AKT and CDK5/p35 Mediate Brain-derived Neurotrophic Factor Induction of DARPP-32 in Medium Size Spiny Neurons in Vitro*

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Mature striatal medium size spiny neurons express the dopamine and cyclic AMP-regulated phosphoprotein, 32 kDa (DARPP-32), but little is known about the mechanisms regulating its levels or the specification of fully differentiated neuronal subtypes. Cell extrinsic molecules that increase DARPP-32 mRNA and/or protein levels include brain-derived neurotrophic factor (BDNF), retinoic acid, and estrogen. DARPP-32 induction by BDNF in vitro requires phosphatidylinositol 3-kinase (PI3K), but inhibition of phosphorylation of protein kinase B/Akt does not entirely abolish expression of DARPP-32. Moreover, the requirement for Akt has not been established. Using pharmacologic inhibitors of PI3K, Akt, and cyclin-dependent kinase 5 (cdk5) and constitutively active and dominant negative PI3K, Akt, cdk5, and p35 viruses in cultured striatal neurons, we measured BDNF-induced levels of DARPP-32 protein and/or mRNA. We demonstrated that both the PI3K/Akt/mammalian target of rapamycin and the cdk5/p35 signal transduction pathways contribute to the induction of DARPP-32 protein levels by BDNF and that the effects are on both the transcriptional and translational levels. It also appears that PI3K is upstream of cdk5/p35, and its activation can lead to an increase in p35 protein levels. These data support the presence of multiple signal transduction pathways mediating expression of DARPP-32 in vitro, including a novel, important pathway via by which PI3K regulates the contribution of cdk5/p35.

The medium size spiny neuron (MSN) is the primary projection neuron of the striatum (caudate and nucleus accumbens) and accounts for over 95% of the striatal neurons (1). All MSNs receive glutamatergic input from the cortex. In addition, they receive dopamine input from the substantia nigra or ventral tegmental area and are therefore dopaminergic, usually expressing only a single dopamine receptor subtype (2, 3). Cell-specific MSN gene transcription is altered during the pathogenesis and treatment of many neuropsychiatric diseases, including Huntington disease, Parkinson disease, drug addiction, affective disorders, attention deficit hyperactivity disorder, and schizophrenia (4–7). Despite the obvious clinical significance of gene regulation in MSNs and the requirement for cell-specific transcription in the development of the nervous system, few details are available regarding how the MSN phenotype is specified at the molecular level. Indeed, little is known about the molecular mechanisms underlying the specification of most central nervous system neuronal subtypes. Delineation of these pathways presents a major challenge to current molecular neurobiology.

DARPP-32 (dopamine and cyclic AMP-regulated phosphoprotein, 32 kDa) is a homologue of protein phosphatase inhibitor-1 that plays a key role in integrating incoming first messenger signals. A host of transmitters and neuropeptides converges upon DARPP-32 and its state of phosphorylation, and in turn, levels of phospho-DARPP-32 provide moment-to-moment control of the signaling "tone" of over a dozen known protein phosphorylation pathways (8, 9). DARPP-32 is one of the best known MSN-enriched molecules, and it is the most commonly used marker of this neuronal subtype in developmental and adult studies (6, 10–12). Although dopamine is one signal controlling the phosphorylation state of DARPP-32, its expression is independent of dopamine input (13, 14). Dopamine, brain-derived neurotrophic factor (BDNF), retinoic acid (12, 15, 16), and estrogen (17) are extracellular factors known to regulate maturation of aspects of the nigrostriatal system in vivo and in vitro, and the other three factors have been shown to regulate DARPP-32 protein levels either in vitro and/or in vivo (18–22).

