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β1-Adrenergic receptors activate two distinct signaling pathways in striatal neurons

John Meitzen, Jessie I. Luoma, Christopher M. Stern and Paul G. Mermelstein

Department of Neuroscience and Graduate Program in Neuroscience, University of Minnesota, Minneapolis, Minnesota, USA

Abstract

Monoamine action in the dorsal striatum and nucleus accumbens plays essential roles in striatal physiology. Although research often focuses on dopamine and its receptors, norepinephrine (NE) and adrenergic receptors are also crucial in regulating striatal function. While noradrenergic neurotransmission has been identified in the striatum, little is known regarding the signaling pathways activated by β-adrenergic receptors in this brain region. Using cultured striatal neurons, we characterized a novel signaling pathway by which activation of β1-adrenergic receptors leads to the rapid phosphorylation of cAMP response element binding protein (CREB), a transcription-factor implicated as a molecular switch underlying long-term changes in brain function. NE-mediated CREB phosphorylation requires β1-adrenergic receptor stimulation of a receptor tyrosine kinase, ultimately leading to the activation of a Ras/Raf/MEK/MAPK/MSK signaling pathway. Activation of β1-adrenergic receptors also induces CRE-dependent transcription and increased *c-fos* expression. In addition, stimulation of β1-adrenergic receptors produces cAMP production, but surprisingly, β1-adrenergic receptor activation of adenylyl cyclase was not functionally linked to rapid CREB phosphorylation. These findings demonstrate that activation of β1-adrenergic receptors on striatal neurons can stimulate two distinct signaling pathways. These adrenergic actions can produce long-term changes in gene expression, as well as rapidly modulate cellular physiology. By elucidating the mechanisms by which NE and β1-adrenergic receptor activation affects striatal physiology, we provide the means to more fully understand the role of monoamines in modulating striatal function, specifically how NE and β1-adrenergic receptors may affect striatal physiology.

Keywords: adrenergic receptor, cyclic AMP response element binding protein, noradrenaline, norepinephrine, nucleus accumbens, striatum.

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Monoamine action on striatal neurons plays essential roles in striatal physiology. While dopamine (DA) and its receptors are the focus of most research in this area, norepinephrine (NE) and adrenergic receptors are also crucial in regulating striatal function. There is abundant expression of α- and β-adrenergic receptors in the striatum (Nicholas *et al.* 1993; Pisani *et al.* 2003; Paschalis *et al.* 2009; Rommelfanger *et al.* 2009; Hara *et al.* 2010), and dysregulation of striatal NE signaling plays important roles in both drug addiction and Parkinson's Disease (Fornai *et al.* 2007; Rommelfanger and Weinshenker 2007; Weinshenker and Schroeder 2007; Aston-Jones and Kalivas 2008; Sofuoglu and Sewell 2009). Despite this recognition, the intracellular signaling mechanisms by which NE modulates striatal neurons are not well understood. While over 30 years ago activation of striatal β-adrenergic receptors was demonstrated to increase cAMP concentrations (Forn *et al.* 1974; Harris 1976), since that time there have been few follow-up studies examining NE-mediated signaling in striatal neurons. This is particularly true regarding β-adrenergic receptors (Hara *et al.* 2010). Thus, many questions remain regarding striatal NE signaling. Our work has focused on answering three of those questions.

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Address correspondence and reprint requests to John Meitzen, PhD, Department of Neuroscience, University of Minnesota, 6-145 Jackson Hall, 321 Church St SE, Minneapolis, MN 55455, USA.
E-mail: meitz010@umn.edu

Abbreviations used: 6-C, 6-chloro-PB; AC, adenylyl cyclase; BDNF, brain-derived neurotrophic factor; CREB, cAMP response element binding protein; DA, dopamine; d.i.v., days *in vitro*; FBS, fetal bovine serum; ISO, isoproterenol; LC, locus ceruleus; MAP2, microtubule-associated protein 2; MEK, Mitogen-activated protein kinase kinase; MEM, minimum essential medium; MSK, mitogen and stress-activated protein kinase; NE, norepinephrine; PBS, phosphate-buffered saline; pCREB, phosphorylated CREB; PD, Parkinson's disease; PKA, Protein kinase A; qPCR, quantitative PCR; RTK, receptor tyrosine kinase.

Specifically, whether cAMP accumulation is the singular action of β-adrenergic receptors in this brain region, whether activation of β-adrenergic receptors affects transcription factors and activity-dependent gene expression, and which of the β-adrenergic receptors and associated signaling pathways mediate these changes in cellular physiology.

We find that NE activation of β1-adrenergic receptors stimulates two distinct signaling pathways: one novel and one canonical. The novel pathway leads to the rapid phosphorylation of cAMP response element binding protein (CREB), a transcription-factor that functions as a molecular switch underlying neural plasticity (Lonze and Ginty 2002; Carlezon *et al.* 2005). β1-adrenergic receptor-mediated CREB phosphorylation is initiated by stimulation of a receptor tyrosine kinase (RTK). Transactivation of the RTK by β1-adrenegic receptors leads to stimulation of a signaling cascade that includes Ras, Raf, Mitogen-activated protein kinase kinase (MEK), MAPK, and mitogen and stress-activated protein kinase (MSK). In addition to CREB phosphorylation, we also observed an increase in CRE-dependent transcription and *c-fos* gene expression. The second signaling pathway is the previously defined canonical pathway in which stimulation of β1-adrenergic receptors leads to an increase in cAMP production. Interestingly, increases in cAMP were not functionally linked to rapid CREB phosphorylation. These findings indicate that NE can act on striatal neurons via different signaling pathways to stimulate both long-term changes in gene expression, as well as rapidly modulate cellular physiology. These data provide a new framework in which to understand monoamine signaling in striatal neurons, whereby NE and adrenergic receptors can modulate striatal physiology.

