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Dopamine depletion alters phosphorylation of striatal proteins in a model of Parkinsonism

Abigail M. Brown[1], **Ariel Y. Deutch**[2,3,4,5], and **Roger J. Colbran**[1,3,5]

[1]Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232–0615, USA

[2]Departments of Psychiatry and Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232–0615, USA

[3]Center for Molecular Neuroscience, Vanderbilt University School of Medicine, Nashville, TN 37232–0615, USA

[4]Center for Integrative and Cognitive Neuroscience, Vanderbilt University School of Medicine, Nashville, TN 37232–0615, USA

[5]Vanderbilt Kennedy Center for Research on Human Development, Vanderbilt University School of Medicine, Nashville, TN 37232–0615, USA

Abstract

Nigrostriatal dopamine depletion disrupts striatal medium spiny neuron morphology in Parkinson's disease and modulates striatal synaptic plasticity in animal models of parkinsonism. We demonstrate that long-term nigrostriatal dopamine depletion in the rat induces evolving changes in the phosphorylation of striatal proteins critical for synaptic plasticity. Dopamine depletion increased the phosphorylation of the alpha isoform of calcium–calmodulin-dependent protein kinase II (CaMKIIα) at Thr^{286}, a site associated with enhanced autonomous kinase activity, but did not alter total levels of CaMKIIα or other synaptic proteins. Dopamine depletion decreased CaMKIIα levels in postsynaptic density-enriched fractions without significant changes in other proteins. The activity of protein phosphatase 1 (PP1), a postsynaptic phosphatase that dephosphorylates CaMKII, is regulated by DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of 32 kDa). Dopamine depletion had no effect on DARPP-32 phosphorylation at Thr^{34}, but increased DARPP-32 phosphorylation at Thr^{75}. Levodopa administration reversed the increased phosphorylation of both CaMKIIα and DARPP-32. Normal ageing increased the levels of PP1(γ1 isoform) but decreased levels of the PP1γ1-targeting proteins spinophilin and neurabin. Elevated phosphorylations of CaMKIIα and DARPP-32 were maintained for up to 20 months after dopamine depletion. However, phosphorylation of the CaMKII–PP1 substrate, Ser^{831} in the

Correspondence: Dr Roger J. Colbran, Room 702 Light Hall, Vanderbilt University School of Medicine, Nashville, TN 37232–0615, USA. roger.colbran@vanderbilt.edu.

Supplementary material

The following supplementary material may be found on: http://www.blackwellpublishing.com/products/journals/suppmat/EJN4190/EJN4190sm.htm

Fig. S1. No significant changes during normal aging in levels of CaMKIIα, GluR1, $PP2A_C$, PP2B or PSD-95, or phosphorylation of CaMKIIα and DARPP-32.

Fig. S2. No significant changes following chronic dopamine depletion in NR1, PP2B or PSD-95.

glutamate receptor GluR1 subunit, was increased only after sustained (9–20 months) dopamine depletion. Interaction of ageing-related changes in PP1 with the dopamine depletion-induced changes in CaMKIIα may account for enhanced GluR1 phosphorylation only after long-term dopamine depletion. These evolving changes may impact striatal synaptic plasticity, Parkinson's disease progression and the changing efficacy and side-effects associated with dopamine replacement therapy.

Keywords

ageing; AMPA receptor; CaMKII; DARPP-32; dendrite; postsynaptic density; PP1

Introduction

Parkinson's disease is a neurodegenerative disorder resulting in striatal dopamine depletion that strikes 1–2% of the population over 60 years of age. Normal ageing induces morphological changes in striatal medium spiny neurons (MSNs) (Ingham *et al.*, 1989), which constitute ≈95% of the total striatal neuron population. However, the loss of nigrostriatal dopamine input results in additional morphological and functional alterations in striatal MSNs. For example, enduring changes in MSN dendrites have been reported in Parkinson's disease (McNeill *et al.*, 1988; Ingham *et al.*, 1998; Arbuthnott *et al.*, 2000; Meshul *et al.*, 2000; Zaja-Milatovic *et al.*, 2005) and in a rat model of parkinsonism induced by 6-hydroxydopamine (6-OHDA) lesion of the substantia nigra (Ingham *et al.*, 1998; Arbuthnott *et al.*, 2000; Meshul *et al.*, 2000). Moreover, nigrostriatal dopamine depletion in animal models affects striatal long-term depression (Partridge *et al.*, 2000) and long-term potentiation (Centonze *et al.*, 1999; Picconi *et al.*, 2004; Norman *et al.*, 2005).