The creation of the DARPP-32-null mouse demonstrated the crucial role of this molecule in many aspects of striatal function (8, 14). The potentiation of target protein phosphorylation, mediated by DARPP-32 regulation of protein phosphatase-1, appears to be a required action of the dopaminergic signaling pathway under physiologic conditions. In the absence of DARPP-32, striatal neurons show marked neurophysiological abnormalities in response to dopamine D1 agonists, including...
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EXPERIMENTAL PROCEDURES

Cell Culture—Timed-mated Swiss-Webster mouse dams were anesthetized with pentobarbital (day of plug = E0–0.5), and the embryos were removed. The striatum (E15–17) was isolated, and the meninges were removed. The tissue was minced with a scalp knife and incubated in Ca\(^{2+}\)/Mg\(^{2+}\)-free Hank’s balanced salt solution for 8 min at 37 °C in a clinical rotator (40 rpm). The incubation mixture was replaced with 0.01% trypsin/Ca\(^{2+}\)/Mg\(^{2+}\)-free Hank’s balanced salt solution, incubated for 8 min, and rinsed twice in Leibovitz’s medium (L-15). It was then suspended in Dulbecco’s minimum essential medium with 10% fetal calf serum, glucose (6 mg/ml), glutamine (1.4 mM), and penicillin/streptomycin (100 units/ml). Cells were triturated through a glass bore pipette and plated onto Lab Tek culture wells (75,000 cells/well) for immunocytochemistry or 12-well plates (2 × 10\(^5\) cells/well) for Western blotting, coated with polymerized polyornithine (0.1 mg/ml in 15 mM borate buffer, pH 8.4), and air-dried. One hour later, the media were replaced with Neurobasal/B27 with additives, with glutamine and penicillin/streptomycin, as above. In the absence of anti-mitotic agents, these cultures contain 90–95% neurons, almost all of which are γ-aminobutyric acid-positive (18). An extra slide was plated each time and stained with enolase and γ-aminobutyric acid to confirm uniformity of the cultures between experiments. Treatments included roscovitine and deguelin (Sigma), butyrolactone (Biomol), and LY294,002, rapamycin, and wortmannin (Calbiochem).

Cell Immunocytochemistry—Cultures were immersion-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and processed using the anti-rabbit secondary antibody and the immunoperoxidase/ABC method (Elite Vectastain, Vector Laboratories, Burlingame, CA). Neuronal purity was assessed by staining parallel cultures with neuron-specific enolase (1:5000; Polysciences). The polyclonal anti-DARP-32 antibody (Chemicon, Temecula, CA) was used at 1:2000 dilution.

Western Blot Analysis—For analysis of phosphoproteins and as a control their respective nonphosphorylated isoforms, total cellular protein was prepared by lysis in boiling sample buffer (20% glycerol, 62.5 mM Tris-HCl, pH 6.9, 1% SDS, 5% β-mercaptoethanol, and 0.025% bromphenol blue), followed by sonication, 15 min of centrifugation, and recovery of the supernatants. An equal volume of lysate was loaded in each well on a gel, run on 10% SDS-polyacrylamide gels, and protein content verified by visualization of total protein by Ponceau Red after transfer to nitrocellulose and/or by demonstration of an equal amount of a “marker” protein, e.g. Akt or actin, in each lane. For analysis of holoproteins, nonphosphorylated, e.g. DARPP-32, cells and tissues were harvested in 137 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1% v/v Nonidet P-40, and 10% v/v glycerol, supplemented with 1 × Mini-Complete protease inhibitors (Roche Diagnostics). Equal amounts of protein were loaded in each lane following measurement of protein concentrations with the BCA assay (Pierce) and verified by visualization of total protein by Ponceau Red after transfer to nitrocellulose and/or by demonstration of an equal amount of a marker protein. Blots were developed with PerkinElmer Life Sciences. Densitometric values are obtained using Biosoft ScanAnalysis for Apple (Biosoft, Inc.).
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Ferguson, MO) and/or by direct UVP Imaging. Effects of treatments were calculated by ANOVA, with Bonferroni post test. Results were considered significant at $p < 0.05$. Each lane contained either 25 or 50 μg from a single well from a 12-well plate, seeded with 2 × 10⁶ neurons at the time of plating, and ultimately yielding 75–100 μg of total protein. Antibodies used included the following: DARPP-32 (1:3000; Chemicon, Temecula, CA); phospho-Ser-473-Akt and Akt (1:1000; Cell Signaling Technology, Beverly, MA); p35 and cdk5 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA); and actin (1:3000; Sigma).