Materials and methods

Cell culture

Striatal neurons were cultured from 1- to 2-day-old Sprague-Dawley male rat pups as previously described (Mermelstein *et al.* 2000; Groth *et al.* 2008). All protocols were approved by the Animal Care and Use Committee at the University of Minnesota. Chemicals were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise. Following decapitation, the dorsal striatum and nucleus accumbens (striatum) were isolated in ice-cold modified Hank's balanced salt solution containing 20% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and (in mM) 4.2 $NaHCO_3$ and 1 HEPES, pH 7.35, 300 mOsm. The tissue was then washed and digested for 5 min in a trypsin solution (type XI; 10 mg/mL) containing 137 mM NaCl, 5 mM KCl, 7 mM Na_2HPO_4, 25 mM HEPES, and 1500 U of DNase, pH 7.2, 300 mOsm. After additional washes, tissue was dissociated and pelleted twice by centrifugation (180 *g* for 10 min) to remove contaminants. Cells were then plated onto 10 mm coverslips (treated with Matrigel to promote adherence; BD Biosciences, San Jose, CA, USA) and incubated for 20 min at 24°C. Two milliliters of minimum essential medium (MEM; Invitrogen, Grand Island, NY, USA) containing 28 mM glucose, 2.4 mM $NaHCO_3$, 0.0013 mM transferrin (Calbiochem, La Jolla, CA, USA), 2 mM glutamine, and 0.0042 mM insulin with 1% B-27 supplement (Invitrogen) and 10% FBS, pH 7.35, 300 mOsm, were added to each coverslip. To inhibit glial growth, 1 mL of medium was replaced with a solution containing 4 μM cytosine 1-β-D-arabinofuranoside and 5% FBS 24 h after plating. Seventy-two hours later, 1 mL of medium was replaced with modified MEM solution containing 5% FBS. Gentamicin (2 μg/mL; Invitrogen) was added to all media solutions to eliminate bacterial growth.

Drugs

The drugs used from Tocris (Ellisville, MO, USA) were: tetrodotoxin (1 μM); D(−)-2-amino-5-phosphonopentanoic acid (25 μM); propanolol (30 μM); betaxolol (10 μM); melittin (1 μM); gallein (75 μM); SQ22536 (90 μM); H89 (5 μM); KT5720 (3 μM); Protein kinase inhibitor 14-22 amide (1 μM); GW5074 (10 μM); SL0101-1 (10 μM); U0126 (10 μM); PD98059 (25 μM); K252a (100 nM); 8CPT-2Me-cAMP (50 μM); 1-(1,1-Dimethylethyl)-1-(4-methylphenyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (5 μM); thapsigargin (1 μM); pertussis toxin (500 ng/mL). The drugs used from Sigma were: NE (25 μM, unless otherwise stated); isoproterenol (10 μM); RP-cAMPs (10 μM); 6-Chloro-PB hydrobromide (500 nM). The drug used from Molecular Probes was: BAPTA-AM (10 μM). The drugs used from Alomone Labs (Jerusalem, Israel) were: recombinant human neurotrophin-3 and neurotrophin-4/5 (NT-3 and NT-4/5, 100 ng/mL), and recombinant human Brain-derived neurotrophic factor (BDNF) (100 ng/mL). The drug used from Cayman Chemical (Ann Arbor, MI, USA) was: farnesyl thiosalicylic acid (25 μM). The drugs used from Ascent Scientific (Princeton, NJ, USA) were: yohimbine (10 μM) and prazosin (5 μM). M119 (5 μM) was a gift of Dr. Kirill Martemyanov (University of Minnesota).

Immunocytochemistry

Immunocytochemistry protocols followed those described previously (Mermelstein *et al.* 2001; Boulware *et al.* 2007). Briefly, cultured striatal neurons [9–10 days *in vitro* (d.i.v.)] were incubated in a Tyrode's solution containing tetrodotoxin (1 μM) and D(−)-2-amino-5-phosphonopentanoic acid (25 μM) at 24°C for 1.5–2.0 h. Unless stated otherwise, cell stimulations (and drug exposure durations before fixation) were performed as follows: vehicle (10 min); all agonists/activators, such as NE and isoproterenol (10 min); 20 mM K+ (5 min). All inhibitors/antagonists were applied 30 min prior to stimulation and then concurrently with stimulation, except for melittin which was applied 15 min prior to stimulation, and pertussis toxin, which was applied 24 h prior to stimulation (Glass and Felder 1997; Boulware *et al.* 2005). Cells were fixed for 20 min after stimulation using ice-cold 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA, USA) in phosphate-buffered saline (PBS) containing 4 mM EGTA. After three PBS washes, permeabilization of cells was achieved by a 5-min incubation in a 0.1% Triton X-100 (VWR Scientific, West Chester, PA, USA) solution. After three more washes, cells were blocked at 37°C for 30 min in PBS containing 1% bovine serum albumin and 2% goat serum (Jackson Immuno-Research, West Grove, PA, USA). The cells were then incubated at 37°C for 1 h in block solution containing a monoclonal antibody directed against serine 133 phosphorylated CREB (pCREB; 1:500; Upstate Biotechnology, Lake Placid, NY, USA), and to identify

individual cell morphology, a polyclonal antibody targeting microtubule-associated protein 2 (MAP2; 1:500; Calbiochem). Cells were then washed three times and incubated for 1 h at 37°C in block solution containing FITC- and CY5-conjugated secondary antibodies for visualization of MAP2 and pCREB, respectively (Jackson ImmunoResearch). After washing off excess secondary antibody, cells were mounted using the antiquenching and mounting medium Citifluor (Ted Pella, Redding, CA, USA). Nuclear fluorescent intensities for pCREB ($n \geq 30$ cells per group) were acquired using a Leica DM5500Q confocal system. Data acquired from the Yokogawa system were quantified using MetaMorph software (version 6.0; Universal Imaging, Downington, PA, USA). Data acquired from the Leica system were quantified with Leica LAS AF (version 1.9.0; Leica, Deerfield, IL, USA).

Following established protocols, the confocal excitation and detection settings (i.e., laser intensity, image acquisition time, etc.) for each experiment were determined using coverslips stimulated with 20 mM K^+. Inter-coverslip variability was accounted for by subjecting two coverslips to each treatment. For image acquisition, at least 30 neurons were selected randomly across both coverslips using MAP2 fluorescence, allowing the experimenter to remain blind to pCREB intensities. Data were acquired from coverslips in a random order. Images were captured through the approximate midline of each cell. During data analysis, the MAP2 staining was used to draw a region of interest outlining the nucleus of each neuron, allowing the experimenter to remain blind to pCREB intensity. The region of interest was then transferred to the pCREB image, and average fluorescence intensities within the nucleus were recorded. For all images, background from a region of the image that did not contain pCREB fluorescence was subtracted from the average pCREB fluorescence intensity. Each experiment was performed at least three times to verify results.

cAMP assay

We measured cAMP concentrations in cultured striatal neurons (9–10 d.i.v.) using a Parameter cAMP kit (R&D Systems, Minneapolis, MN, USA). Cell stimulations were as described before.

