

α -Synuclein activation of protein phosphatase 2A reduces tyrosine hydroxylase phosphorylation in dopaminergic cells

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Summary

α -Synuclein is an abundant presynaptic protein implicated in neuronal plasticity and neurodegenerative diseases. Although the function of α -synuclein is not thoroughly elucidated, we found that α -synuclein regulates dopamine synthesis by binding to and inhibiting tyrosine hydroxylase, the rate limiting enzyme in dopamine synthesis. Understanding α -synuclein function in dopaminergic cells should add to our knowledge of this key protein, which is implicated in Parkinson's disease and other disorders. Herein, we report a mechanism by which α -synuclein diminishes tyrosine hydroxylase phosphorylation and activity in stably transfected dopaminergic cells. Short-term regulation of tyrosine hydroxylase depends on the phosphorylation of key seryl residues in the amino-terminal regulatory domain of the protein. Of these, Ser40 contributes significantly to tyrosine hydroxylase activation and dopamine synthesis. We observed that α -synuclein overexpression caused reduced Ser40 phosphorylation in

MN9D cells and inducible PC12 cells. Ser40 is phosphorylated chiefly by the cyclic AMP-dependent protein kinase PKA and dephosphorylated almost exclusively by the protein phosphatase, PP2A. Therefore, we measured the impact of α -synuclein overexpression on levels and activity of PKA and PP2A in our cells. PKA was unaffected by α -synuclein. PP2A protein levels also were unchanged, however, the activity of PP2A increased in parallel with α -synuclein expression. Inhibition of PP2A dramatically increased Ser40 phosphorylation only in α -synuclein overexpressors in which α -synuclein was also found to co-immunoprecipitate with PP2A. Together the data reveal a functional interaction between α -synuclein and PP2A that leads to PP2A activation and underscores a key role for α -synuclein in protein phosphorylation.

Key words: MN9D, PC12, dopamine, PP2A, Parkinson's disease

Introduction

α -Synuclein (α -Syn), a protein highly enriched in presynaptic terminals (Maroteaux et al., 1988), is implicated in normal brain function (George et al., 1989) as well as disease (reviewed by Perez and Hastings, 2004). Knowledge about α -Syn is expanding and a chaperone function is probably based in part on its homology to the chaperone protein 14-3-3 (Ostrerova et al., 1999), which binds to and regulates the activity of many cellular proteins. α -Syn interacts with and affects the activity of the enzymes phospholipase D (Ahn et al., 2002), protein kinase C and the extracellular regulated kinases (ERK) (Ostrerova et al., 1999). We discovered a functional interaction between α -Syn and the dopamine (DA) biosynthetic enzyme tyrosine hydroxylase (TH) (Perez et al., 2002), another key enzyme that is also regulated by 14-3-3. However, unlike 14-3-3, which binds to and stimulates TH enzymatic activity (Ichimura et al., 1988), we found that α -Syn binding to TH inhibits its activity (Perez et al., 2002). In our current studies we explore the mechanisms by which α -Syn inhibits TH activity.

The regulation of TH activity in the short term depends on

the phosphorylation of key seryl residues (Ser19, Ser31 and Ser40) in the amino-terminal regulatory domain (Campbell et al., 1986; Haycock, 1990). Of these, phosphorylated Ser40 is a major contributor to DA synthesis in vivo (Ramsey et al., 1996). In previous studies we saw a significant reduction in total TH phosphorylation in cells overexpressing α -Syn, however, at that time we were unable to identify which residue(s) of TH were affected (Perez et al., 2002). More recently, antibodies specific for TH P-Ser40 became available allowing us to evaluate the impact of α -Syn on the phosphorylation state of that specific seryl residue. In preliminary studies we observed that cells with higher α -Syn levels had reduced Ser40 phosphorylation leading us to explore the mechanism by which α -Syn contributed to P-Ser40 dephosphorylation.

Reversible phosphorylation requires the activity of kinases and phosphatases. Although multiple kinases can phosphorylate TH on Ser40 in vitro, including cyclic GMP-dependent protein kinase (Rodriguez-Pascual et al., 1999; Roskoski et al., 1987), CamKII (Campbell et al., 1986; Yamauchi and Fujisawa, 1981),

PKC (Albert et al., 1984; Vulliet et al., 1985), MSK-1 (Toska et al., 2002), MAPKAP kinases 1 and 2 (Sutherland et al., 1993), it is the cAMP-dependent protein kinase PKA (Joh et al., 1978) that predominantly phosphorylates TH Ser40 in vivo (Campbell et al., 1986; Wu et al., 1992). It is well documented that dephosphorylation of TH Ser40 occurs *almost exclusively* by the phosphatase PP2A (Berresheim and Kuhn, 1994; Haavik et al., 1989; Leal et al., 2002), which reduces TH activity both in dopaminergic cells (Haavik et al., 1989) and in rat striatum (Bevilaqua et al., 2003). To test the means by which α -Syn reduces TH phosphorylation we explored the impact of α -Syn overexpression on PKA and PP2A in dopaminergic cells expressing different levels of α -Syn. Herein, we report our novel findings implicating α -Syn in the activation of PP2A.

Materials and Methods

Cell culture

The MN9D dopaminergic cells express abundant TH (Choi et al., 1991) and synthesize DA (Choi et al., 1992). MN9D cells were grown in Dulbecco's modified Eagle's medium (DMEM; D5648; Sigma, St Louis, MO, USA) as recommended by Alfred Heller and Lisa Won (University of Chicago, USA) who generously provided the cells. Low-passage number MN9D cells were grown as previously described (Ugarte et al., 2003). Control MN9D cells with low endogenous levels of α -Syn, included both untransfected parental MN9D cells and MN9D cells stably transfected with either pcDNA3 or GFP vector (Perez et al., 2002). PC12 cells were grown on collagen-coated plates in RPMI 1640 medium (Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 50 units/ml penicillin, 50 μ g/ml streptomycin, 10% horse serum (Gibco-Invitrogen, Carlsbad, CA, USA) and 5% fetal bovine serum (Hyclone, Logan, UT, USA). All transfected cells were maintained in media containing 200 μ g/ml G418 (Invitrogen, Carlsbad, CA). Plasmid-transfected control MN9D and PC12 cells expressed comparable low levels of α -Syn.