Viral Transduction—Viral infections were performed after cells had attached for 24–48 h. Vector was added in fresh medium, and the medium was changed again 18 h later. BDNF (10 ng/ml) was added when appropriate after another 24–48 h to allow time for expression of proteins from the virus. Cells were harvested or fixed from 24 to 72 h later, i.e. 72–96 h following addition of virus, depending on the experiment. The goal with each vector was to obtain maximal level of infection/expression without toxicity, as measured by the Molecular Probes Live/Dead assay (data not shown), used as per the manufacturer’s instructions. PI3K (constitutively active p110 = caPI3K and dominant negative p85 = dnPI3K) constructs were in herpesvirus (courtesy of Drs. Robert Kalb and Rachel Neve (Yale University, New Haven, CT) (37, 38)). The wild type p35 and dominant negative Cdk5 plasmids (received from Dr. Nancy Ip, Hong Kong University of Science and Technology) (32) and the dominant negative AKT-CAAX (where AA is aliphatic amino acid) construct (39) were transferred into adenoviral expression vectors (ViraPower adenoviral expression system; Invitrogen). DARPP-32 levels were not significantly altered in the presence of control virus (Null virus), and expression of the protein from the virus was confirmed by Western blotting. Multiplicity of infection (m.o.i.) ranged from 2 to 10:1.

Semi quantitative RT-PCR—Total RNA was isolated from 3 × 10⁶ cells with the use of an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol (RNeasy mini handbook). After the final step, mRNA samples were pre-treated with DNase (RNase-free DNase; Promega, Madison, WI) at 37 °C for 30 min with 1 unit of DNase for 1 μg of RNA in 10 μl of 1× buffer. For each cDNA synthesis reaction, 1 μg of total RNA was reverse-transcribed with Cells-to-cDNA™ II kit (Ambion, Austin, TX). The amount of synthesized cDNA, ~50 ng, was then used for simultaneous amplification of DARPP-32 and β-actin in a Gene Amp PCR System 9700 (Applied Biosystems) using Platinum PCR SuperMix (Invitrogen) (n = 3 separate platings). The primers were designed with Primer Express 3.0 software (Applied Biosystems) based on published cDNA sequences (GenBank™ accession numbers NM144828 and NM007393, respectively). Primer sequences were as follows: DARPP-32 upstream (5’-CACCCACCAAAATCGAAGAGA-3’, corresponding to nucleotide residues 540–560); DARPP-32 downstream (5’-CGAAGCTCCCTAATCCTACCTC-3’, corresponding to nucleotide residues 676–655); β-actin upstream (5’-CGATGCGCTGAGCTTTT-3’, corresponding to nucleotide residues 846–864); β-actin downstream (5’-TGATGCCACAGGTTCAC-3’, corresponding to nucleotide residues 904–886). The expected lengths of the RT-PCR products were 137 bp for DARPP-32 and 59 bp for β-actin. Primers for each gene were located in different exons to distinguish from amplification of contaminating genomic DNA. The thermocycle parameters were as follows: 3 min at 94 °C, followed by 20 cycles of 30 s at 94 °C, 15 s at 53 °C, and 1 min at 72 °C, and final incubation for 7 min at 72 °C. Control reactions were performed to verify that no amplification would occur without cDNA. The number of cycles was determined empirically by sampling product between 10 and 40 cycles to select the approximate midpoint of linear amplification for the PCRs (not shown). Eight microliters from each PCR amplification were loaded onto 4% agarose gels, and the intensity of DNA bands was estimated using UVP Biol Image 1 system (UVp, Inc, Upland, CA) and Lab Works Image Acquisition and Analysis software version 4.00. The abundance of DARPP-32 PCR fragments was normalized by intensity of the β-actin PCR fragment. Statistical analysis was performed as for Western blotting.