Luciferase-based gene reporter assays

Cultured neurons were transfected on 8 d.i.v. with a luciferase-based reporter (1 μg of DNA per coverslip) of CRE-dependent transcription using a calcium phosphate-based method (Deisseroth *et al.* 1998) or Optifect (Invitrogen). Once transfected, cells were incubated in serum-free Dulbecco's modified Eagle's medium (Invitrogen) with 1% B-27. On 10 d.i.v., cells were stimulated for 4 h, lysed, and then assayed for luciferase expression using standard protocols and a luminometer (Monolight 3010; PharMingen, San Diego, CA, USA). Each treatment group within a single experiment was comprised of at least eight coverslips, and all experiments were replicated at least three times.

PCR

Quantitative PCR (qPCR) was performed using standard protocols (Mermelstein *et al.* 2000; Boulware *et al.* 2007). mRNA was extracted and reverse transcribed from cultured striatal neurons or the striatum of adult rats using a standard kit (RNAeasy Mini kit; QauntiTect kit; Qiagen, Valencia, CA, USA). In select experiments, cultures were exposed to NE, isoproterenol (ISO), or vehicle in MEM (Invitrogen) for 1 h prior to mRNA extraction. Striatal tissue was stored in RNAlater (Qiagen). qPCR amplification was performed using QuantiFast SYBR Green PCR master mix (Qiagen). All qPCR was performed and analyzed using an Opticon 2 (Bio-Rad, Hercules, CA, USA) and standardized to the ribosome-related genes *s15* and *rpl13a*. The critical cycle threshold was set at 25 SDs above baseline. PCR for individual cDNA samples was performed in triplicate, and overall experiments were repeated at least three times. The thermal cycling program used with QuantiFast SYBR was: an initial denaturing step at 95°C for 6 min, followed by at least 30 cycles consisting of a 10 s denaturing step at 95°C, annealing/extension step for 30 s at 60°C, and a measurement of fluorescent intensity. At the end of each cycling program, a melting curve was run. PCR products were sequenced for verification of product identity.

The primer sequences used were as follows. The upper and lower sequences for *adrb1* (GenBank accession number: NM_012701) were: 5′-ACCCCAAGTGCTGCGATTTCGT-3′ and 5′-GCTCGCAGCTGTCGATCTTCTT-3′. The primer sequences for *adrb2* (GenBank accession number: NM_012492.2) were 5′-TTCTGTGCCTTCGCCGGTCTTCTT-3′ and 5′-ATGCCAGGGGCTTCCTCACAAA-3′. The primer sequences for *adrb3* (GenBank accession number: NM_013108.1) were 5′-AACTCTGCCTTCAACCCGCTCA-3′ and 5′-TGAGTTCTGCCTGGACGCAACA-3′. The primer sequences for *c-fos* (GenBank accession number: NM_022197.2) were 5′-TGCCAGATGTGGACCTGTCTGGTT-3′ and 5′-TATAGGTAGTGCAGCTGGGAGTGC-3′. The primer sequences for *rpl13a* (GenBank accession number: NM_173340) were 5′-TGCTGCCGCACAAGACCAAA-3′ and 5′-AACTTTCTGGTAGGCTTCAGCCGC-3′. The primer sequences for *s15* (GenBank accession number: BC094409) were 5′-CCGAAGTGGAGCAGAAGAAG-3′ and 5′-CTCCACCTGGTTGAAGGTC-3′ (Groth *et al.* 2007).

Statistics

Experiments were analyzed using ANOVAS and Tukey's multiple comparison *post hoc* tests, Student's *t* test, or non-linear curve fits using Prism 4.03 (GraphPad Software, La Jolla, CA, USA). Statistical differences between all treatment groups are depicted within each figure as different alphabetical characters. Probability values < 0.05 were considered *a priori* as significant. Data are presented as mean ± SEM.

Results

NE induces CREB phosphorylation in striatal neurons

Our initial experiments were designed to test the hypothesis that NE would rapidly induce CREB phosphorylation in cultured striatal neurons. A 10-min exposure of striatal neurons to 25 μM NE increased CREB phosphorylation (Fig. 1a and b). NE stimulation triggered CREB phosphorylation in a dose-dependent manner, at concentrations consistent with activation of adrenergic receptors (Fig. 1c). Since 25 μM NE was maximally effective in eliciting CREB phosphorylation, this concentration was used for the remainder of the studies. NE-induced CREB phosphorylation in striatal neurons occurred rapidly, within 5 min of NE administration, with maximal responses observed after a 10 min stimulus (Fig. 1d). In addition, CREB remained

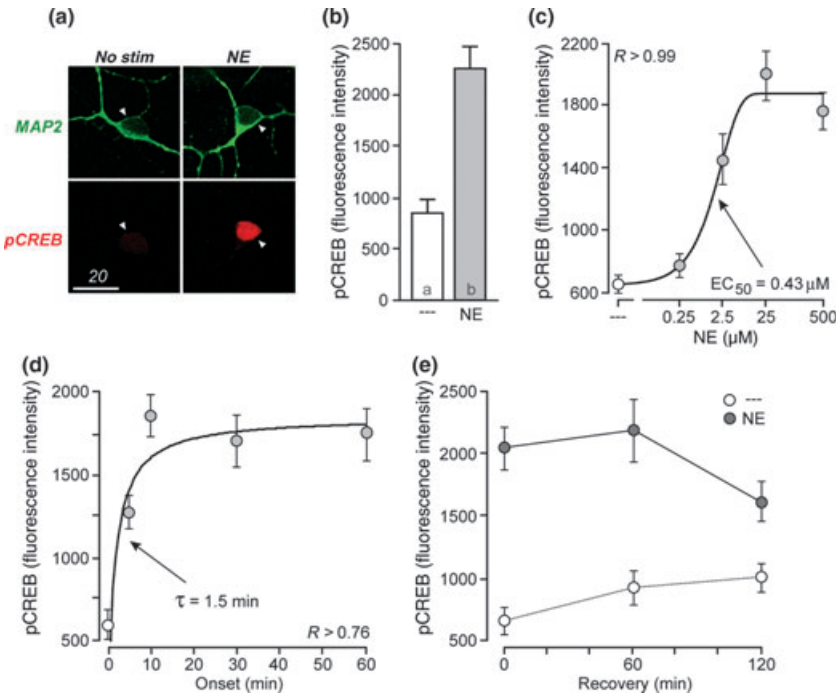

Fig. 1 Norepinephrine (NE) induces cAMP response element binding protein (CREB) phosphorylation in striatal neurons. (a) Example confocal images of cultured striatal neurons immunolabeled with microtubule-associated protein 2 (MAP2; green) and phosphorylated CREB (pCREB; red). Striatal neurons exhibited heightened nuclear staining for pCREB after a 10-min application of norepinephrine (NE; 25 μM). No Stim (i.e. no stimulation) neurons were treated with vehicle only. Scale bar, 20 μM. (b) Quantification of the immunolabeling demonstrated that NE significantly increased CREB phosphorylation ($t = 5.489$). Letters within each bar indicate statistically different groups. (c) NE increased CREB phosphorylation in a concentration-dependent manner, with an EC_{50} = 0.43 μM. (d) Time course of the onset of NE-induced CREB phosphorylation. (e) Time course of the recovery of CREB phosphorylation following a 10-min exposure to 25 μM NE.

phosphorylated within striatal neurons for at least 120 min after a 10-min exposure to NE (Fig. 1e), providing ample time in which CREB-dependent changes in gene expression could occur (see below).

