the NAc. We then used an in vitro model (primary cell cultures of NAc/striatum) to explore potential intracellular mechanisms of this effect. For comparison, we also examined the in vitro effects of fluoxetine (FLX), a selective serotonin reuptake inhibitor (SSRI) with clinical efficacy similar to DMI (Frazer, 1997).

Fig. 1. DMI regulates molecular consequences of CREB activity within the NAc. A, prodynorphin mRNA levels within the NAc shell were significantly elevated 24 h after exposure to forced swimming. A treatment regimen of DMI (10 mg/kg) that produces antidepressant-like effects in the FST completely blocked the induction of prodynorphin mRNA but had no effect in rats exposed to a sham swim session (“Sham”; see Materials and Methods). Data are expressed as relative levels of prodynorphin mRNA, corrected for β-actin mRNA content. *, P < 0.05 compared with sham-treated rats; ψ, P < 0.05 comparing FST and DMI + FST, Fisher’s protected t tests, 6 to 13 rats per group. B, visualization of prodynorphin and β-actin amplicons from Q-PCR.
To determine the mechanisms by which DMI and FLX might
activate phospho-CREB (P-CREB) in NAc tissue, we treated
primary neuronal cultures with DMI or FLX for 1 h (intended to correspond
with cells in culture for 7 days, and treatments were done in triplicate
and the number of living (green) and dead (red) cells was counted.

Primary Cultures of NAc and Striatum. Primary cultures
were prepared as described previously (Chartoff et al., 2003). In
brief, striata (which contain NAc and striatal tissue) were dissected
from E18 Sprague-Dawley rat fetuses in 1× Hank’s balanced salt
solution (Invitrogen) using a stereomicroscope. Striata were resus-
pended in 2 ml of defined medium (50% Ham’s F12/Dulbecco’s mod-
ified Eagle’s medium and 50% Dulbecco’s modified Eagle’s medium
(Mediotech, Herndon, VA) that contained the following supplements
per liter of medium: 4 g of dextrose, 1× B27 (Invitrogen), 10 ml of
penicillin-streptomycin liquid (Sigma-Aldrich), and 7.5 mM HEPES
(Sigma-Aldrich). Using a Pasteur pipette, the tissue was mechani-
cally dissociated and the cells resuspended to 1
106 cells/ml. For
Western blot analysis, cells were plated in 12-well plates (1 × 106
cells/well). For the live/dead assay, cells were plated on round glass
coverslips (15-mm diameter; Electron Microscopy Sciences, Ft.
Washington, PA) that fit inside 12-well plates. Coverslips were ster-
ilized by soaking overnight in concentrated nitric acid, washing three
times with sterile H2O, and baking for 30 min. Coverslips and 12-
well plates (BD Biosciences, Franklin Lakes, NJ) were pretreated
with 1 ml of sterile polyethyleneimine (1:500 in H2O; Sigma-Aldrich)
for 24 h, washed twice with sterile H2O, and coated with 2.5% fetal
bovine serum (Sigma-Aldrich) in PBS for 4 h. The serum was aspi-
rated just before plating of the cells. All experiments were performed
with cells in culture for 7 days, and treatments were done in tripli-
cate using cells obtained from at least two independent dissections.

Studies of P-CREB Induction. To study the effects of antide-
nessants on phospho-CREB (P-CREB) in NAc tissue, we treated
neuronal cultures with DMI or FLX for 1 h (intended to correspond
with immediate effects) or 24 h (intended to correspond with longer-
term effects). For studies investigating the effects of DMI on basal
P-CREB levels, DMI was added to the tissue culture media either 24
or 1 h before cell harvesting. For studies investigating the effects of
antidepressant drugs on the activation of P-CREB by cellular depo-
larization, DMI or FLX was added to the media either 21 or 1 h
before KCl (40 mM), and the cells were harvested 4 h after. This
course is based on previously published work (Schwaninger et al.,
1995). To determine the mechanisms by which DMI and FLX might
be attenuating CREB function, several pathways known to activate
CREB were investigated. DMI was administered 1 h before SKF
82958 (DA D1-selective agonist), FPL 64176 (L-type Ca2+ channel
activator), PMMA (protein kinase C activator), NMDA, or thapsigar-
gin, (sarcoplasmic/endoplasmic reticulum Ca2+-ATPase inhibitor),
which increases levels of free cytosolic Ca2+ (Treiman et al., 1998), and
the cells were harvested 15 min later.