RESULTS

dnPI3K, caPI3K, and Wortmannin Confirm the Requirement for Activation of PI3K for Induction of DARPP-32 by BDNF—We demonstrated previously in vivo that BDNF is required for normal ontogeny of DARPP-32 and in vitro that BDNF induces the expression of DARPP-32 protein. Furthermore, inhibition of PI3K by 50 μM LY294,002 reduced the induction of DARPP-32 by greater than 50% in cultured striatal neurons in the presence of BDNF, while entirely inhibiting the phosphorylation of Akt(Ser-473) (18, 19, 35). LY294,002 is an inhibitor of PI3K and was used at doses previously used in primary neuronal cultures (35). To confirm that activation of PI3K is required for the increase in DARPP-32 protein following treatment, we repeated these experiments using a second chemical inhibitor of PI3K, wortmannin, and transduction with a dominant negative form of p85, a subunit of PI3K. We confirmed our previous results with LY294,002 and also noted that the “control” lanes versus “LY294,002” had lower levels of DARPP-32 (Fig. 1A).

Transduction with a dnPI3K p85 subunit almost entirely inhibited the induction of DARPP-32 by BDNF and, importantly, also lowered base-line levels below those of control (Fig. 1A). To confirm that wortmannin was inhibiting PI3K in this system, we demonstrated that BDNF-induced phosphorylation of Akt was inhibited in its presence (Fig. 1B). Levels of pAkt could not be quantified as they were essentially undetectable in the presence of wortmannin. As wortmannin is highly labile in tissue culture media (40, 41), we conducted experiments in which fresh wortmannin was added to the cultures every 8 h, and the cells were harvested at 24 h. Under these conditions, the increase in DARPP-32 protein following treatment with BDNF was decreased by an average of 50% in the presence of wortmannin plus BDNF (Fig. 1C), despite the total abolition of phosphorylation of Akt by BDNF.

Finally, transduction with a constitutively active p110 subunit of PI3K was sufficient to increase levels of DARPP-32 protein (Fig. 1D). Quantitation was performed with the addition of 1 μl of virus (m.o.i. = 5). We concluded that PI3K is both nec-
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FIGURE 1. PI3K is necessary and sufficient for induction of DARPP-32 (D32) alone and by BDNF. Medium size spiny neurons were grown in vitro and Western blots analyzed as described under "Experimental Procedures." Panels display representative gels, and results are summarized in the bar graphs. A, select wells were transduced with dn-p85 virus at 1 DIV and/or treated with LY294,002 (25 μM) and/or BDNF (10 ng/ml) at 5 DIV. All cells were harvested at 6 DIV. B, select wells were treated at 1 DIV with wortmannin (50 μM) and 1 h later with BDNF (10 ng/ml). Cells were harvested 30 min after addition of BDNF. Phosphorylation of Akt (Ser-473) (pAkt) was undetectable in most experiments after treatment with wortmannin, so quantitation was not performed. C, select wells were treated at 1 DIV with wortmannin (50 μM), every 8 h for 24 h, and/or BDNF (10 ng/ml). Wells that did not receive wortmannin were treated with identical amounts of media every 8 h. Cells were harvested at 2 DIV. D, selected wells were transduced with ca-p110 virus at 1 DIV. Media were changed at 2 DIV, and cells harvested at 6 DIV. BDNF (10 ng/ml) was added at 5 DIV. Quantitation was performed for 1.0 μl of virus (m.o.i. = 5). In all cases, the amount of DARPP-32 is expressed relative to Control (untreated) = 1, always plated and harvested at the same times as the experimental samples. Data are presented as means ± S.E. (n = 3–6, each representing a separate plating). All statistics were performed with one-way ANOVA followed by Bonferroni post hoc test (*, p < 0.05; ***, p < 0.001). ca = constitutively active; D32 = DARPP-32; dn = dominant negative; wort = wortmannin.