Generation of stably transfected inducible PC12 cell lines

α -Syn was cloned into pcDNA3 as previously described (Stefanis et al., 2001) followed by subcloning into the SKSP shuttle cloning vector using *HindIII-XhoI*. From this vector, *SfiI* and *PmeI* were used to subclone α -Syn into the ecdysone-inducible PBWN vector, downstream of the response element. These constructs form the basis of the 'bomb system' for inducible transgene expression (Suhr et al., 1998; Suhr et al., 2001) and were generously provided by Fred Gage and Steve Suhr at the Salk Institute (La Jolla, CA, USA). PC12 cells were transfected with PBWN- α -Syn using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), following the manufacturer's recommendations. One week after transfection, selection was begun in 500 μ g/ml G418 and individual clones selected. Tebufenozide, which is a molt-inducing insecticide that mimics the action of ecdysone (Addison, 1996), was used for induction of α -Syn expression. PC12 clone, Sm1 (plasmid) was only slightly inducible after 2.0 μ M tebufenozide for 72 hours (Fig. 1A, right side, lane 2), making these cells excellent baseline controls for our highly inducible clone, Sm4 (α -Syn; Fig. 1A, right side, lane 3), which expressed high levels of α -Syn expression after tebufenozide treatment (100 nM-2.0 μ M, 24-72 hours). Untransfected (UT) PC12 cells were utilized as additional baseline controls. The data presented on PC12 plasmid and α -Syn cells were obtained from induced cells.

Immunoblotting, antibodies and densitometry

Cell lysates prepared in 1% NP40 buffer containing protease

inhibitors were sonicated for ~5 seconds and particulates were eliminated by centrifugation at 15,000 *g* for 10 minute at 4°C as previously described (Perez et al., 1999). Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL, USA) and spectrophotometry. Proteins in Laemmli sample buffer were boiled and 20 μ g of protein were separated by 10% or 12% Tris-glycine SDS-PAGE and transferred to nitrocellulose. Prestained protein standards were used to determine the relative molecular mass of proteins. Equivalent sample loading was further confirmed using Ponceau S staining. Immunoblots were blocked in 5% nonfat dry milk in Tris-buffered saline, then incubated with primary antibodies at 4°C overnight. Primary antibodies included anti-synuclein-1 antibody (Syn-1, α -synuclein 610786, BD Bioscience-Transduction Laboratories), TH phospho-ser40 (Chemicon AB5935, Temecula, CA, USA), total TH (Chemicon MAB318, Temecula, CA, USA), PKA catalytic subunit (Calbiochem 539231, La Jolla, CA, USA) PP2A catalytic subunit antibody (Upstate 1D6, Lake Placid, NY, USA). Secondary antibodies were peroxidase-coupled anti-mouse or anti-rabbit (Calbiochem, La Jolla, CA, USA). Data were visualized on Biomax-MR film (Kodak, Rochester, NY, USA) after chemiluminescence (Dupont NEN, Boston, MA, USA). The optical densities of the bands were quantitated using MCID (Imaging Research Inc., St Catharines, Ontario, Canada) or ImageQuant software (Amersham Biosciences). All data measuring phosphorylated Ser40 levels, were normalized to total TH.

Co-immunoprecipitation

For co-immunoprecipitation all steps were carried out at 4°C. Adult rat striata were collected, weighed and homogenized in 5 volumes of ice-cold co-IP buffer, which contained 50 mM Tris pH 7.4, 100 mM NaCl, 5 mM EDTA, 0.3% Triton X-100, 10% glycerol plus aprotinin, leupeptin, 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), β -glycerolphosphate, and dithiothreitol to inhibit protease and phosphatase activities. Supernatants were collected after centrifugation at 17,000 *g* (Sorvall RC5B, Kendro Laboratory Products, Newtown, CT, USA). A control aliquot of each supernatant was separated and frozen prior to co-IP for total protein determinations. Samples were pre-cleared for 1 hour with 10 μ l 1% BSA plus 25 μ l each of protein A- and protein G-Sepharose beads (Zymed Laboratories, S. San Francisco, CA, USA). Immunoprecipitating antibodies (5 μ g) were coupled to Sieze™ X beads according to the manufacturer's instructions (Pierce, Rockford, IL, USA). Equal aliquots of homogenate (5.0 mg/ml total protein) were incubated with antibodies or pre-absorbed antibodies. Immune complexes were eluted, separated on 10 or 15% Tris-glycine SDS-PAGE gels, transferred to nitrocellulose, reacted with the same primary antibodies described above, and visualized by chemiluminescence. MN9D and PC12 cell extracts were prepared using the same buffers and conditions described above except that antibodies were not coupled to Sieze™ X beads. As the α -synuclein and PP2A antibodies are both of mouse origin, we saw IgG heavy (55 kDa) and light chains (25 kDa) in addition to antigens and some non-specific bands in some experiments (indicated by asterisks in the figure).

PKA assay

Lysates of MN9D cells were prepared, protein concentrations determined, and PKA activity measured using a kit according to the manufacturer's instructions (cat. no. 17-134, Upstate Biotechnology, Lake Placid, NY, USA). [³²P]ATP from NEN-Perkin Elmer Life Sciences (Boston, MA, USA) was utilized for the assay and PKA activity was measured by scintillation counting with activity expressed as pmol phosphate incorporated/minute/ μ g protein.

PP2A assay

PP2A immunoprecipitation and activity were determined using a non-radioactive kit according to the manufacturer's instructions (cat. no. 17-127, Upstate Biotechnologies, Lake Placid, NY, USA). Lysates of MN9D and PC12 cells were prepared in 20 mM imidazole-HCl, 2 mM EDTA, 2 mM EGTA, pH 7.0 with aprotinin, benzamide and AEBSF (all from Sigma-Aldrich, St Louis, MO, USA). Protein concentrations were determined using BCA and 0.5-1.0 mg protein was immunoprecipitated using an anti-PP2A catalytic subunit antibody (cat. no. 06-222, Upstate, Lake Placid, NY, USA) and protein A-Sepharose beads (Zymed Laboratories, South San Francisco, CA, USA). Equivalent immunoprecipitation of PP2A from all samples was confirmed by immunoblot. Immunoprecipitated PP2A was then tested for activity in a 10-minute reaction at 37°C, in which phosphopeptide (K-R-pT-I-R-R) dephosphorylation was assayed spectrophotometrically at 650 nm using Malachite Green. PP2A activity was determined for all samples relative to a phosphate standard curve with activity expressed as pmol incorporated phosphate/minute/ μ g protein.