Western Blot Analysis. Primary rat NAc striatal cultures were
harvested in Nu-PAGE lithium dodecyl sulfate sample buffer (In-
vitrogen) and 50 mM dithiothreitol as described previously (Chartoff et al.,
2003). Cell lysates were sonicated and centrifuged briefly at
4°C and then heated to 70°C for 10 min before gel electrophoresis.
Ten microliters of each sample were loaded onto Nu-PAGE Novex 4
at 12% Bis-Tris gels (Invitrogen) for separation by gel electrophore-
sis. Proteins were subsequently transferred to polyvinylidene fluo-
ride membrane (PerkinElmer Life and Analytical Sciences, Waltham, MA).
Non-specific binding sites on the membranes were blocked
for 2 h at room temperature in blocking buffer (5% nonfat dry
milk in PBS and 0.1% Tween 20 (PBS-T)). Blots were then incubated
in primary antibody [1:4000 monoclonal anti-Ser133-phospho-CREB
(P-CREB) or 1:4000 anti-CREB (Cell Signaling Technology Inc.,
Beverly, MA)] PBS-T overnight at 4°C. Blots were washed four
times for 15 min each in PBS-T and then incubated in secondary
antibody [1:5000 goat anti-rabbit, or anti-mouse, horseradish peroxy-
dase-linked IgG (Vector Laboratories, Burlingame, CA)] for 2 h
at room temperature. Blots were washed four times for 15 min each in
PBS-T, followed by immunological detection with Chemilumines-
cence Reagent Plus (PerkinElmer Life and Analytical Sciences). P-
CREB or CREB antibodies were stripped from the blots by incuba-
tion with stripping buffer (62.5 mM Tris, 2% SDS, and 100 mM
beta-mercaptoethanol, pH 6.8) for 15 min at 50°C. Blots were subse-
sequently re-blocked and probed with 1:20,000 anti-beta-actin (Sigma).
SeeBlue Plus 2 (Invitrogen) prestained standards were run for mol-
ecular mass estimation. P-CREB and CREB bands were detected
at 43 kDa, and beta-actin was detected at 42 kDa.

Protein immunoblots were analyzed using Kodak 1D Image Anal-
ysis software (Carestream Health, Rochester, NY). Relative optical
densities were determined for each band of interest. To control for
loading differences of protein, the optical density of each band was
normalized with the corresponding optical density of beta-actin. To
allow for comparisons of blots from independent dissections, data
were normalized to the vehicle-treated controls in each experiment.
Data are expressed as the mean -fold induction compared with ve-
hicle control ± S.E.M. and were analyzed using one-way ANOVA
followed by Fisher’s protected t tests.

Live/Dead Assay. To determine whether DMI is cytotoxic to
striatal cultures, a Live/Dead Viability/Cytotoxicity Kit (Invitrogen)
was used. Cells were plated on round glass coverslips as described
above and treated as described in the figure legends. At the appro-
priate time after drug treatments, cells were washed twice with PBS.
The coverslips containing the cells were then inverted onto 150-μl drops
of a dye mixture containing 1 mM calcein AM and 1 mM etidium homodimer-1
and allowed to incubate for 30 min at room temperature in a darkened chamber containing a water-soaked Kim-
wipe to prevent the coverslips from drying. Coverslips were then
washed briefly in PBS and placed cell-side down onto 20-μl drops
of PBS on microscope slides. Visualization (Axioskop 2 plus; Zeiss
GmbH, Jena, Germany) and digital image capture (Openlab 3.0.7
software; Improvision, Coventry, UK) of cells were done immedi-
ately, because the coverslips dried out after 1 h. Live cells fluoresced
green as a result of esterase-mediated cleavage of cell-permeant
calcium AM to fluorescent calcein and were detected with an
enhanced green fluorescent protein filter. Dead cells fluoresced red as
a result of the entry of etidium homodimer-1 into cells with dam-
aged membranes and subsequent binding to nucleic acid; they were
detected with a Texas Red filter. To quantify the percentage of live
cells, ImageJ 1.33a software (http://rsb.info.nih.gov/ij/) was used.
A 1020 × 760-pixel box was randomly overlaid onto each digital image,
and the number of living (green) and dead (red) cells was counted.
The number of green plus red cells was used as the total cell popu-
lation, and data are expressed as the percentage of live cells. The
live/dead assay was performed on cells from two separate dissec-
tions, and for each dissection, the treatments were administered in
duplicate. Data are expressed as the mean percentage of live cells ±
S.E.M. and were analyzed using one-way ANOVA. As followed by Fisher-
’er’s protected t tests.
Results