FIGURE 2. Akt is necessary and sufficient for induction of DARPP-32 (D32), alone and by BDNF. Medium size spiny neurons were grown in vitro and Western blots analyzed as described under "Experimental Procedures." Panels display representative gels, and results are summarized in the bar graphs. A, select wells were treated at 1 DIV with deguelin (Deg) (10−7 to 10−4 M) and/or BDNF (10 ng/ml), and cells were harvested at 2 DIV. Blot and quantification were performed at 10−5 M (p = 0.002). B and C, select wells were transduced with dn- or caAkt1 (m.o.i. = 10) at 1 DIV and/or treated with BDNF (10 ng/ml) at 4 DIV. Cells were harvested at 5 DIV. In all cases, the amount of DARPP-32 is expressed relative to Control (untreated) = 1, always plated and harvested at the same times as the experimental samples. Data are presented as means ± S.E. (n = 3–6, each representing a separate plating). All statistics were performed with one-way ANOVA followed by Bonferroni post hoc test (*, p < 0.05; **, p < 0.01). D32 = DARPP-32; dn = dominant negative; ca = constitutively active; end = endogenous.

necessary and sufficient for induction of the DARPP-32 protein, with or without BDNF, in MSNs in vitro. In addition, PI3K appears to be necessary for maintaining base-line levels of DARPP-32 protein in cultures containing B27, but its inhibition does not totally eliminate expression of DARPP-32 and, in several cases, also does not entirely inhibit the increase in DARPP-32 protein in the presence of BDNF. Deguelin, dnAkt, and Rapamycin Prevent Induction of DARPP-32 by BDNF, and caAkt Induces DARPP-32 Protein—

Based on the requirement for PI3K, we sought to determine whether activation of Akt, a well characterized downstream effector of PI3K, is necessary and/or sufficient for induction of DARPP-32 by BDNF. There are three Akt isoforms, Akt1, Akt2, and Akt3, that are 80% homologous (42). It is frequently assumed, but not always proven, that inhibition of one with a dominant negative construct likely inhibits the others. We therefore first treated the cultures with a pan-Akt inhibitor, deguelin (10−9 to 10−7 M) (43) in the presence and absence of BDNF. Of note, although controversial, deguelin appears to inhibit PI3K-dependent and -independent activation of Akt (43). We found that the BDNF-induced increase in DARPP-32 protein was markedly reduced in the presence of deguelin, but interestingly, base-line levels were not significantly altered (Fig. 2A). We also transduced cultures with a dnAkt virus expressing Akt1-CAAX (39). We first found that Akt1-CAAX (m.o.i. = 10) entirely prevented phosphorylation of endogenous Akt by BDNF (data not shown). We also found that it reduced the induction of DARPP-32 protein by BDNF but did not reduce base-line levels of DARPP-32 protein (Fig. 2B). Finally, transduction with a constitutively active Akt1, in which the pleckstrin homology domain has been deleted and an Src myristoylation sequence has been added (44), increased levels of DARPP-32 protein by 3-fold (Fig. 2C). We concluded that Akt1 is necessary for BDNF induction of DARPP-32 protein in
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**FIGURE 3.** mTOR mediates the BDNF induction of DARPP-32 (D32). A and B, rapamycin, an inhibitor of mTOR, inhibits the BDNF-induced increase in DARPP-32 protein. A, cells were harvested for Western blotting. B, medium size spiny neurons were grown in vitro, and select wells were treated with BDNF (10 ng/ml) and or rapamycin (10 nm) at 1 DIV, and all wells were fixed for 30 min in freshly prepared 4% paraformaldehyde at 2 DIV and immunostained with anti-DARPP-32 antibody. C = control (untreated); B = BDNF; R = rapamycin. Note that treatment with rapamycin also decreases the induction in neuronal size mediated by BDNF, although not quantitated in this study. Data are presented as means ± S.E. (n = 3–6, each representing a separate plating). All statistics were performed with one-way ANOVA followed by Bonferroni post hoc test (**, p < 0.01). rap = rapamycin.