Okadaic acid treatment

Okadaic acid binds to the catalytic subunit of PP2A and inhibits its activity. Although okadaic acid at high concentrations can inhibit PP1 as well as PP2A, it is well documented that dephosphorylation of TH Ser40 occurs almost exclusively by PP2A, not by PP1 activity (Berresheim and Kuhn, 1994; Dunkley et al., 2004; Haavik et al., 1989; Leal et al., 2002). Furthermore, we treated cells with low to high dose okadaic acid (5 nM-1 μ M) dissolved in DMSO (0.13 μ M) (Garcia et al., 2002; Haavik et al., 1989) for 1 hour to assess the impact on PP2A inhibition in the presence of α -Syn overexpression and saw a similar effect. For baseline TH Ser40 phosphorylation, cells were treated with 0.13 μ M DMSO for 1 hour without okadaic acid. Cell lysates were prepared, protein concentrations determined, and 20 μ g of protein from each condition was separated by SDS-PAGE for immunoblotting and densitometry. Equal protein loading was also confirmed with Ponceau S staining of blots prior to antibody incubation. Phosphorylated Ser40 levels were normalized to total TH for all treatment conditions, providing an internal standard for each measure of P-Ser40 on TH. For α -Syn inducible PC12 cells, data were also normalized to relative α -Syn levels within treatment conditions.

Statistical analyses

Independent sample *t*-tests, linear regression, and one way ANOVA were performed using SPSS (SPSS Inc., Chicago IL, USA) or Instat (Graphpad, San Diego, CA, USA) software. Post hoc analyses were performed by the method of Tukey-Kramer for data significant at *P*<0.05 or better. Experiments were repeated a minimum of two to three times on separate occasions with some experiments being performed five or more times. Data are presented as the mean \pm s.e.m. for all treatments.

Results

Increased α -Syn expression reduces Ser40 phosphorylation in dopaminergic cells

Ser40 phosphorylation is a major contributor to both TH activity and DA synthesis, and using a highly specific antibody to label P-Ser40 we measured Ser40 phosphorylation in stably transfected α -Syn-overexpressing MN9D cells compared to untransfected (UT) cells and plasmid transfected (plasmid) control MN9D cells. Equal amounts of protein were separated by SDS-PAGE and analyzed by immunoblotting for TH P-Ser40 levels followed by reprobing for total TH to normalize

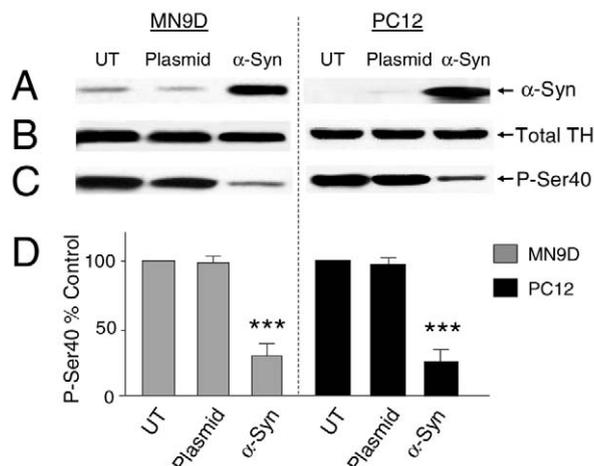


Fig. 1. α -Syn overexpression inhibits TH Ser40 phosphorylation. Representative immunoblots from a single experiments comparing 20 μ g total protein from MN9D and PC12 cells. (Left column) (A) α -Syn expression is low in UT (lane 1) and plasmid-transfected MN9D cells (lane 2), but increased >10-fold in stably transfected α -Syn MN9D cells (lane 3). (B) Total TH protein is abundant in all MN9D cells, although, in this experiment, a reduction in total TH is apparent in the α -Syn-overexpressing cells, although that was not always noted for α -Syn MN9D cells. (C), P-Ser40 levels are virtually identical for UT (lane 1) and plasmid transfected cells (lane 2), but significantly reduced in the α -Syn MN9D cells (lane 3). (Right column) Similar to MN9D cells (A) α -Syn expression is low in UT (lane 1) and plasmid-transfected PC12 cells (lane 2), but increased ~20-fold in stably transfected α -Syn PC12 cells (lane 3) at 72 hours after induction. (B) Total TH protein is equally abundant in all PC12 cells regardless of α -Syn levels. (C) P-Ser40 levels are virtually identical for control PC12 cells (UT, lane 1; plasmid transfected PC12 cells, lane 2) but significantly reduced in the α -Syn PC12 cells (lane 3). (D) P-Ser40 data from several experiments were normalized to total TH and expressed as percentage relative to control UT MN9D (left) and PC12 (right) cells. Untransfected cells (UT), plasmid-transfected control cells (plasmid), and stably transfected α -Syn-overexpressing cells (α -Syn). Values are mean \pm s.e.m. from three independent experiments for each cell line. ****P*<0.001.

P-Ser40 data, and for α -Syn to confirm α -Syn expression levels. Some α -Syn is expressed in all MN9D cells but high levels are only apparent in α -Syn-transfected cells relative to UT or plasmid-transfected MN9D control cells (Fig. 1A, left side). All MN9D cells also expressed abundant TH (Fig. 1B, left side); however, we sometimes saw a trend toward reduced TH levels in the α -Syn-overexpressing cells. To control for any variability in total TH levels we normalized P-Ser40 levels to total TH in all experiments. We observed a significant reduction in P-Ser40 only in α -Syn-overexpressing cells (Fig. 1C, lane 3, left side). When we plotted the data from multiple experiments, there was a significant decrease in P-Ser40 only in the α -Syn-overexpressing MN9D cells (Fig. 1D, left graph). These data revealed that when α -Syn levels increased, P-Ser40 levels decreased significantly in MN9D cells, suggesting that one means by which α -Syn inhibits TH activity is by inhibiting TH Ser40 phosphorylation.

To confirm that the effect on P-Ser40 was associated with α -Syn levels in dopaminergic cells we generated additional clonal cell lines in which the expression of α -Syn was under the control of an inducible promoter. Using these induced

PC12 cell lines we again measured the impact of α -Syn on P-Ser40. UT and plasmid PC12 cells had little α -Syn compared to the α -Syn-overexpressing PC12 cells, which had up to 20-fold more α -Syn than plasmid control cells when cells were induced for 72 hours (Fig. 1A, right side). Total TH was equivalent in all PC12 cells (Fig. 1B, right side) confirming that the increase in α -Syn did not alter TH expression in stably transfected PC12 cells. When we compared P-Ser40 levels between UT, plasmid and α -Syn PC12 cells we observed that while controls maintained equally high P-Ser40 levels, the α -Syn PC12 cells had reduced P-Ser40 levels (Fig. 1C, right side) similar to that observed in α -Syn-overexpressing MN9D cells (Fig. 1C, left side). When data from multiple experiments were plotted we again saw a large decrease in P-Ser40 levels only the α -Syn PC12 cells (Fig. 1D, right side) similar to the effect observed in MN9D cells (Fig. 1D, left side). To further probe the relationship between α -Syn overexpression and reduced TH Ser40 phosphorylation, we treated inducible α -Syn PC12 cells with different amounts of inducer. When analyzed by linear regression we identified a significant negative correlation between α -Syn and P-Ser40 ($r=-0.93$, $n=15$, $P=0.0017$). Taken together the data from both MN9D cells and inducible PC12 cells indicate that the phosphorylation of TH Ser40 is negatively regulated by α -Syn in dopaminergic cells. This observation led us to further explore how α -Syn contributed to TH dephosphorylation.