Both synaptic and morphological plasticity in hippocampal neurons are modulated by intracellular signalling proteins, such as calcium–calmodulin-dependent protein kinase II (CaMKII) and related proteins (Smart & Halpain, 2000; Winder & Sweatt, 2001; Lisman *et al.*, 2002; Colbran & Brown, 2004). The association of CaMKII, protein phosphatase 1 (PP1), spinophilin and neurotransmitter receptors with synapses and postsynaptic densities (PSDs) also is dynamically regulated (Malinow, 2003; Colbran, 2004a; Colbran, 2004b; Griffith, 2004; Schulman, 2004). The morphological and functional effects of striatal dopamine depletion presumably result from changes in the expression and / or function of dendritic cytoskeletal and signalling proteins.

Acute dopamine signalling modulates both *N*-methyl-D-aspartate (NMDA)- and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors (Svenningsson *et al.*, 2004). Although the effects of chronic dopamine insufficiency associated with Parkinson's disease are poorly understood, studies in animal models have demonstrated modest changes in levels of some glutamate receptor subunits and of PSD-95 in synaptic membranes (Porter *et al.*, 1994; Oh *et al.*, 1999; Dunah *et al.*, 2000; Betarbet *et al.*, 2004; Picconi *et al.*, 2004), as well as altered phosphorylation of the NMDA receptor NR2B, NR2A and NR1 subunits (Menegoz *et al.*, 1995; Oh *et al.*, 1999; Dunah *et al.*, 2000). However, most of these previous studies analysed whole striatal samples from relatively

young animals (3–6 months), typically within 6 weeks of dopamine depletion. Notably, Parkinson's disease is a progressive disease induced by chronic striatal dopamine depletion, most often in elderly individuals. Normal ageing modifies synaptic plasticity in the striatum and in other brain regions (Ou *et al.*, 1997; Rosenzweig & Barnes, 2003), and also affects intracellular signalling proteins (Norris *et al.*, 1998; Foster *et al.*, 2001; Foster *et al.*, 2003). Thus, the full manifestation of Parkinson's disease may result from the combined effects of dopamine depletion and normal ageing.

Here, we report novel changes in signalling pathways of the dorsolateral striatum that occur in a graded manner following 6-OHDA lesion of the nigrostriatal pathway. Enduring elevations in the phosphorylation of CaMKII and dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) are seen within 3 weeks, whereas phosphorylation of GluR1 increases only after several months of dopamine depletion.

Materials and methods

6-OHDA lesion surgery

Male Sprague-Dawley rats (Harlan; Indianapolis, IN, USA) were housed under a 12 : 12 light : dark cycle with food and water freely available. Experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH), under the oversight of the Institutional Animal Care and Use Committee. Rats (3 months old) were anaesthetized with ketamine and xylazine, and 6-OHDA HBr (4 μg / μL free base in 0.02% ascorbate) was infused into two sites in the substantia nigra (AP, −5.3; L, 2.3 and 1.0; DV, −8.3; Paxinos & Watson, 1986), at a rate of 0.25 μL / min. Shamlesioned rats received injections of vehicle alone. Age-matched noninjected rats were also studied. At the times indicated after surgery, rats were lightly anaesthetized with isoflurane, decapitated and the brain removed. Data reported here were obtained from five batches of 6–8 rats each that were killed 3 and 6–12 weeks, and 9, 11 and 18–20 months after 6-OHDA lesion surgery. For presentation purposes, we pooled data obtained from animals 3–12 weeks and 9–11 months after surgery. There were no statistically significant differences between batches of animals within each set of pooled data. Moreover, differences observed in the pooled data were observed in both individual groups of animals at each time point.

L-DOPA treatment

Rats 6–12 weeks after 6-OHDA lesion were treated with levodopa (L-DOPA) methyl ester and benserazide (50 and 12.5 mg / kg, i.p.) or benserazide alone every 12 h for 9 days, then were killed (as indicated above) 16 h after the last injection. This L-DOPA treatment paradigm induced a characteristic increase in the contralateral rotation frequency by the 9th day of treatment (data not shown), consistent with previous reports (Schwarting & Huston, 1996). This repeated injection paradigm was designed to mimic the effects of repeated L-DOPA administration to Parkinson's disease patients.