β1-adrenergic receptor activation mediates NE-induced CREB phosphorylation

Both α- and β-adrenergic receptors are present in the striatum and nucleus accumbens. α-Adrenergic receptors are primarily found on pre-synaptic terminals (Rommelfanger *et al.* 2009), whereas β-adrenergic receptors are primarily found on post-synaptic membranes and cell bodies (Pisani *et al.* 2003; Paschalis *et al.* 2009; Hara *et al.* 2010). Because β-adrenergic receptors are found on the post-synaptic membrane and cell bodies, we first tested whether these receptors mediate NE-induced CREB phosphorylation. Using RT-PCR, we found that β1, β2, and β3-adrenergic receptor mRNA is expressed in both cultured striatal neurons (Fig. 2a) and adult striatal tissue (data not shown). We found that exposure to 30 μM propanolol, a panspecific β-adrenergic receptor antagonist, blocked NE-induced CREB phosphorylation (Fig. 2b). Because striatal neurons express all three β-adrenergic receptor subtypes, we then exposed neurons to 10 μM betaxolol, a β1-adrenergic receptor antagonist, and found that this also blocked NE-induced CREB phosphorylation (Fig. 2b). Exposure to 10 μM ISO, a β1 and β2-adrenergic receptor agonist, mimicked the effect of NE (Fig. 2c). The effects of ISO and NE were not additive (Fig. 2c). ISO stimulated CREB phosphorylation in a dose-dependent manner, with 10 μM ISO being maximally effective in eliciting CREB phosphorylation. This concentration was used for the remainder of the study. The magnitude of ISO-induced CREB phosphorylation was comparable with that induced by the DA D1 receptor agonist 6-Chloro-PB (6-C; Fig. 2d), and the effects of ISO and 6-C were not additive (Fig. 2d). The effects of ISO were also blocked by betaxolol (Fig. 2e) and propanolol (Fig. 2f), and did not differ between dorsal striatum and nucleus accumbens enriched cultures (data not shown). To test the specificity of ISO stimulation of β-adrenergic receptors, we exposed striatal neurons to yohimbine, an α2-adrenergic receptor antagonist, and prazosin, an α1 and α2B-adrenegic receptor and melatonin MT3 receptor antagonist. Neither yohimbine (Table 1) nor prazosin (Table 1) blocked the effects of ISO, indicating that the effect of ISO is not mediated via α-adrenergic receptors. Of note, the remainder of the studies used ISO because of its specificity in activating β-adrenergic receptors.

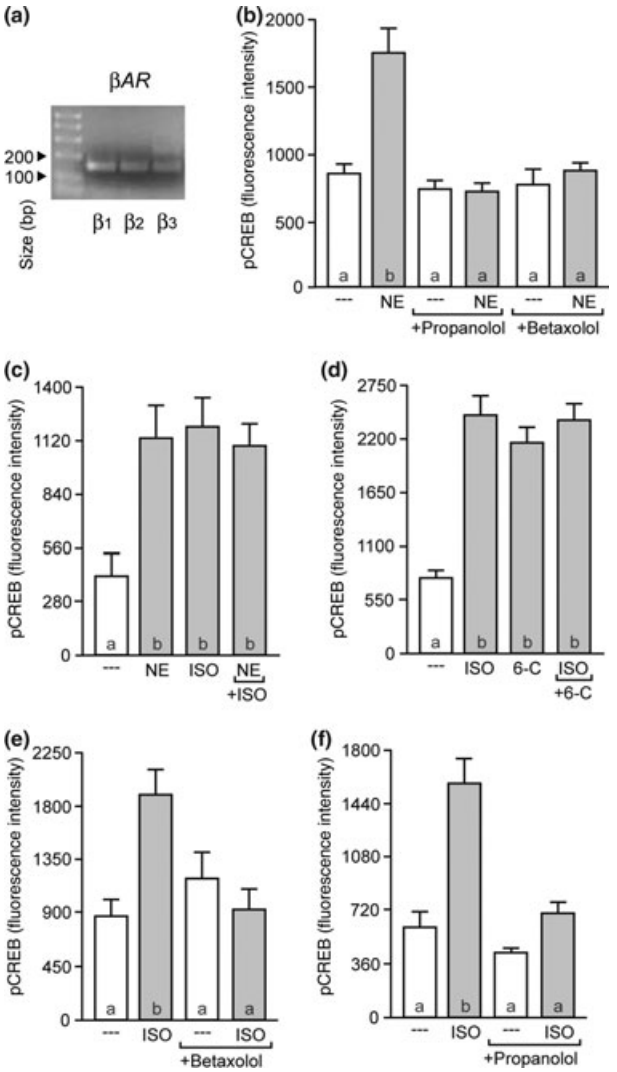

Fig. 2 β1-Adrenergic receptors mediate norepinephrine (NE)-induced cAMP response element binding protein (CREB) phosphorylation. (a) RT-PCR detection of β1-, β2-, and β3-adrenergic receptor mRNA in striatal cultures. Products were verified by sequencing. (b) The effects of NE were blocked after inhibition of β-adrenergic receptors with the panspecific β-adrenergic receptor antagonist propanolol or the β1-adrenergic receptor specific antagonist betaxolol ($F = 16.50$). (c) The β1- and β2-adrenergic receptor agonist isoproterenol (ISO) mimics NE action, and the effects of ISO and NE are not additive ($F = 4.337$). (d) The effects of ISO and the D1 dopamine receptor agonist 6-chloro-PB (6-C) were also comparable and not additive ($F = 18.19$). (e) Betaxolol blocks the effects of ISO ($F = 6.87$). (f) Propanolol blocks the effects of ISO ($F = 13.69$).