Overexpression of α -Syn does not alter PKA protein levels or activity

Since PKA is the major kinase mediating Ser40 phosphorylation and because α -Syn is so strongly implicated in enzymatic inhibition, e.g. ERK2, PLD2, TH (Iwata et al., 2001; Jenco et al., 1998; Perez et al., 2002) we first hypothesized that the reduction in TH Ser40 phosphorylation was probably occurring by α -Syn inhibition of PKA. To test this we measured PKA protein levels in UT, plasmid transfected, and α -Syn-overexpressing MN9D cells and found

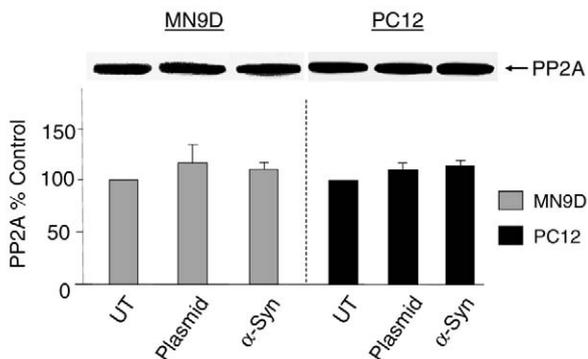


Fig. 2. α -Syn overexpression does not alter PP2A protein levels in MN9D or PC12 cells. (Top) A representative immunoblot for MN9D and PC12 cells reacted with the PP2A antibody. (Below) Bar graph showing that UT, plasmid and α -Syn MN9D and PC12 cells had essentially identical PP2A levels. Untransfected cells (UT), plasmid-transfected control cells (plasmid), and stably transfected α -Syn-overexpressing cells (α -Syn). Data are expressed as the mean percent change relative to UT cells \pm s.e.m. of combined data from three independent experiments.

them to be equivalent (data not shown). We then measured PKA activity and confirmed similar activity in all cells regardless of α -Syn levels (data not shown) revealing that the reduction in P-Ser40 in α -Syn-overexpressing cells was not due to PKA inhibition. We then turned our attention to PP2A, the phosphatase that dephosphorylates P-Ser40 on TH (Haavik et al., 1989; Leal et al., 2002).

Overexpression of α -Syn does not alter PP2A protein levels

We measured PP2A levels from control and α -Syn-overexpressing MN9D and PC12 cells by immunoblotting (Fig. 2). Equal amounts of total protein were evaluated for all conditions and revealed that PP2A levels in UT, plasmid and α -Syn MN9D cells were unchanged (Fig. 2, left side) as were PP2A levels in the PC12 cell lines (Fig. 2, right side). These data reveal that α -Syn overexpression did not alter PP2A protein levels in our dopaminergic cells as can be further appreciated when graphs of data from several experiments are examined (Fig. 2).

α -Syn increases PP2A activity and binds to the PP2A catalytic domain

We measured PP2A activity from the MN9D and induced PC12 cells using a well-established immunoprecipitation and phosphatase activity protocol (Begum and Ragolia, 1996). As we found identical data for control UT and plasmid cells in all previous experiments, we utilized plasmid cells as baseline controls for this series of experiments. We first confirmed that equal amounts of PP2A had been immunoprecipitated with an antibody to the PP2A catalytic subunit (Fig. 3A). We then measured the activity of the immunoprecipitated PP2A and found a doubling of PP2A activity in α -Syn MN9D cells compared to plasmid transfected control MN9D cells (Fig. 3B,

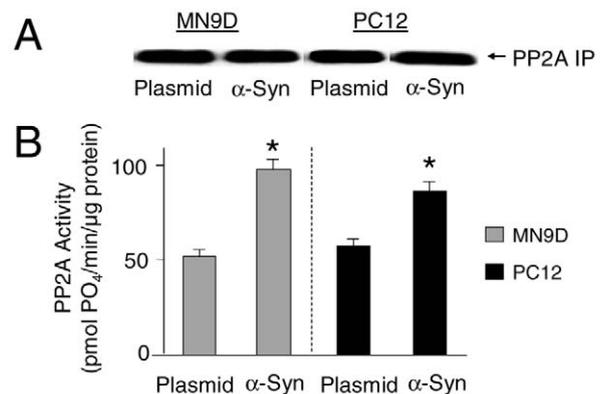


Fig. 3. α -Syn contributes to PP2A activation in MN9D and PC12 cells. (A) Cell lysates from MN9D cells and from induced PC12 cells were immunoprecipitated for the PP2A catalytic subunit. A representative immunoblot shows equivalent amounts of PP2A immunoprecipitated from all samples. (B) The activity of the immunoprecipitated PP2A was measured for MN9D cells (gray bars) and for induced PC12 cells (black bars). Plasmid, plasmid-transfected control cells; α -Syn; stably transfected α -Syn-overexpressing cells. Values are mean \pm s.e.m. from three independent experiments. * $P<0.05$.

left side). We observed a similar increase in PP2A activity in α -Syn PC12 cells (Fig. 3B, right side).

We then tested for an interaction between α -Syn and PP2A in our cells by a co-immunoprecipitation assay. α -Syn was found to co-immunoprecipitate with PP2A from MN9D cells (not shown) and from inducible PC12 cells (Fig. 4A) confirming that soluble α -Syn interacts with PP2A as measured using the Syn-1 antibody for co-immunoprecipitation. We tested the association between α -Syn and PP2A in rat striatum (Fig. 4B), confirming that the proteins also interact when α -Syn and PP2A are expressed at endogenous levels.

Taken together these data imply that α -Syn interacts with PP2A to significantly increase PP2A activity in both MN9D and PC12 dopaminergic cells and that this activation of PP2A contributed to a reduction in P-Ser40 levels on TH in our cells.