Dorsolateral striatal tissue homogenates

Punches (1.15 mm ID) of dorsolateral striatum were removed from both hemispheres of 1.0-mm-thick coronal slices at the level of the crossing of the anterior commissure, and flash-

frozen on dry ice within 2 min of decapitation. Tissue punches were stored at −80 °C. We confirmed that this procedure minimized postmortem changes in CaMKII phosphorylation (Suzuki *et al.*, 1994; Lengyel *et al.*, 2001).

Whole striatal extracts and subcellular fractionation

Whole extracts were prepared by sonicating striatal tissue punches in 200–300 μL 2% SDS with 10 μg / mL leupeptin and 1 μg / mL pepstatin. PSD-enriched fractions were prepared by homogenizing frozen striatal punches in ice-cold 7 mM Tris–HCl, pH 7.5, containing EDTA, 0.2 mM; EGTA, 0.2 mM; sucrose, 320 mM; benzamidine, 1 mM; aprotinin, 10 μg / mL; leupeptin, 10 μg / mL; pepstatin, 10 μM; microcystin, 1 μM; cypermethrin, 0.5 nM; and $NaVO_4$, 1 mM; using a Kontes tissue homogenizer. After centrifugation (1000 ***g*** for 7 min at 4 °C) to separate a pellet fraction (P1) enriched in nuclei, Triton X-100 (1% final) was added to the supernatant fraction (S1). After mixing gently for 30 min at 4 °C, S1 samples were re-centrifuged (100 000 ***g*** for 1 h at 4 °C) to separate a supernatant (S2) enriched in cytosolic and detergent-soluble membrane proteins, and a pellet (P2) enriched in cytoskeletal elements including PSDs. Protein concentrations of samples were determined by the method of Lowry (Lowry *et al.*, 1951).

Antibodies

The following primary antibodies were used for immunoblotting: goat anti-CaMKII α / β (McNeill & Colbran, 1995; 1 : 4000), mouse anti-CaMKIIα (ABR, 1 : 4000), rabbit anti-phospho-Thr^{286}-CaMKIIα (Promega, 1 : 2500), rabbit anti-DARPP-32 (Cell Signalling, 1 : 4000), rabbit anti-phospho-Thr^{34}-DARPP-32 (Cell Signalling, 1 : 250), rabbit anti-phospho-Thr^{75}-DARPP-32 (Cell Signalling, 1 : 500), rabbit anti-GluR1 (Upstate, 1 : 4000), rabbit anti-phospho-Ser^{831}-GluR1 (Upstate, 1 : 500), rabbit anti-phospho-Ser^{845}-GluR1 (Upstate, 1 : 2000), rabbit anti-neurabin (1 : 2500) (MacMillan *et al.*, 1999), mouse anti-NR1 (Chemicon, 1 : 3000), rabbit or mouse anti-NR2B (Molecular Probes, 1 : 500), sheep anti-PP1γ1 (1 : 1000; Colbran *et al.*, 2003), mouse anti-PSD-95 (Upstate, 1 : 1000), rabbit anti-spinophilin (1 : 2000; MacMillan *et al.*, 1999) and mouse anti-tyrosine hydroxylase (ImmunoStar, 1 : 1000). Secondary antibodies were from Jackson Immunoresearch (goat anti-rabbit AP, 1 : 1000), Sigma (rabbit anti-mouse AP, 1 : 2000), Promega (goat anti-mouse HRP, 1 : 2000; goat anti-rabbit HRP, 1 : 4000), Vector Laboratories (rabbit anti-goat AP, 1 : 2000) or Alpha-Quest (rabbit anti-goat HRP, 1 : 4000).

Immunoblots

Samples (20–40 μg protein per lane) were fractionated by SDS-PAGE and transferred to nitrocellulose membranes, which were stained with Ponceau-S (Sigma) and then digitally scanned. After blocking, membranes were probed with the indicated primary antibodies: phosphorylation site-specific primary antibodies were incubated overnight at 4 °C, whereas other primary antibodies were incubated for 2 h at room temperature. Membranes were then washed and incubated for 1 h at room temperature with the alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibodies. Alkaline phosphatase-conjugated secondary antibodies were detected with 5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt (BCIP; Pierce) and nitroblue tetrazolium chloride (NTB; Pierce). Horseradish

peroxidase-conjugated secondary antibodies were detected with enhanced chemiluminescence (Perkin Elmer) and multiple X-ray film exposures to ensure that signals were within the linear range. In some cases primary and HRP-conjugated secondary antibodies were stripped from the membrane by incubation in stripping buffer (Tris–HCl, 62.5 mM, pH 7.5; SDS, 2%; and 2-mercaptoethanol, 0.8%) for 1 h at 50 °C. Stripping efficiency was confirmed by subsequent incubation with secondary antibody and development. Membranes were then incubated in PBST blocking buffer for 1 h prior to reprobing with different primary antibodies.