Gαs/olf is implicated in β1-adrenergic receptor signaling

The next experiments were designed to elucidate the G-protein responsible for β1-adrenergic receptor-induced CREB phosphorylation. Similar to the D1 DA receptor and adenosine A2A receptor (Herve *et al.* 1993, 2001; Kull *et al.* 2000), striatal β1-adrenergic receptors are usually described as activating the Gs family of G-proteins, including Golf and Gs (Hara *et al.* 2010). To test whether β1-adrenergic receptor-induced CREB phosphorylation is mediated by Gs/olf, we first exposed neurons to melittin (1 μM). Melittin inhibits the Gs family of G-proteins, although it has other actions, including activation of the Gi G-protein family, Phospholipase A2, and possibly Phospholipase C (Fukushima *et al.* 1998; Raghuraman and Chattopadhyay 2007). We also note that the only study to date that has examined the effects of melittin used synaptic membranes, not intact neurons (Fukushima *et al.* 1998). Melittin was found to block ISO-induced CREB phosphorylation (Fig. 3a). As a positive control, we also exposed neurons to melittin and the D1 DA receptor agonist 6-Chloro-PB. As predicted, melittin blocked 6-chloro-PB-induced CREB phosphorylation (Fig. 3b). To eliminate the possibility that ISO-induced CREB phosphorylation was mediated by stimulating a Gi G-protein, we pre-treated neurons with the Gi/o inhibiter pertussis toxin. Pertussis toxin did not block ISO-induced CREB phosphorylation (Table 1). Given that G-protein-coupled receptors can stimulate intracellular signaling pathways via both Gα and Gβγ G-protein subunits, we next tested whether β1-adrenergic receptor-induced CREB phosphorylation was mediated via Gβγ (possibly following dissociation from Gαs/olf). We found that the Gβγ inhibitors gallein (Table 1; 75 μM) and M119 (Table 1; 5 μM) did not affect ISO-induced CREB phosphorylation. These same compounds were effective in blocking the actions of corticotropin releasing factor on CREB phosphorylation (Stern and Mermelstein, unpublished data), although we note that M119 and gallein individually do not interfere with all Gβγ pathways. Future studies will

Table 1 Drugs that did not block isoproterenol (ISO)-induced CREB phosphorylation

Drug	Action
Yohimbine	α2-adrenergic receptor antagonist
Prazosin	α1 and α2B-adrenegic receptor antagonist, melatonin MT3 receptor antagonist
Pertussis Toxin	Gi/o G-protein inhibitor
Gallein	Gβγ G-protein subunit inhibitor
M119	Gβγ G-protein subunit inhibitor
KT5720	PKA inhibitor
Rp-cAMPs	PKA inhibitor
PKI 14-22 amide	PKA inhibitor
BAPTA-AM	Cell-permeable calcium chelator
Thapsigargin	Depletes intracellular calcium stores
Calcium-free media	Removes external calcium
PP1	Src kinase family inhibitor
SL0101-1	RSK inhibitor

None of the drugs listed in this table blocked ISO-induced cAMP response element binding protein (CREB) phosphorylation.

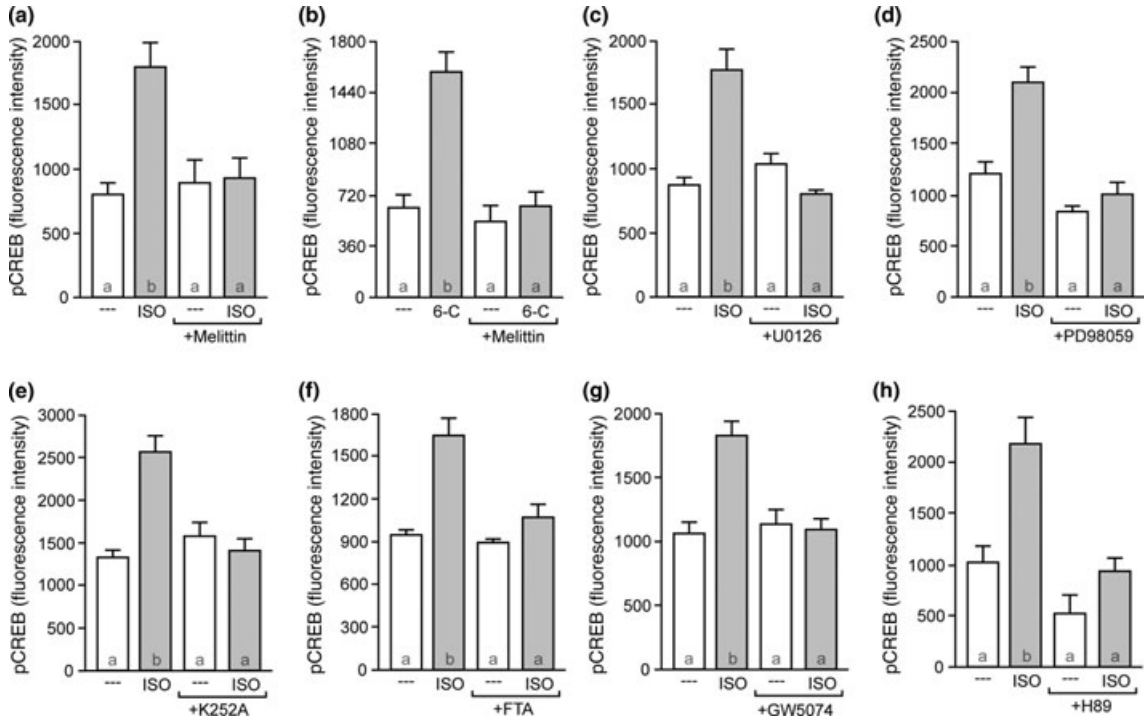

Fig. 3 Isoproterenol (ISO)-mediated cAMP response element binding protein (CREB) phosphorylation via Gαs/olf, MEK, TRK, RAS, RAF, MSK signaling. (a) Melittin, a Gαs family antagonist and Gαi/o agonist, blocked ISO-induced CREB phosphorylation ($F = 8.096$). The Gαi/o inhibitor pertussis toxin did not block ISO-induced CREB phosphorylaion. (b) As a positive control, we exposed neurons to melittin and the D1 receptor agonist 6-Chloro-PB. Melittin also blocked CREB phosphorylation induced by 6-chloro-PB ($F = 14.28$). (c) The effect of ISO was eliminated after inhibition of MEK with U0126 ($F = 18.34$). (d) The effect of ISO was eliminated after inhibition of MEK with PD98059 ($F = 16.11$). ISO-induced CREB phosphorylation was not blocked by the PKA inhibitors KT5720, Rp-cAMPs, or PKI 14-22 amide. (e) The receptor tyrosine kinase inhibitor K252A also blocked ISO-mediated CREB phosphorylation ($F = 15.09$). (f) The Ras inhibitor farnesylthiosalicylic acid blocked CREB phosphorylation ($F = 17.57$). (g) The effect of ISO was also blocked after inhibition of Raf with GW5074 ($F = 9.671$). (h) The MSK inhibitor H89 blocked CREB phosphorylation ($F = 11.73$), while the RSK inhibitor SL0101-1 did not.

need to combine both genomic and biochemical experiments to further identity the exact G-proteins involved.