Inhibiting PP2A produces robust phosphorylation of TH Ser40 only in α -Syn cells

To assess whether TH Ser40 phosphorylation would increase after PP2A activity was inhibited, we treated control and α -Syn-overexpressing cells with the phosphatase inhibitor, okadaic acid, which inhibits PP2A with an IC₅₀ of 10 nM. We prepared cell extracts from MN9D and PC12 cells and measured increases in P-Ser40 levels after okadaic acid

treatment (5 nM-1.0 μ M). Okadaic acid treatment resulted in significant increases in P-Ser40 levels in all cells relative to vehicle-treated parallel cultures. This increase was expected because dephosphorylated Ser40 residues are present on TH in all cells. When α -Syn MN9D cells were treated with low dose okadaic acid (10-100 nM) large significant increases in P-Ser40 levels were noted in α -Syn-overexpressing cells (Fig. 5A). A large increase in P-Ser40 was also observed in PC12 α -Syn cells (Fig. 5B, $P < 0.01$). When we quantified the relative increases in P-Ser40 after okadaic acid treatment for MN9D and PC12 cells, we found nearly identical effects in both dopaminergic cell lines (MN9D=2.2 \pm 0.72; PC12=2.84 \pm 0.57, $P > 0.05$). We noted that the magnitude of the increase in P-Ser40 levels when PP2A activity was inhibited was greatest in α -Syn-overexpressing cells (Fig. 5A,B), which had low baseline levels of P-Ser40 (see Fig. 1C, lane 3) and in which PP2A activity was significantly elevated (Fig. 3B). Since it is well-documented that Ser40 dephosphorylation occurs almost exclusively by PP2A (Berresheim and Kuhn, 1994; Dunkley et al., 2004; Haavik et al., 1989; Leal et al., 2002) our findings indicate that (1) TH Ser40 is dephosphorylated by PP2A in dopaminergic cells, and (2) the Ser40 residue on TH remains accessible to PP2A even when α -Syn is overexpressed. All in all, these data provide the first indication that α -Syn contributes to PP2A activation, which has potential relevance to synucleinopathies.

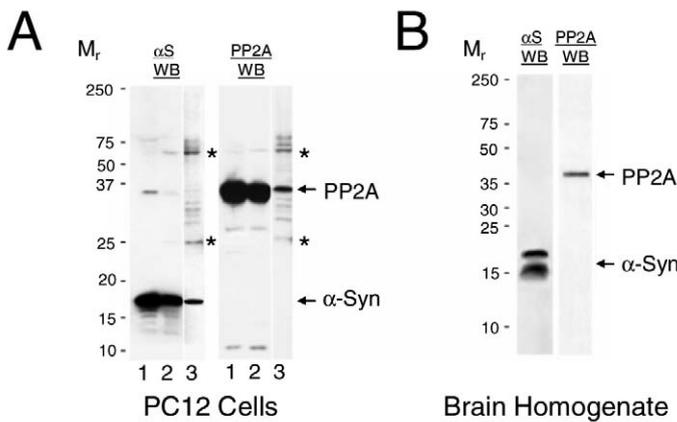


Fig. 4. Interaction of α -Syn and PP2A in cells and in rat brain. (A) α -Syn PC12 cell lysates were co-immunoprecipitated and proteins were separated by SDS-PAGE and the western blots (WB) were reacted with α -Syn antibody (α -S WB) or PP2A antibody (PP2A WB). Left blot shows the levels of α -Syn in cells from the initial homogenate (lane 1), in the homogenate after co-immunoprecipitation (co-IP; lane 2), and α -Syn immunoprecipitated with the Syn-1 antibody (lane 3). Right blot in A was prepared from the same co-IP sample with the levels of PP2A in the initial homogenate (lane 1), PP2A in the homogenate after co-IP (lane 2), and PP2A co-immunoprecipitated along with α -Syn using the Syn-1 antibody (lane 3). Non-specific bands, two of which appear to be IgG bands (asterisks) are evident in both blots. (B) The α -Syn WB reveals α -Syn immunoprecipitated from rat striatum (left lane) and in the PP2A WB the PP2A that co-immunoprecipitated with α -Syn using the Syn-1 antibody (right lane). Relative molecular mass (M_r , $\times 10^{-3}$) was determined from prestained standards. α -S WB, α -synuclein Syn-1 antibody-reacted western blot; PP2A WB, PP2A antibody-reacted western blot.

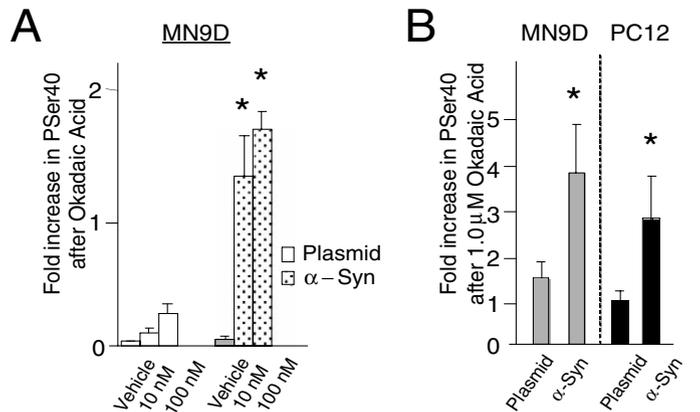


Fig. 5. Blockade of PP2A activity with okadaic acid demonstrates a role for PP2A in Ser40 dephosphorylation in MN9D and PC12 cells. PP2A inhibition by 10 nM-1.0 μ M okadaic acid for 1 hour, caused an increase in P-Ser40 levels in all conditions when compared to parallel vehicle-treated controls for each condition. The baseline value was set to zero to demonstrate the fold increase in P-Ser40 levels above baseline for each condition. (A) Okadaic acid at low doses (0-100 nM) produced small changes in P-Ser40 in plasmid control MN9D cells but large significant increases in P-Ser40 in α -Syn-overexpressing MN9D cells. Vehicle-treated cells were essentially unchanged from baseline at these concentrations of okadaic acid. (B) With higher dose okadaic acid, even more robust increases in P-Ser40 were noted for α -Syn MN9D and induced α -Syn PC12 cells, suggesting that low baseline P-Ser40 phosphorylation levels in α -Syn overexpressors had probably occurred through effects on PP2A activation. Values are mean \pm s.e.m. of two to six independent experiments. * $P < 0.01$.

Discussion

To further elucidate normal α -Syn function and the mechanism by which α -Syn regulates TH phosphorylation and DA synthesis we performed the studies described above and made the remarkable discovery that α -Syn contributes to the regulation of PP2A phosphatase activity, leading to PP2A activation.