Quantification of immunoblots and statistical analyses

Optical densities of total protein loaded in each gel lane (Ponceau S-stained membranes) and specific immunoblotted proteins were measured using NIH Image 1.6 (http://www.rsb.info.nih.gov/nihimage). Immunoblotted protein band densities in each lane were normalized to total protein loaded in the corresponding lane to yield a 'normalized immunoblot signal'. For analysis of PSD-enriched fractions, the relative total mass of the specific protein in each sample was obtained by correcting the normalized immunoblot signals for the volume of P2 fraction loaded on each lane and the total volume of the corresponding P2 fraction.

Only rats with > 90% depletion of striatal tyrosine hydroxylase (TH) relative to the intact contralateral striatum were included in statistical analyses. Statistical comparisons of normalized immunoblot signals were performed using either one- or two-way ANOVA with Scheffé's *post hoc* tests or *post hoc t*-tests, as indicated. For graphing purposes, the mean normalized immunoblot signal obtained from the intact hemisphere of 6-OHDA-lesioned rats was set at 100% and values from individual samples were expressed relative to this value.

Results

Dopamine depletion increased Thr^{286} phosphorylation of CaMKIIα

Striatal TH levels were decreased by ≈95% at 3–12 weeks after unilateral 6-OHDA injections into the substantia nigra (Fig. 1A). No changes in total levels of several synaptic proteins were observed, including NMDA receptor NR2B subunit, synaptophysin, CaMKIIα and CaMKIIβ (Fig. 1A and B). In contrast, Thr^{286} phosphorylation of CaMKIIα was significantly increased by dopamine depletion relative to both the contralateral intact striatum and to control striatum from sham-lesioned or nonlesioned rats (Fig. 1B).

To test the effects of dopamine replacement on CaMKIIα phosphorylation, 6-OHDA-lesioned rats were treated with either vehicle or L-DOPA for 9 days (see Materials and methods). In rats that received vehicle injections, Thr^{286} phosphorylation was significantly elevated in the lesioned striatum relative to intact striatum. However, Thr^{286} phosphorylation in both hemispheres of L-DOPA-injected rats was similar to that in the intact striatum from vehicle-injected rats; i.e. L-DOPA treatment completely reversed the increase in Thr^{286} phosphorylation (Fig. 1C). However, L-DOPA did not significantly change the total levels of TH, CaMKIIα, CaMKIIβ, PP1γ1 or spinophilin (data not shown).

PSD-enriched cytoskeletal fractions from the striatum of 6-OHDA-lesioned rats killed 3 weeks after surgery were also analysed by immunoblotting. As seen in whole striatal extracts, Thr^{286} phosphorylation of CaMKIIα in the PSD-enriched fractions was significantly elevated. However, total levels of PSD-associated CaMKIIα (but not CaMKIIβ) were slightly but significantly decreased (Fig. 2).

Effect of dopamine depletion on striatal phosphatases and PP1 regulatory proteins

Increased Thr^{286} phosphorylation of CaMKIIα following dopamine depletion may result from altered regulation of striatal protein phosphatases, major targets of dopamine signalling (Svenningsson *et al.*, 2004). We observed no significant changes in total levels of $PP1\gamma_1$ or protein phosphatase 2A (PP2A) catalytic subunits, or of the PP1 scaffolding proteins spinophilin and neurabin 3–12 weeks postoperatively (Fig. 3A). Moreover, there were no significant changes in the amounts of $PP1\gamma_1$ or spinophilin associated with the PSD-enriched cytoskeletal fraction (Fig. 2). Interestingly, while levels of total DARPP-32 remained unchanged, the phosphorylation of DARPP-32 at Thr^{75} (but not Thr^{34}) was markedly increased in dopamine-depleted striatum (Fig. 3B). The repeated L-DOPA injection paradigm (see Materials and methods) completely reversed the increase in Thr^{75} phosphorylation (Fig. 3C).