DMI Blocks Stress-Induced Prodynorphin mRNA Expression. To examine the possibility that the antidepressant actions of DMI involve disruption of CREB function within the NAc, we examined its effects on local expression of prodynorphin, a gene known to be regulated by CREB (Douglas et al., 1994; Cole et al., 1995). Stress is a major trigger for depression (Nestler et al., 2002), and it has been shown that FS stress activates CREB within the NAc (Pliakas et al., 2001) and induces the release of dynorphin (McLaughlin et al., 2003). Thus DMI—which reduces FS-induced depressive-like behavior (Cryan et al., 2002)—may act in part to attenuate stress-induced neuroadaptations such as CREB-regulated increases in prodynorphin. We found that prodynorphin mRNA levels within the NAc depended upon treatment ($F_{3,29} = 4.091; P < 0.05$) (Fig. 1A): forced swimming induced increased expression of prodynorphin within the NAc that was sustained for at least 24 h ($P < 0.05$), and this effect was blocked by a treatment regimen of DMI (10 mg/kg injection) that produces antidepressant-like effects in the FST (Carlezon et al., 2002). In contrast, DMI had no effect on prodynorphin mRNA levels in rats not exposed to forced swim stress. Visualization confirmed that the prodynorphin and β-actin amplicons were the expected size on a polyacrylamide gel (Fig. 1B).

Effects of DMI on CREB Function in Dissociated NAc/Striatal Cultures. The NAc receives relatively minor NE input (Delfs et al., 1998), raising the possibility that the ability of DMI to block FS stress-induced prodynorphin in the NAc is due either to indirect circuit effects or to direct effects on NAc function that are independent of NE reuptake blockade. To test whether DMI can act directly on postsynaptic cells in the NAc to modulate intracellular signaling, we used primary cultures of dissociated E18 rat NAc and striatal tissue. We treated these cultures for 1 hr or 24 hr with DMI and measured the relative levels of CREB protein or CREB phosphorylated at serine residue 133 (P-CREB). One-hour treatment with DMI decreased basal P-CREB levels ($F_{5,209} = 7.59; P < 0.01$) without affecting total CREB protein ($F_{5,21} = 0.15; 	ext{not significant}$) (Fig. 2A). Twenty-four-hour treatment with DMI had a bimodal effect on P-CREB levels ($F_{5,171} = 22.0; P < 0.01$) (Fig. 2B): intermediate doses of DMI in-
creased (P < 0.01) basal P-CREB, whereas a high dose (3 μM) decreased it (P < 0.01). It is noteworthy that the high dose of 24-h DMI (3 μM) increased levels of CREB protein (F_{5,57} = 6.79; P < 0.01) (Fig. 2B). Inasmuch as it has been reported previously that long-term treatment with antidepressant drugs increased both CREB mRNA levels and CREB function in the hippocampus (Nibuya et al., 1996), our data indicate that CREB-mediated plasticity is complex and probably brain area-dependent (Carlezon et al., 2005).

The decrease in P-CREB observed with 24-h DMI (3 μM) treatment could be due to cellular toxicity. High concentrations of DMI (20 mM) have been shown to induce cell death in a neural cell line (Post et al., 2000). Although the doses of DMI used in our studies did not approach this level, the propensity of DMI to cause toxicity in primary NAc and striatal cultures is unknown. We found that DMI had no effect on cell viability when cultures were treated for 24 h with the highest concentration of the drug (3 μM), whereas a brief treatment with 70% methanol caused 100% cell death (F_{2,6} = 35.4; P < 0.01) (Fig. 2C). Likewise, we observed no effects of 1-h DMI (3 μM) treatment on cell viability (data not shown).

To examine whether DMI regulates CREB under conditions of heightened neuronal activation—similar to what may occur in the brain during periods of stress—we depolarized the cell cultures with KCl (40 mM). This has been shown to activate CRE (cAMP response element)-mediated gene transcription by influx of Ca^{2+} through L-type voltage-dependent Ca^{2+} channels (Sheng et al., 1990). Addition of KCl to the cultures induced robust increases in P-CREB, which were dose-dependently attenuated by both 1-h DMI pretreatment (F_{5,155} = 34.1; P < 0.01) (Fig. 3A; solid black bars) and 24-h DMI pretreatment (F_{5,128} = 20.4; P < 0.01) (Fig. 3B; solid black bars).