We noted that increased α -Syn expression produced a several-fold decrease in TH Ser40 phosphorylation in the MN9D and PC12 cells with elevated α -Syn levels. This was further assessed by measuring a dose effect of α -Syn on P-Ser40 reduction in the inducible PC12 cells in which we found a significant negative correlation between elevated α -Syn levels and a diminution of P-Ser40. Thus, using two independent dopaminergic cellular models we showed that only when α -Syn levels were elevated, whether by constitutive or inducible overexpression, did we see significant decreases in P-Ser40 levels. Because α -Syn reportedly inhibits multiple enzymatic activities (Iwata et al., 2001; Jenco et al., 1998; Perez et al., 2002) we had anticipated identifying a role for α -Syn as an inhibitor of PKA, which proved not to be the case. We found no change in either PKA protein levels or PKA activity in cells overexpressing α -Syn, confirming that the decrease in Ser40 phosphorylation in α -Syn-overexpressing cells was not due to an effect of α -Syn on PKA kinase activity. We therefore turned our attention to PP2A, the enzyme that is responsible for P-Ser40 dephosphorylation on TH.

Haavik and colleagues originally showed that PP2A, the major serine/threonine phosphatase that regulates many signaling pathways in mammalian cells, is responsible for greater than 90% of the dephosphorylation of TH at Ser40 (Haavik et al., 1989). Incubation of adrenal chromaffin cells with okadaic acid in the aforementioned study, dramatically increased TH phosphorylation and TH activity, firmly establishing PP2A as a regulator of both Ser40 phosphorylation and TH activity in dopaminergic cells. More recently, using PP1- and PP2A-specific inhibitors to measure TH dephosphorylation in brain, Dunkley and colleagues (Leal et al., 2002) reconfirmed the role of PP2A in both TH activity and phosphorylation state. In our studies we found that PP2A protein levels were not altered by α -Syn overexpression in dopaminergic cells, yet PP2A activity was significantly increased in α -Syn-overexpressing cells. To further verify that the effects on Ser40 were associated with changes in PP2A activity we treated cells with okadaic acid, and found that Ser40 phosphorylation became significantly elevated in cells overexpressing α -Syn that had low baseline P-Ser40 levels. These data implicate PP2A activation as the mediator of P-Ser40 dephosphorylation in our α -Syn cell lines. These findings in dopaminergic cells with increased α -Syn levels confirm that (1) PP2A is more active, and (2) a dramatic increase in P-Ser40 phosphorylation is achieved by blocking PP2A activity. Additionally, the data strongly suggest that α -Syn-mediated activation of PP2A may have reduced both TH activity and DA synthesis in our earlier studies (Perez et al., 2002).

Co-localization of the various PP2A subunits is required for PP2A activation and is thought to occur by interactions of the various subunits with molecules such as chaperones. An active

PP2A enzyme consists of a heterotrimer of the structural A subunit, a catalytic C subunit, and a regulatory B subunit (Dobrowsky et al., 1993). The A and C subunits are ubiquitously expressed (Mayer et al., 1991) and form the catalytic complex (PP2A/C), which interacts with at least three different families of regulatory B subunits, as well as with certain tumor antigens (Mumby and Walter, 1993). The regulatory B subunits of PP2A are known to be temporally expressed during development (Csortos et al., 1996; Mayer et al., 1991; McCright and Virshup, 1995; Ruediger et al., 1991) and neuron-specific isoforms have also been identified (Mayer et al., 1991). The substrate specificity of PP2A appears to be determined by the regulatory B subunits (Cegielska et al., 1994; Csortos et al., 1996) and there is evidence that B subunits are associated with targeting the PP2A/C catalytic complexes to various intracellular sites such as microtubules (McCright et al., 1996; Sontag et al., 1995) and mitochondria (Ruvolo et al., 2002) suggesting that PP2A complexes are actively trafficked by their associated interacting proteins, one of which may be α -Syn. We have discovered that α -Syn and PP2A interact with each other in soluble fractions of brain and dopaminergic cells as measured by co-immunoprecipitation. Membrane bound PP2A in brain is reportedly less active (Sim et al., 1998), thus the interaction of PP2A with α -Syn within the cytosol may serve to stimulate PP2A activity. This interaction of α -Syn with PP2A may affect PP2A conformation or trafficking and subsequently contribute to its activation.

α -Syn oxidative modification or aggregation occurs in neurodegenerative diseases such as Alzheimer's disease, a condition in which PP2A is also implicated (Trojanowski and Lee, 1995; Zhao et al., 2003). Based on our novel findings these are the first data to identify an association between α -Syn and PP2A that affects PP2A activity and may contribute to neuronal homeostasis, which if disrupted may be detrimental.

The impact of α -Syn on TH P-Ser40 may involve other means of regulating the phosphorylation on this site. For example, α -Syn can directly interact with 14-3-3 (Ostrerova et al., 1999) and with TH (Perez et al., 2002). It is known that 14-3-3 can activate TH (Ichimura et al., 1988) by 14-3-3 binding first to TH Ser19 then to TH Ser40 (Kleppe et al., 2001). There is additional evidence that the binding of 14-3-3 to TH phosphorylated on Ser19 and Ser40 stabilizes its conformation and enhances TH activity (Bevilaqua et al., 2001). An interaction of α -Syn with 14-3-3 may contribute to dissociation of 14-3-3 from TH to permit PP2A physical access to the Ser40 site with subsequent effects on TH phosphorylation and DA synthesis. Further studies are required to identify the precise manner by which α -Syn acts to stimulate PP2A activity. However, regardless of how it does so, we provide novel evidence that α -Syn interacts with and contributes to the activation of PP2A, a major brain phosphatase. Our findings also underscore the importance of further elucidating normal α -Syn function because (1) many substrates require PP2A for dephosphorylation, and (2) α -Syn is implicated in multiple synucleinopathies.