MEK and TRK inhibitors block CREB phosphorylation

At this point in our research, we expected β1-adrenergic receptors to stimulate CREB phosphorylation through the canonical adenylyl cyclase (AC)/cAMP/Protein kinase A (PKA) pathway (Lands *et al.* 1967; Ursino *et al.* 2009). We were therefore surprised to find that specific PKA blockers did not block ISO-induced CREB phosphorylation. We used three separate PKA inhibitors: KT5720 (Table 1; 3 μM), RP-cAMPs (Table 1; 10 μM), and protein kinase inhibitor 14-22 amide (Table 1; 1 μM). Instead, CREB phosphorylation was blocked by inhibitors of MEK, including U0126 (Fig. 3c; 10 μM) and PD98059 (Fig. 3d; 25 μM). Evidently, a signaling pathway distinct from the canonical pathway was responsible for β1-adrenergic receptor-mediated CREB phosphorylation.

β1-Adrenergic receptors could activate MEK signaling via several different routes, including transactivation of a RTK (Lowes *et al.* 2002). Indeed, we found that the RTK inhibitor K252a (100 nM) blocked ISO-mediated CREB phosphorylation (Fig. 3e). To determine whether activation of the RTK by ISO was mediated through the release of NTs, we examined the time course of NT-induced CREB phosphorylation. We reasoned that if NT release was downstream of β1-adrenergic receptor activation, the time course of NT-mediated CREB phosphorylation would be at least as fast as ISO-induced CREB phosphorylation. To test this hypothesis, we utilized BDNF, known to elicit CREB phosphorylation via TrkB activation (Finkbeiner *et al.* 1997; Arthur *et al.* 2004). We found that ISO-induced CREB phosphorylation occurs more rapidly than BDNF-induced CREB phosphorylation. As mentioned previously, ISO-mediated CREB phosphorylation occurs within 5 min of drug administration. In comparison, BDNF did not produce a significant increase in CREB phosphorylation under these conditions (pCREB fluorescence intensity: vehicle: 857 ± 107; ISO: 1908 ± 166, $p < 0.05$ vs. vehicle and BDNF; BDNF: 1134 ± 140; $F = 14.87$; BDNF concentration: 100 ng/mL). BDNF-mediated CREB phosphorylation was first observed 15 min following NT administration. As an additional test,

we found the effects of NT-3 (100 ng/mL) and NT-4 (100 ng/mL) to parallel those of BDNF (data not shown). While there are several possibilities to account for the slower time course of NT-mediated CREB phosphorylation, the data suggest to us that at least several RTKs responsible for signaling to CREB (including those activated by β1-adrenergic receptors) are not on the extracellular surface (Rajagopal *et al.* 2004). However, the definitive testing of this hypothesis requires further study (see Discussion).

Calcium or src kinase family inhibitors do not block CREB phosphorylation

Both DA (Iwakura *et al.* 2008) and adenosine (Lee and Chao 2001; Assaife-Lopes *et al.* 2010) receptors can transactivate RTKs in neurons via a calcium and/or src kinase-dependent mechanism that is independent of NT binding. We next tested whether this same mechanism also underlies adrenergic receptor-mediated signaling to CREB. We find that blocking calcium action with the cell-permeable calcium chelator BAPTA-AM (Table 1; 10 μM), by depleting intracellular stores with thapsigargin (Table 1; 1 μM), or with incubation in calcium-free media (Table 1) did not affect ISO-mediated CREB phosphorylation. We also find that the src kinase family inhibitor 1-(1,1-Dimethylethyl)-1-(4-methylphenyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (Table 1; 5 μM) did not block CREB phosphorylation.

Ras, Raf, and MSK are necessary for CREB phosphorylation

In our next series of experiments, we determined what signaling molecules lie between the RTK and MEK. RTKs typically activate the MEK pathway via the Ras/Raf signal transduction cascade. Consistent with this hypothesis, the Ras inhibitor farnesyl thiosalicylic acid blocked ISO-mediated CREB phosphorylation (Fig. 3f; 25 μM). The same was true for the Raf inhibitor, GW5074 (Fig. 3g; 10 μM).

CREB phosphorylation because of activation of MEK/MAPK signaling has been intensely studied (Carlezon *et al.* 2005). MAPK signaling ultimately leads to activation of either 40S ribosomal protein S6 kinase and/or MSK, two kinases believed to directly phosphorylate CREB. To determine whether either of these kinases mediated β1-adrenergic receptor-induced CREB phosphorylation, cultures were treated with inhibitors of either 40S ribosomal protein S6 kinase (SL0101-1; 10 μM) or MSK (H89; 5 μM). SL0101-1 did not affect ISO-mediated CREB phosphorylation (Table 1), whereas H89 did block the actions of ISO (Fig. 3h). At the 5 μM concentration used, H89 also inhibits PKA, creating a potential confound. However, since three more specific PKA inhibitors failed to block CREB phosphorylation (Table 1), along with the knowledge that MAPK signaling often leads to activation of MSK, we attribute the effect of H89 to inhibition of MSK, and not PKA.

β1-Adrenergic receptor activation induces cAMP production

While the preceding experiments indicate that NE induces rapid CREB phosphorylation via a novel signaling pathway, the question remained whether we could also observe activation of the canonical β-adrenergic receptor/Gαs/AC/cAMP pathway. We tested this by measuring cAMP concentrations following β1-adrenergic receptor activation. ISO was found to increase cAMP concentrations in our cultured striatal neurons, an effect that was blocked by betaxolol and propanolol (Fig. 4a, and data not shown). ISO-mediated increases in cAMP concentrations were also blocked by the AC inhibitor SQ22536 (Fig. 4a; 90 μM). However, SQ22536 did not affect CREB phosphorylation (Fig. 4b), indicating that cAMP accumulation was not functionally linked to rapid CREB signaling. Previous reports have indicated that global activation of AC in striatal neurons using forskolin will in fact produce CREB phosphorylation (Liu and Graybiel 1996). Consistent with these studies, we found that a 10-min exposure of our striatal