One of the ways in which DMI might block KCl-stimulated increases in P-CREB is by disruption of Ca^{2+} signaling. It has been shown previously that antidepressant drugs inhibit KCl- and voltage-stimulated calcium channel-induced increases in intracellular Ca^{2+} levels in neuronal cultures (Cai and McCaslin, 1992; Choi et al., 1992; Shimizu et al., 1994). We found that 1-h pretreatment with DMI had no effect on the induction of P-CREB by the L-type voltage-dependent Ca^{2+} channel activator FPL 64176 (F_{2,18} = 38.1; P < 0.01), NMDA (F_{2,26} = 13.4; P < 0.01), or the protein kinase C (PKC) activator PMA (F_{2,15} = 21.7; P < 0.01) (Table 1). In contrast, 1-h (F_{1,28} = 18.9; P < 0.01) pretreatment with DMI inhibited P-CREB induced by thapsigargin (100 nM; blocks uptake of Ca^{2+} into intracellular stores), and 24-h (F_{3,74} = 5.713; P < 0.01) pretreatment with DMI (3 μM) attenuated (trend, P = 0.091) thapsigargin-induced P-CREB (Fig. 4, A and B; black bars). Finally, 1-h pretreatment with DMI had no effect on P-CREB induced by the D1 agonist SKF 82958 (F_{2,9} = 13.9; P < 0.01) (Table 1), further suggesting a specific role for Ca^{2+} in mediating the effects of DMI.

Effects of FLX on CREB Function in Dissociated NAc/Striatal Cultures. To determine whether the ability of DMI to reduce CREB function within the NAc and striatum through inhibition of signaling pathways activated by intracellular Ca^{2+} release was specific to DMI or generalizable to other classes of antidepressant drugs, we tested the effects of FLX, an SSRI, on KCl- and thapsigargin-stimulated P-CREB in primary cultures of the NAc and striatum. We found that both 1-h (F_{1,62} = 30.897; P < 0.01) and 24-h (F_{3,62} = 13.389; P < 0.01) pretreatments with FLX attenuated KCl-induced P-CREB (Fig. 5, A and B; black bars). Likewise, both 1-h (F_{3,44} = 10.428; P < 0.01) and 24-h (F_{3,83} = 7.032; P < 0.01) pretreatments with FLX inhibited thapsigargin-induced P-CREB (Fig. 5, C and D; black bars). Similar to our findings with DMI, 1-h FLX reduced basal P-CREB (Fig. 5C, inset) (F_{2,24} = 12.30; P < 0.001). Twenty-four-hour FLX, however, slightly increased basal P-CREB at the highest dose tested (Fig. 5D, inset) but did not show the bimodal effect on P-
CREB observed with DMI ($F_{2,27} = 1.25$; ns). Similar to DMI, 1-h FLX had no effect on FPL 64176-induced P-CREB (data not shown). Hence, despite distinct pharmacological profiles, DMI and FLX both seem to act via a common mechanism within the NAc and striatum.

**Discussion**

The NE reuptake inhibitor DMI decreases CREB function and prodynorphin mRNA expression in the NAc, supporting the hypothesis that increased CREB function in the NAc contributes to depressive-like behaviors (Carlezon et al., 2005). We demonstrate this in two distinct but complementary ways. First, we found that swim stress-induced activation of prodynorphin within the NAc is blocked by a DMI regimen that produces antidepressant-like effects in the FST. Second, we used primary cultures of embryonic NAc and striatal tissue to show that DMI reduced CREB function. The SSRI FLX had similar effects in studies designed to treat depression. We demonstrate this in two distinct but complementary ways. First, we found that swim stress-induced activation of CREB observed with DMI ($F_{2,27} = 1.25$; ns). Similar to DMI, 1-h FLX had no effect on FPL 64176-induced P-CREB (data not shown). Hence, despite distinct pharmacological profiles, DMI and FLX both seem to act via a common mechanism within the NAc and striatum.

**Effects of DMI and FLX in Vitro.** To understand the mechanism by which DMI blocks swim stress-induced prodynorphin expression, we used dissociated cultures of NAc and striatal tissue to examine the effects of DMI on CREB and CREB phosphorylation—a marker of CREB activation (Mayr and Montminy, 2001). In humans and in animal models of depression, the therapeutic effects of antidepressant drugs occur primarily after prolonged administration. To be sensitive to this important issue, we assessed CREB function in response to 1 h (corresponds to short-term effects) and 24 h (corresponds to long-term effects) treatments. We show that both regimens of DMI treatment reduce baseline and KCl-stimulated CREB phosphorylation in primary cultures of NAc and striatal tissue. We demonstrate that DMI can have direct, NE-independent actions within this novel in vitro model that are not due to toxicity. Our data further support the hypothesis that DMI reduces CREB function and CREB-regulated target gene expression within the NAc. Considering that CREB protein levels remain unaltered by DMI at doses that significantly affect P-CREB, our in vivo finding that DMI reduces stress-activated prodynorphin expression within the NAc is probably due to attenuated CREB phosphorylation rather than reduced CREB levels.