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References

- Addison, J. A.** (1996). Safety testing of tebufenozide, a new molt-inducing insecticide, for effects on nontarget forest soil invertebrates. *Ecotox. Environ. Safe.* **33**, 55-61.
- Ahn, B. H., Rhim, H., Kim, S. Y., Sung, Y. M., Lee, M. Y., Choi, J. Y., Wolozin, B., Chang, J. S., Lee, Y. H., Kwon, T. K. et al.** (2002). Alpha-Synuclein interacts with phospholipase D isozymes and inhibits pervanadate-induced phospholipase D activation in human embryonic kidney-293 cells. *J. Biol. Chem.* **277**, 12334-12342.
- Albert, K. A., Helmer-Matyjek, E., Nairn, A. C., Muller, T. H., Haycock, J. W., Greene, L. A., Goldstein, M. and Greengard, P.** (1984). Calcium/phospholipid-dependent protein kinase (protein kinase C) phosphorylates and activates tyrosine hydroxylase. *Proc. Natl. Acad. Sci. USA* **81**, 7713-7717.
- Begum, N. and Ragolia, L.** (1996). cAMP counter-regulates insulin-mediated protein phosphatase-2A inactivation in rat skeletal muscle cells. *J. Biol. Chem.* **271**, 31166-31171.
- Berresheim, U. and Kuhn, D. M.** (1994). Dephosphorylation of tyrosine hydroxylase by brain protein phosphatases: a predominant role for type 2A. *Brain Res.* **637**, 273-276.
- Bevilaqua, L. R., Graham, M. E., Dunkley, P. R., von Nagy-Felsobuki, E. I. and Dickson, P. W.** (2001). Phosphorylation of Ser(19) alters the conformation of tyrosine hydroxylase to increase the rate of phosphorylation of Ser(40). *J. Biol. Chem.* **276**, 40411-40416.
- Bevilaqua, L. R., Cammarota, M., Dickson, P. W., Sim, A. T. and Dunkley, P. R.** (2003). Role of protein phosphatase 2C from bovine adrenal chromaffin cells in the dephosphorylation of phospho-serine 40 tyrosine hydroxylase. *J. Neurochem.* **85**, 1368-1373.
- Campbell, D. G., Hardie, D. G. and Vulliet, P. R.** (1986). Identification of four phosphorylation sites in the N-terminal region of tyrosine hydroxylase. *J. Biol. Chem.* **261**, 10489-10492.
- Cegielska, A., Shaffer, S., Derua, R., Goris, J. and Virshup, D. M.** (1994). Different oligomeric forms of protein phosphatase 2A activate and inhibit simian virus 40 DNA replication. *Mol. Cell. Biol.* **14**, 4616-4623.
- Choi, H. K., Won, L. A., Kontur, P. J., Hammond, D. N., Fox, A. P., Wainer, B. H., Hoffmann, P. C. and Heller, A.** (1991). Immortalization of embryonic mesencephalic dopaminergic neurons by somatic cell fusion. *Brain Res.* **552**, 67-76.
- Choi, H. K., Won, L., Roback, J. D., Wainer, B. H. and Heller, A.** (1992). Specific modulation of dopamine expression in neuronal hybrid cells by primary cells from different brain regions. *Proc. Natl. Acad. Sci. USA* **89**, 8943-8947.
- Csortos, C., Zolnierowicz, S., Bako, E., Durbin, S. D. and DePaoli-Roach, A. A.** (1996). High complexity in the expression of the B' subunit of protein phosphatase 2A0. Evidence for the existence of at least seven novel isoforms. *J. Biol. Chem.* **271**, 2578-2588.
- Dobrowsky, R. T., Kamibayashi, C., Mumby, M. C. and Hannun, Y. A.** (1993). Ceramide activates heterotrimeric protein phosphatase 2A. *J. Biol. Chem.* **268**, 15523-15530.
- Dunkley, P. R., Bobrovskaya, L., Graham, M. E., von Nagy-Felsobuki, E. I. and Dickson, P. W.** (2004). Tyrosine hydroxylase phosphorylation: regulation and consequences. *J. Neurochem.* **91**, 1025-1043.
- Garcia, L., Garcia, F., Llorens, F., Unzeta, M., Itarte, E. and Gomez, N.** (2002). PP1/PP2A phosphatases inhibitors okadaic acid and calyculin A block ERK5 activation by growth factors and oxidative stress. *FEBS Lett.* **523**, 90-94.
- George, R. J., Haycock, J. W., Johnston, J. P., Craviso, G. L. and Waymire, J. C.** (1989). In vitro phosphorylation of bovine adrenal chromaffin cell tyrosine hydroxylase by endogenous protein kinases. *J. Neurochem.* **52**, 274-284.
- Haavik, J., Schelling, D. L., Campbell, D. G., Andersson, K. K., Flatmark, T. and Cohen, P.** (1989). Identification of protein phosphatase 2A as the major tyrosine hydroxylase phosphatase in adrenal medulla and corpus striatum: evidence from the effects of okadaic acid. *FEBS Lett.* **251**, 36-42.
- Haycock, J. W.** (1990). Phosphorylation of tyrosine hydroxylase in situ at serine 8, 19, 31, and 40. *J. Biol. Chem.* **265**, 11682-11691.
- Ichimura, T., Isobe, T., Okuyama, T., Takahashi, N., Araki, K., Kuwano, R. and Takahashi, Y.** (1988). Molecular cloning of cDNA coding for brain-specific 14-3-3 protein, a protein kinase-dependent activator of tyrosine and tryptophan hydroxylases. *Proc. Natl. Acad. Sci. USA* **85**, 7084-7088.
- Iwata, A., Maruyama, M., Kanazawa, I. and Nukina, N.** (2001). Alpha-synuclein affects the MAPK pathway and accelerates cell death. *J. Biol. Chem.* **276**, 45320-45329.
- Jenco, J. M., Rawlingson, A., Daniels, B. and Morris, A. J.** (1998). Regulation of phospholipase D2: selective inhibition of mammalian phospholipase D isoenzymes by alpha- and beta-synucleins. *Biochemistry* **37**, 4901-4909.
- Joh, T. H., Park, D. H. and Reis, D. J.** (1978). Direct phosphorylation of brain tyrosine hydroxylase by cyclic AMP-dependent protein kinase: mechanism of enzyme activation. *Proc. Natl. Acad. Sci. USA* **75**, 4744-4748.
- Kleppe, R., Toska, K. and Haavik, J.** (2001). Interaction of phosphorylated tyrosine hydroxylase with 14-3-3 proteins: evidence for a phosphoserine 40-dependent association. *J. Neurochem.* **77**, 1097-1107.
- Leal, R. B., Sim, A. T., Goncalves, C. A. and Dunkley, P. R.** (2002). Tyrosine hydroxylase dephosphorylation by protein phosphatase 2A in bovine adrenal chromaffin cells. *Neurochem. Res.* **27**, 207-213.
- Maroteaux, L., Campanelli, J. T. and Scheller, R. H.** (1988). Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal. *J. Neurosci.* **8**, 2804-2815.
- Mayer, R. E., Hendrix, P., Cron, P., Matthies, R., Stone, S. R., Goris, J., Merlevede, W., Hofsteenge, J. and Hemmings, B. A.** (1991). Structure of the 55-kDa regulatory subunit of protein phosphatase 2A: evidence for a neuronal-specific isoform. *Biochemistry* **30**, 3589-3597.
- McCright, B. and Virshup, D. M.** (1995). Identification of a new family of protein phosphatase 2A regulatory subunits. *J. Biol. Chem.* **270**, 26123-26128.
- McCright, B., Rivers, A. M., Audlin, S. and Virshup, D. M.** (1996). The B56 family of protein phosphatase 2A (PP2A) regulatory subunits encodes differentiation-induced phosphoproteins that target PP2A to both nucleus and cytoplasm. *J. Biol. Chem.* **271**, 22081-22089.
- Mumby, M. C. and Walter, G.** (1993). Protein serine/threonine phosphatases: structure, regulation, and functions in cell growth. *Physiol. Rev.* **73**, 673-699.
- Ostrerova, N., Petrucelli, L., Farrer, M., Mehta, N., Choi, P., Hardy, J. and Wolozin, B.** (1999). alpha-Synuclein shares physical and functional homology with 14-3-3 proteins. *J. Neurosci.* **19**, 5782-5791.
- Perez, R. and Hastings, T.** (2004). Could a loss of alpha-synuclein function put dopaminergic neurons at risk? *J. Neurochem.* **89**, 1318-1324.
- Perez, R. G., Soriano, S., Hayes, J. D., Ostaszewski, B., Xia, W., Selkoe, D. J., Chen, X., Stokin, G. B. and Koo, E. H.** (1999). Mutagenesis identifies new signals for beta-amyloid precursor protein endocytosis, turnover, and the generation of secreted fragments, including Abeta42. *J. Biol. Chem.* **274**, 18851-18856.
- Perez, R. G., Waymire, J. C., Lin, E., Liu, J. J., Guo, F. and Zigmund, M. J.** (2002). A role for alpha-synuclein in the regulation of dopamine biosynthesis. *J. Neurosci.* **22**, 3090-3099.
- Ramsey, A. J., Hillas, P. J. and Fitzpatrick, P. F.** (1996). Characterization of the active site iron in tyrosine hydroxylase. Redox states of the iron. *J. Biol. Chem.* **271**, 24395-24400.
- Rodriguez-Pascual, F., Ferrero, R., Miras-Portugal, M. T. and Torres, M.** (1999). Phosphorylation of tyrosine hydroxylase by cGMP-dependent protein kinase in intact bovine chromaffin cells. *Arch. Biochem. Biophys.* **366**, 207-214.
- Roskoski, R., Jr, Vulliet, P. R. and Glass, D. B.** (1987). Phosphorylation of tyrosine hydroxylase by cyclic GMP-dependent protein kinase. *J. Neurochem.* **48**, 840-845.
- Ruediger, R., Van Wart Hood, J. E., Mumby, M. and Walter, G.** (1991). Constant expression and activity of protein phosphatase 2A in synchronized cells. *Mol. Cell. Biol.* **11**, 4282-4285.
- Ruvolo, P. P., Clark, W., Mumby, M., Gao, F. and May, W. S.** (2002). A functional role for the B56 alpha-subunit of protein phosphatase 2A in ceramide-mediated regulation of Bcl2 phosphorylation status and function. *J. Biol. Chem.* **277**, 22847-22852.
- Sim, A. T., Collins, E., Mudge, L. M. and Rostas, J. A.** (1998). Developmental regulation of protein phosphatase types 1 and 2A in post-hatch chicken brain. *Neurochem. Res.* **23**, 487-491.
- Sontag, E., Nunbhakdi-Craig, V., Bloom, G. S. and Mumby, M. C.** (1995). A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. *J. Cell Biol.* **128**, 1131-1144.
- Stefanis, L., Kholodilov, N., Rideout, H. J., Burke, R. E. and Greene, L. A.** (2001). Synuclein-1 is selectively up-regulated in response to nerve growth factor treatment in PC12 cells. *J. Neurochem.* **76**, 1165-1176.
- Suhr, S. T., Gil, E. B., Senut, M. C. and Gage, F. H.** (1998). High level transactivation by a modified Bombyx ecdysone receptor in mammalian cells without exogenous retinoid X receptor. *Proc. Natl. Acad. Sci. USA* **95**, 7999-8004.
- Suhr, S. T., Senut, M. C., Whitelegge, J. P., Faull, K. F., Cuizon, D. B.**