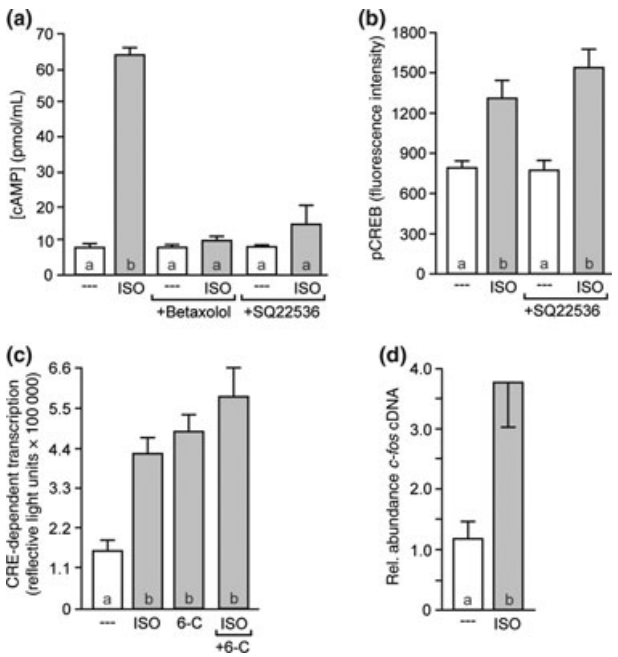

Fig. 4 β1-Adrenergic receptor activation induces cAMP formation, although cAMP signaling is not functionally linked to rapid cAMP response element binding protein (CREB) phosphorylation. (a) Isoproterenol (ISO)-induced cAMP formation is blocked by the β1-adrenergic receptor antagonist betaxolol and the adenylyl cyclase inhibitor SQ22536 ($F = 117.7$). (b) ISO-induced CREB phosphorylation is not affected by pre-treatment with SQ22536 ($F = 14.40$). (c and d) Activation of β1-adrenergic receptors stimulate CRE-dependent transcription and c-fos gene transcription. (c) Application of ISO increased CRE-dependent transcription, as did 6-C. The effects of ISO and 6-C were not additive ($F = 12.72$). (d) Exposure to ISO produced an approximate 4-fold increase in *c-fos* expression ($t = 3.33$).

neurons to 25 μM forskolin also induced CREB phosphorylation (data not shown), indicating consistent findings across laboratories. These data suggest that while β1-adrenergic receptors activate AC, either the pool of enzymes activated by these receptors is not functionally linked to rapid CREB phosphorylation, or that a cAMP threshold is not achieved through direct stimulation with ISO.

As an additional test that cAMP accumulation following ISO administration is not functionally linked to rapid CREB signaling, we activated Epac, a cAMP-sensitive guanine nucleotide-exchange factor that is known to link the cAMP and MEK signaling pathways. Exposure to the Epac activator, 8CPT-2Me-cAMP (50 μM) did not affect CREB phosphorylation (data not shown).

β1-Adrenergic receptors stimulate CRE-dependent transcription and *c-fos* expression

Because phosphorylation of CREB on serine 133 is necessary but not sufficient for activation of CRE-dependent transcription, we applied isoproterenol to cultured striatal neurons transfected with a luciferase-based CRE reporter construct in order to monitor changes in CRE-dependent transcription. We found that isoproterenol increased CRE-dependent transcription (Fig. 4c), as did the D1 DA agonist 6-Chloro-PB. The effects of isoproterenol and 6-Chloro-PB were not additive. Similar results were also observed with NE stimulation (data not shown).

We then tested whether activation of β1-adrenergic receptors induces changes in mRNA expression by exposing cultured striatal neurons to isoproterenol and then measuring changes in *c-fos* cDNA using qPCR. The *c-fos* gene was chosen because it is a known target of CREB that plays a significant role in striatal plasticity (Konradi *et al.* 1994). Administration of ISO produced an approximate fourfold increase in the abundance of *c-fos* (Fig. 4d), indicating that activation of β1-adrenergic receptors drives changes in gene expression in striatal neurons.

Discussion

This study found that NE acts on striatal neurons to stimulate both novel and canonical signaling pathways that induce rapid CREB phosphorylation, affect gene expression, and initiate cAMP production. While both rapid CREB phosphorylation and cAMP accumulation are induced by activating β1-adrenergic receptors, the signaling pathways diverge to induce cAMP accumulation through canonical signaling, and rapid CREB phosphorylation via a RTK/Ras/Raf/MEK/MAPK/MSK pathway (Fig. 5). These findings establish that β1-adrenergic receptors can activate multiple signaling pathways in striatal neurons, including those that affect striatal plasticity and function through changes in gene expression. These data provide a potential new mechanism underlying the influence of NE and adrenergic receptors on striatal function.

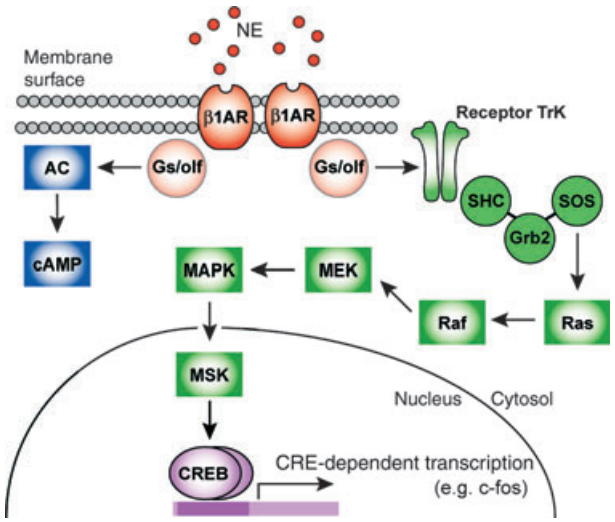

Fig. 5 β1-Adrenergic receptors activate multiple pathways in striatal neurons. β1-adrenergic receptor stimulation induces canonical cAMP production and the activation of a receptor tyrosine kinase. Receptor tyrosine kinase activation leads to the stimulation of the Ras/Raf/MEK/MAPK/MSK signaling pathway, ultimately leading to rapid cAMP response element binding protein (CREB) phosphorylation. We note that receptor tyrosine kinases can be present on both the cellular and internal membranes.

A novel signaling pathway for NE action in the striatum

β-Adrenergic receptors are classically described as stimulating the AC/cAMP/PKA pathway (Lands *et al.* 1967; Ursino *et al.* 2009), with β-adrenergic activation of cAMP in the striatum having been described since the 1970s (Forn *et al.* 1974; Harris 1976, 1978; Daly *et al.* 1981). Here, we describe a new action of NE in the striatum: β1-adrenergic receptors can rapidly induce CREB phosphorylation and downstream gene expression via activation of a RTK. This finding adds to recent research in other systems, which has found that a number of different pathways can be activated by β-adrenergic receptors depending on the specific receptor and cell type (Giembycz and Newton 2006; Hein 2006; Chen *et al.* 2007; Grimm and Brown 2009; Ursino *et al.* 2009).