Twenty-four-hour pretreatment with DMI had bimodal ef-
The ability of DMI to disrupt CREB function within the NAc seems to involve, at least in part, effects on intracellular Ca\textsuperscript{2+} storage. DMI attenuates the ability of thapsigargin-induced increases in intracellular Ca\textsuperscript{2+} to phosphorylate CREB. Thapsigargin inhibits sarco-endoplasmic reticulum Ca\textsuperscript{2+}-AT-Pase (SERCA) pumps, which actively pump Ca\textsuperscript{2+} into intracellular stores (SER). SERCA inhibition results in increased cytosolic Ca\textsuperscript{2+} levels, which in turn can trigger waves of Ca\textsuperscript{2+} release through activation of ryanodine receptors on the SER. The resultant calcium “waves” can activate calcium-regulated signal transduction pathways that lead to CREB phosphorylation. In our in vitro studies with NAc and striatal neuronal cultures, DMI had no effect on P-CREB stimulated by the L-type Ca\textsuperscript{2+} channel agonist PPL64176 or NMDA, suggesting that DMI does not modulate Ca\textsuperscript{2+} entry through these specific ion channels. This is in apparent contrast to several previous studies showing that tricyclic antidepressants suppress Ca\textsuperscript{2+} entry through voltage-sensitive Ca\textsuperscript{2+} channels and inhibit KCl-stimulated increases in intracellular Ca\textsuperscript{2+} levels.

Fig. 5. Effects of FLX on KCl- and thapsigargin-induced P-CREB in primary cultures of NAc and striatal tissue. The ratio of P-CREB/β-actin was determined for each sample and normalized to the control group ratio to yield a -fold induction. Representative Western blots are shown below each panel. A, a 3-h treatment with KCl (40 mM) significantly increased P-CREB; a 1-h pretreatment with FLX dose-dependently decreased KCI-stimulated P-CREB. B, likewise, a 24-h pretreatment with FLX decreased KCI-stimulated P-CREB. **, P < 0.01 compared with vehicle (Veh); ##, P < 0.01 compared with KCl alone. Fisher’s protected t tests, n = 2 to 3 experiments with treatments given in triplicate. C, a 15-min treatment with thapsigargin (100 nM) significantly increased P-CREB; a 1-h pretreatment with FLX blocked thapsigargin-induced P-CREB. A 1-h pretreatment with FLX 1 h before Veh dose-dependently decreased basal P-CREB (inset). D, 24-h pretreatment with FLX also decreased thapsigargin-induced P-CREB. A 24-h pretreatment with FLX 24 h before Veh had no significant effect on basal P-CREB (inset). **, P < 0.01 compared with vehicle (Veh); #, P < 0.05; ##, P < 0.01 compared with thapsigargin alone (Thaps), Fisher’s protected t tests, n = 2 to 3 experiments with treatments given in triplicate.
that originate from extracellular sources (Cai and McCaslin, 1992; Choi et al., 1992; Shimizu et al., 1994). However, DMI did not completely block KCl-stimulated P-CREB, which is consistent with a partial role for intracellular stores in CREB activation. In addition, our studies are unique in that we are examining P-CREB regulation rather than Ca\(^{2+}\) entry, and our findings suggest that DMI-mediated decreases in P-CREB depend, at least in part, on targeting of intracellular Ca\(^{2+}\) stores. We also show that DMI does not affect P-CREB stimulated by the PKC activator PMA. PKC is activated by Ca\(^{2+}\) and has been shown to phosphorylate CREB (Mao et al., 2007). In addition, it has been shown previously that DMI does not block CaM kinase IV-induced CRE-mediated gene transcription (Schwaninger et al., 1995). Finally, we show that DMI has no effect on P-CREB stimulated by the D1 receptor agonist SKF 82958, which is consistent with the previous finding that DMI did not alter forskolin-stimulated CRE-mediated gene transcription (Schwaninger et al., 1995). Together, these findings raise the possibility that the effects of DMI are not due to general inhibition of Ca\(^{2+}\) - or cAMP-mediated signaling cascades but rather to direct actions at SERCA pumps or at receptors that control release of Ca\(^{2+}\) from the SER (e.g., ryanodine receptors).