**FIGURE 4.** Induction of DARPP-32 (D32) by BDNF occurs at both the transcriptional and translational levels. Medium size spiny neurons were grown in vitro, and select wells were treated with BDNF (10 ng/ml), LY294,002 (LY) (25 μM) (A), DN (dominant negative)-Akt (m.o.i. = 10) (B), and/or rapamycin (10 nm) (C) at 1 DIV, and all wells were harvested for semi-quantitative RT-PCR analysis, as described under “Experimental Procedures.” In all cases, the amount of DARPP-32 is expressed relative to Control (untreated) = 1, always plated and harvested at the same times as the experimental samples. Data presented as means ± S.E. (n = 3–6, each representing a separate plating). All statistics were performed with one-way ANOVA followed by Bonferroni post-hoc test (*, p < 0.05; **, p < 0.01; and ***, p < 0.001). rap = rapamycin.

**vivo** and alone is sufficient to increase DARPP-32 protein levels in MSNs **in vitro**.

To further investigate the role of Akt in the regulation of DARPP-32 protein levels, we treated MSNs **in vitro** with rapamycin (10 nm), an inhibitor of mammalian target of rapamycin (mTOR). mTOR is a major effector of PI3K/Akt, regulating both transcription and translation (45), although its role in the regulation of translation is more prominent. To confirm activation of mTOR, we first showed that p70 S6 kinase, a downstream mediator of mTOR, is phosphorylated in MSNs **in vitro** following treatment with BDNF (data not shown). Treatment with rapamycin resulted in a total inhibition of the BDNF-induced increase in DARPP-32 (Fig. 3A), but base-line levels were maintained to a large degree. It is important to note that BDNF treatment did not increase levels of holo-Akt (e.g. Fig. 2B). Typical neurons are shown in Fig. 3B, visually demonstrating that BDNF increased immunostaining for DARPP-32 (B = BDNF, p35 pathway, because it has been demonstrated to be regulated by neuregulins and members of the neurotrophin family and is implicated in many processes in neuronal and neuromuscular development (32–34). Treatment of cultured medium size spiny neurons with BDNF resulted in an increase in p35 protein (Fig. 5A), particularly when the cultures were treated more than 24 h after plating and plated at higher density, i.e. in those undergoing viral transduction. We have demonstrated previously that DARPP-32 levels **in vitro** are not density-dependent (18). Furthermore, the increase in p35 was prevented by dn-p85 (Fig. 5A), and p35 never increased in the presence of dnPI3K (Fig. 5A) or LY294,002 (data not shown). Treatment with calP110 alone also increased levels of p35 protein (Fig. 5B). We concluded that activation of PI3K by BDNF was leading to the increase in p35.

To further evaluate whether activation of cdk5 by BDNF was downstream of activation of PI3K, we examined phosphoryla-
FIGURE 5. cdk5/p35 are required for induction of DARPP-32 (D32) by BDNF in vitro, and p35 alone is sufficient for DARPP-32 induction. Medium size spiny neurons were grown in vitro and Western blots or RNA analyzed as described under “Experimental Procedures.” Panels display representative gels, and results are summarized in the bar graphs. A and B, select wells were transduced with dn-p85 or ca-p110 at 1 DIV and/or treated with BDNF (10 ng/ml) at 4 DIV, and harvested at 24 h after addition of roscovitine or LY294,002 1 h prior to addition of BDNF. Cells were harvested at 30 min after treatment with BDNF and were pretreated with roscovitine or LY294,002, 1 h prior to addition of BDNF. D–F, select wells were treated with butyrolactone (10 μM) or roscovitine (10 μM), inhibitors of cdk5, at 1 DIV, and in some cases BDNF (10 ng/ml), and harvested at 24 h after addition of BDNF for analysis by Western blotting (D and E) or semi-quantitative RT-PCR (F). G and H, select wells were transduced with dnCdk5 (m.o.i. = 5) or wt-p35 (m.o.i. = 5) at 1 DIV and/or treated with BDNF (10 ng/ml) at 4 DIV, and harvested for Western blot analysis at 5 DIV. In all cases, the amount of DARPP-32 (D32) is expressed relative to Control (untreated) = 1, always plated and harvested at the same times as the experimental samples. Data are presented as means ± S.E. (n = 3–6, each representing a separate plating). All statistics were performed with one-way ANOVA followed by Bonferroni post hoc test (*, p < 0.05; **, p < 0.01; and ***, p < 0.001). LY = LY294,002; rosc = roscovitine; butyr = butyrolactone; wt = wild-type; dn = dominant negative.