One interesting aspect of this novel pathway is the link between the β1-adrenergic receptor and RTK signaling. Our working hypothesis is that the β1-adrenergic receptor transactivates RTKs via an intracellular pathway that is independent of NT binding (Lee *et al.* 2002; Lowes *et al.* 2002), perhaps similar to that linking the D1 DA receptor to TrkB receptors in cultured striatal neurons (Iwakura *et al.* 2008) or that linking adenosine receptors to Trk receptors located on Golgi membranes in cultured basal forebrain or hippocampal neurons (Lee and Chao 2001; Rajagopal *et al.* 2004). Indeed, in another system, the β2-adrenergic receptor transactivates Trk via intracellular mechanisms (Maudsley *et al.* 2000). While we favor the intracellular pathway

hypothesis, we acknowledge that other possible mechanisms could link the β1-adrenergic receptor to Trk. One such mechanism is a direct physical coupling of β1-adrenergic receptors to Trk. Another possibility is a β-arrestin-dependent pathway (Reiner *et al.* 2010). Alternatively, β1-adrenergic receptor activation could lead to BDNF or other NT release which then activates a Trk receptor (Chen *et al.* 2007). We do not favor this hypothesized mechanism given the rapidity of ISO-induced CREB phosphorylation compared with that induced by NTs, including BDNF (Finkbeiner *et al.* 1997). Future experiments will more explicitly test these various hypotheses, and attempt to identify the specific Trk. That said, it is difficult to directly test this hypothesis because of a lack of specific inhibitors of RTKs, and knockdown or inhibition of these receptors have pronounced effects on cell viability (Ghosh *et al.* 1994).

NE and DA signaling in the nucleus accumbens and striatum

Although DA is the principal neuromodulator studied in the context of striatal physiology, NE and adrenergic receptors are also present in the nucleus accumbens and striatum. The brainstem noradrenergic cell groups A1 and A2 project to the nucleus accumbens (Berridge *et al.* 1997; Delfs *et al.* 1998; Tong *et al.* 2006), while noradrenergic cell bodies in the locus ceruleus (LC) project to many brain regions, including the striatum (Moore and Bloom 1979). These projections are sparser than those associated with DA release, but NE signaling within the striatum is highly relevant. Baseline striatal NE concentrations are approximately half of that of DA, and as with DA, striatal NE concentrations are significantly elevated following injection of psychostimulants, as measured using *in vivo* microdialysis techniques (Li *et al.* 1998; McKittrick and Abercrombie 2007). In parallel, NE neurotransmission is disrupted in Parkinson's disease (PD; Rommelfanger and Weinshenker 2007). Dopamine has also been shown to activate α- and β-adrenergic receptors (Malenka and Nicoll 1986; Cornil and Ball 2008). As such, it should not be surprising that α- and β-adrenergic receptors are abundantly present in the striatum, on both medium spiny projection neurons and cholinergic interneurons (Nicholas *et al.* 1993; Pisani *et al.* 2003; Paschalis *et al.* 2009; Rommelfanger *et al.* 2009). The extent to which β1-adrenergic receptors co-localize with D1 and D2 DA receptor expressing neurons is unknown, although the non-additive effects of ISO and 6-Chloro-PB (Fig. 2d) suggest that β1-adrenergic receptors at least co-localize with D1 receptors.

NE and striatal pathologies

Norepinephrine has long been studied in the context of drug addiction and PD. NE was in fact the first candidate for the essential 'reward transmitter', but then fell out of favor in the late 1970s. The importance of NE has since re-emerged following the development of more sophisticated models of drug addiction (Weinshenker and Schroeder 2007; Aston-Jones and Kalivas 2008; Sofuoglu and Sewell 2009). Exposure to many drugs of abuse enhance NE neurotransmission throughout the nervous system, including within the striatum (Li *et al.* 1998; McKittrick and Abercrombie 2007). For instance, NE signaling is required for the full amphetamine-induced increase in locomotor activity, as well as maximal behavioral sensitization following repeated drug exposure (Kostowski *et al.* 1982; Archer *et al.* 1986; Mohammed *et al.* 1986; Harris *et al.* 1996; Weinshenker *et al.* 2002; Vanderschuren *et al.* 2003). Furthermore, experiments in DA transporter knockout mice find substantial DA-independent amphetamine-induced locomotion (Sotnikova *et al.* 2005). Later in the addiction cycle, NE signaling is known to affect drug relapse (Davis *et al.* 1975; Weinshenker and Schroeder 2007; Smith and Aston-Jones 2008; Sofuoglu and Sewell 2009). These and other studies in animal models have lead to clinical studies involving drugs that manipulate various aspects of noradrenergic neurotransmission (Szerman *et al.* 2005; Sofuoglu and Sewell 2009).

Norepinephrine has similarly been implicated in PD, as NE-producing neurons in the LC die alongside the DArgic neurons of the substantia nigra pars compacta (Mann and Yates 1983; Mann *et al.* 1983; Rommelfanger and Weinshenker 2007). In non-human primates and other animal models, the symptoms of PD following DA depletion are exacerbated by lesions of the LC (Mavridis *et al.* 1991; Marien *et al.* 1993; Fornai *et al.* 1997; Srinivasan and Schmidt 2003; Rommelfanger *et al.* 2007). It has been suggested that NE plays a neuroprotective role essential for the maintenance of striatal control of motor function, with the activity of surviving LC neurons perhaps compensating for depleted DA concentrations (Marien *et al.* 2004; Rommelfanger and Weinshenker 2007).

Conclusion

Over 30 years ago, NE exposure was found to induce cAMP production in striatal tissue. Since that time, few studies have examined NE and β-adrenergic receptor-mediated signaling in this brain region. Juxtaposed to the paucity of research on this topic, many studies have demonstrated the overall importance of NE neurotransmission in both normal striatal function and the phenotypes of striatal-mediated pathologies. Given this, it is important to understand how NE and adrenergic receptors signals in striatal neurons. In a broader context, that β1-adrenergic receptors trigger multiple signaling pathways may be relevant to other brain regions, given that NE is found across the brain and has been implicated in a host of basic processes and pathologies, including but not limited to sleep (Mitchell and Weinshenker 2009), post-traumatic stress disorder (Krystal and Neumeister 2009) and Alzheimer's disease (Weinshenker 2008).

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