Antidepressant drugs have been shown to inhibit P-CREB and CREB-mediated transcription in vitro (Schwaninger et al., 1995). Considering the present data demonstrating the ability of Ca\(^{2+}\)-stimulating agents to increase P-CREB and the known ability to P-CREB to regulate prodynorphin (Cole et al., 1995), our finding that DMI inhibits Ca\(^{2+}\)-mediated activation of CREB strongly suggests that prodynorphin expression would be inhibited in NAc/striatal tissue (as it is in vivo). However, there is some evidence in the literature that Ca\(^{2+}\) can have mixed effects on CREB-regulated gene expression. Work done in cultured cortical neurons has shown that Ca\(^{2+}\) can sometimes act to inhibit CREB-mediated transcription by parallel induction of phosphorylation at Ser\(^{142,143}\) which would tend to offset the stimulatory effects of phosphorylation at Ser\(^{133}\) (Kornhauser et al., 2002). In contrast, work done in culture spinal cord neurons has shown that Ca\(^{2+}\) can potentiate prodynorphin expression by binding to and removing the transcriptional repressor DREAM (Cheng et al., 2002). In addition, in cultured myocardial cells 4 h after KCl administration, prodynorphin expression is dramatically increased in a Ca\(^{2+}\)-dependent manner (Ventura et al., 1994). Together, these findings indicate that Ca\(^{2+}\)-stimulated signal transduction pathways are complex and can have a variety of effects on transcription that probably depend on cell type and brain region (see Carlezon et al., 2005).

We hypothesized that if the effects of DMI on intracellular Ca\(^{2+}\) function within the NAc contributes to the antidepressant actions of the drug, then other classes of antidepressants might have similar effects. We found that 1-h and 24-h treatments with FLX also attenuated KCl-stimulated P-CREB in the cell cultures. This effect can be attributed, at least in part, to a decrease in intracellular Ca\(^{2+}\) function: FLX reduced thapsigargin-stimulated P-CREB. Although DMI and FLX had similar effects on P-CREB, there were minor differences. One-hour, but not 24-hour, FLX reduced basal P-CREB levels in the absence of stimulation. In addition, KCl-stimulated P-CREB was less sensitive to FLX than DMI, whereas thapsigargin-stimulated P-CREB appeared more sensitive to FLX than DMI. These differences might be related to the pharmacokinetics of these drugs. In humans, steady-state serum concentrations of DMI and FLX can reach approximately 1000 and 1500 nM (Baldessarini, 2006), which are within the range of doses we have shown to have effects on P-CREB. It is possible that, relative to DMI, slightly higher concentrations of FLX are required to modulate basal P-CREB over the 24-h time course as well as attenuate KCl-stimulated P-CREB. Likewise, the mechanism of action of FLX might be more dependent on regulation of intracellular Ca\(^{2+}\). Future work might identify the mechanism by which these drugs act upon intracellular Ca\(^{2+}\) stores and determine whether this is a point of convergence for all antidepressants.

**Conclusions**

We identify a novel mechanism through which DMI has direct effects on Ca\(^{2+}\)-mediated signaling that lead to decreased CREB function and prodynorphin expression within the NAc. These findings are congruent with evidence that KOR blockade and Ca\(^{2+}\) channel inhibitors produce antidepressant-like effects, and that inhibition of Ca\(^{2+}\) influx within the NAc has reward-facilitating effects (Pucilowski, 1992; Pliakas et al., 2001; Newton et al., 2002; Mague et al., 2003; Chartoff et al., 2006). They are also consistent with work demonstrating that deletion of CREB isoforms throughout the brain produces antidepressant-like effects (Conti et al., 2002), and activation of CREB-regulated target genes within the mesolimbic dopamine system contributes to induction of depressive-like states (Berton et al., 2006). Considering the growing sentiment that altered monoaminergic function alone is unlikely to explain depression or the therapeutic effects of antidepressants (Castrén, 2005; Nestler and Carlezon, 2006), our findings have important implications. First, the therapeutic effects of antidepressants including DMI might involve diminished activity of CREB-regulated target genes within the NAc. Second, such effects may occur independently of—or in addition to—their ability to regulate monoaminergic systems. These data provide early evidence that antidepressants produce some of their therapeutic effects directly within the NAc.

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**References**


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