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In summary, we have demonstrated that PI3K-mediated regulation of either Akt or cdk5 kinases independently increases the level of DARPP-32 protein in medium size spiny neurons in vitro. Moreover, we show that inhibition of Cdk5 does not prevent phosphorylation of Akt mediated by BDNF. Therefore, in medium size spiny neurons in vitro we conclude the following: 1) PI3K is required for base-line and BDNF-induced expression of DARPP-32 protein; 2) PI3K regulates phosphorylation of Akt and levels of p35, a key regulator of Cdk5 activity (49), but cdk5 does not regulate Akt following treatment with BDNF; and 3) both Akt and Cdk5/p35 are sufficient for the induction of DARPP-32, alone and by BDNF.

The mechanism via which Akt regulates levels of DARPP-32 protein appears to involve mTOR, a known downstream target of Akt. Interestingly, the activity of mTOR is down-regulated in the brains of Akt3-null mice (50), in which multiple neuronal mRNAs are up- or down-regulated. At sufficient levels, Akt1 is almost certainly able to transduce signals via similar mecha-
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nisms as does Akt3 in MSNs. This scenario of course would be consistent with data demonstrating the role of Akt1 and mTOR in selected populations of central nervous system progenitor cells (51). PI3K, Akt, and mTOR are able to activate both transcription and translation (45, 52). Levels of DARPP-32 therefore are regulated by both mechanisms, although the initial step mediated by PI3K as determined by inhibition with LY294,002 is largely at the translational level. Our results with semi-quantitative PCR are consistent with the previously reported decrease in DARPP-32 mRNA in the striatum in BDNF-null mice (19). As the effect on DARPP-32 transcription is cell- and transcript-specific (19), downstream the cell-specific effectors of Akt are probably involved. Of note, FoxP1 and FoxP2 are striatial enriched members of the forkhead transcription family (53, 54), and some members of this family are known downstream effectors of neurotrophins and PI3K/Akt (55).

Although induction by PI3K/AKT predominates, the cdk5/p35 system is able to mediate induction of DARPP-32 by BDNF. cdk5/p35 regulates neurogenesis, apoptosis, migration, and differentiation (56). Cross-talk between PI3K and cdk5 has been identified in other systems, including in primary neurons (33) as demonstrated in PC12 and cerebellar granule cells that nerve growth factor induces sustained expression of p35 for greater than 24 h, resulting in activation of cdk5 via extracellular signal-regulated kinase (ERK). This activity is required for nerve growth factor-induced PC12 cell differentiation. In cultured cortical neurons, neuregulin activates cdk5/p35 with subsequent activation of PI3K and Akt via ErbB3 and ErbB2 receptors (34). These studies also demonstrated that in cortical neurons, Cdk5 appears to regulate Akt phosphorylation independent of PI3K. During serum-induced L6 myoblast differentiation (57), LY294,002 blocks the enhancement of cdk5 activity and myogenin expression, suggesting that up-regulation of these factors is coupled to PI3K activation. Overexpression of dominant negative-Akt also reduces cdk5/p35 activity and myogenin expression, indicating that the PI3K-Cdk5 signaling cascade is linked to Akt activation. In our system, the PI3K inhibitor, LY294,002, was also found to inhibit BDNF-induced phosphorylation of ERK, a known inducer of p35 levels (33, 35). However, ERK activation is not required for induction of DARPP-32 by BDNF (35). We therefore anticipated that activation of cdk5 and Akt would be linked downstream of PI3K, but the interaction of these two pathways in MSNs differs from that seen in primary cortical neurons, and there may be a novel pathway via that PI3K regulates Akt and cdk5/p35 via independent, parallel mechanisms.