- and Gage, F. H. (2001). Identities of sequestered proteins in aggregates from cells with induced polyglutamine expression. *J. Cell Biol.* **153**, 283-294.
- Sutherland, C., Alterio, J., Campbell, D. G., Le Bourdelles, B., Mallet, J., Haavik, J. and Cohen, P. (1993). Phosphorylation and activation of human tyrosine hydroxylase in vitro by mitogen-activated protein (MAP) kinase and MAP-kinase-activated kinases 1 and 2. *Eur. J. Biochem.* **217**, 715-722.
- Toska, K., Kleppe, R., Cohen, P. and Haavik, J. (2002). Phosphorylation of tyrosine hydroxylase in isolated mice adrenal glands. *Ann. New York Acad. Sci.* **971**, 66-68.
- Trojanowski, J. Q. and Lee, V. M. (1995). Phosphorylation of paired helical filament tau in Alzheimer's disease neurofibrillary lesions: focusing on phosphatases. *FASEB J.* **9**, 1570-1576.
- Ugarte, S. D., Lin, E., Klann, E., Zigmond, M. J. and Perez, R. G. (2003). Effects of GDNF on 6-OHDA-induced death in a dopaminergic cell line: Modulation by inhibitors of PI3 kinase and MEK. *J. Neurosci. Res.* **73**, 105-112.
- Vulliet, P. R., Woodgett, J. R., Ferrari, S. and Hardie, D. G. (1985). Characterization of the sites phosphorylated on tyrosine hydroxylase by Ca²⁺ and phospholipid-dependent protein kinase, calmodulin-dependent multiprotein kinase and cyclic AMP-dependent protein kinase. *FEBS Lett.* **182**, 335-339.
- Wu, J., Filer, D., Friedhoff, A. J. and Goldstein, M. (1992). Site-directed mutagenesis of tyrosine hydroxylase. Role of serine 40 in catalysis. *J. Biol. Chem.* **267**, 25754-25758.
- Yamauchi, T. and Fujisawa, H. (1981). A calmodulin-dependent protein kinase that is involved in the activation of tryptophan 5-monoxygenase is specifically distributed in brain tissues. *FEBS Lett.* **129**, 117-119.
- Zhao, W. Q., Feng, C. and Alkon, D. L. (2003). Impairment of phosphatase 2A contributes to the prolonged MAP kinase phosphorylation in Alzheimer's disease fibroblasts. *Neurobiol. Dis.* **14**, 458-469.