As the neuronal activator of Cdk5, the role of p35 is further determined by its cleavage state. It is anti-apoptotic as p35, but this molecule is pro-apoptotic as p25 (49). Interestingly, p25 was virtually undetectable in our cultures, even when p35 was overexpressed, likely accounting for the absence of increased cell death. cdk5/p35 has also been shown to negatively regulate transcription, via phosphorylation of mSds3 (58), and to positively regulate transcription, via STAT3 (47). Downstream targets of cdk5 operative in the regulation of DARPP-32 expression remain to be identified, although it is likely on the translational level. This appears to be the first example of neuronal cell type-specific transcription regulated by cdk5/p35.

The decrease in base-line levels of DARPP-32 protein levels in the presence of PI3K inhibitors has specific implications. Growth in Neurobasal/B27 raises DARPP-32 expression relative to other defined conditions or serum (18). Thus, the decrease in base-line DARPP-32 levels in the presence of LY294,002 and dnPI3K implies that other additives in B27 may be acting through the PI3K pathway to increase DARPP-32, implying convergence of multiple signals on a single pathway. Interestingly, B27 contains retinyl acetate, which can substitute for retinoic acid, a known inducer of DARPP-32 mRNA and protein levels in vitro (22).

Overexpression of p35 in the adult mouse brain does not raise the level of DARPP-32 protein (59). Therefore, downstream effects of individual signal transduction pathways may differ in the developing and the mature MSN, and/or there are compensatory, homeostatic mechanisms that maintain DARPP-32 protein at a constant level. Regulation of the Cdk5 pathway by BDNF, however, may still have important consequences in the adult brain. There are multiple pathophysiological conditions, e.g. addiction (reviewed in Ref. 60) and Huntington disease (61), in which a role for BDNF in the striatum is strongly implicated. Thus, BDNF peptide levels increase in the striatum after chronic exposure to psychostimulants and during withdrawal, whereas levels decrease in Huntington disease (HD). Abnormalities of the cdk5/p35 pathway are also implicated in both diseases. p35 levels increase in some models of addiction and may be involved in neuromolecular adaptations to drugs of abuse (62). cdk5 activity is decreased in the brain in mouse models of Huntington disease (63). To date, regulation of this pathway in the striatum has been ascribed to the dopamine pathway and its downstream effectors, e.g. DARPP-32 (64). Our data suggest, however, that the cdk5/p35 system may represent another important point of convergence of multiple pathways, i.e. dopamine and BDNF.

There also exist potential pathophysiological consequences for Akt function, with and without interactions with BDNF, in the same conditions as noted above for cdk5/p35. Akt is dysfunctional in HD (65), and its down-regulation may contribute to cell death in this disease. Transcriptional dysregulation precedes cell death in HD, and DARPP-32 is among the earliest of the identified genes to be down-regulated (66). Interestingly, however, rapamycin attenuates mutant Huntingtin toxicity by enhancing autophagic clearance of polyglutamine aggregates (67). When considering the use of rapamycin as a therapeutic agent in HD, it will therefore be important to determine whether it exacerbates alterations in levels of important MSN molecules. Regarding neuromolecular adaptations to psychostimulants, exposure to cocaine induces a PI3K-independent signaling cascade of changes in gene expression that appear to be critical for addiction and relapse and for amelioration of transcriptional dysregulation in HD.

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