Effects of ageing and striatal dopamine depletion

Because Parkinson's disease is associated with ageing, the effects of ageing on signalling proteins in normal and dopamine-depleted dorsolateral striatum were assessed. There were no significant differences in levels of CaMKIIα, phospho-Thr^{286}-CaMKIIα, phospho-Thr^{34}-DARPP-32, phospho-Thr^{75}-DARPP-32, PP2A, protein phosphatase 2B (PP2B), PSD-95, NMDA receptor subunits or CaMKIIβ in striatum from control rats at 4–6, 12–14 or 21–23 months of age (see Supplementary material, Fig. S1, A, C and D). However, there was a significant increase in $PP1\gamma_1$ and a significant decrease in spinophilin and neurabin in control rats at 21–23 months of age (Fig. 4). In addition, there was a trend for decreasing levels of DARPP-32 with normal ageing, but this was only statistically significant at 12–14 months of age (Fig. 4).

We also analysed dorsolateral striatum from rats killed 9–11 or 18–20 months after 6-OHDA injections (i.e. at 12–14 or 21–23 months of age). Total levels of multiple signalling proteins were unchanged after chronic dopamine depletion (Fig. 5A–C; supplementary Fig. S2). However, the elevated phosphorylation of CaMKIIα at Thr^{286} and of DARPP-32 at Thr^{75} seen 3–12 weeks postoperatively was also detected at both later time points (Fig. 5).

Effects of dopamine depletion on AMPA receptor phosphorylation

Because ageing and chronic dopamine depletion yield complex changes in the levels and phosphorylation of CaMKIIα, $PP1\gamma_1$ and PP1 regulatory proteins, we examined a common downstream target of these enzymes, the GluR1 subunit of the AMPA-type glutamate receptor. There were no changes in the levels of GluR1 or of the phosphorylation of GluR1 at Ser^{831} or Ser^{845} during normal ageing (4–23 months of age; supplementary Fig. S1, B). Dopamine depletion had no significant effect on the levels of total GluR1 in whole striatal extracts at any time point (Fig. 6), or in PSD-enriched fractions (Fig. 2). Phosphorylation of

GluR1 at Ser^{831} was unaltered 3–12 weeks postsurgery but was significantly elevated in dopamine-depleted striatum at both 9–11 and 18–20 months postoperatively (Fig. 6). In contrast, phosphorylation of GluR1 at Ser^{845} was not significantly different between the hemispheres at any time point after surgery (Fig. 6).

Discussion

Dopamine depletion increases Thr^{286} phosphorylation of CaMKIIα

Phosphorylation of CaMKIIα at Thr^{286} was significantly increased within 3 weeks of 6-OHDA lesion surgery in both total homogenates and PSD-enriched subcellular fractions of dorsolateral striatum (Figs 1 and 2). A recent study using whole striatum detected enhanced Thr^{286} phosphorylation of CaMKIIα in a PSD-enriched fraction but not in the total homogenate (Picconi *et al.*, 2004). Notably, dorsolateral striatum receives the densest nigrostriatal dopamine inputs (Nakano *et al.*, 2000), perhaps suggesting that this region will be most severely affected by 6-OHDA lesion of the substantia nigra. Thus, differences between these studies may reflect the analysis of different parts of the striatum, or the choice of different rat strains.

Thr^{286} phosphorylation of CaMKIIα confers autonomous (Ca^{2+}–calmodulin-independent) kinase activity (Hudmon & Schulman, 2002; Colbran & Brown, 2004) and stabilizes CaMKII binding to PSD proteins and synapses in hippocampal neurons (Strack *et al.*, 1997; Yamauchi & Yoshimura, 1998; Shen & Meyer, 1999; Colbran, 2004a). Therefore, it was surprising that total CaMKIIα levels in the PSD-enriched fractions isolated from the dorsolateral striatum were decreased (Fig. 2). Significant changes in other PSD-associated protein levels were not detected, although quantitative ultrastructural studies may reveal more subtle changes in subcellular distribution. In addition, it should be noted that total CaMKIIα levels in PSD fractions from whole striatum were unaltered (Picconi *et al.*, 2004). A similar decrease in hippocampal PSD-associated total CaMKIIα in the face of enhanced Thr^{286} phosphorylation was also recently observed in a mouse model of Angelman's syndrome, which lacks an E6AP-ubiquitin ligase (Weeber *et al.*, 2003). However, it is important to note that CaMKIIα association with PSDs is regulated by additional mechanisms such as autophosphorylation at $Thr^{305\,/\,306}$ (Shen *et al.*, 2000; Elgersma *et al.*, 2002) and a protein kinase C-driven mechanism (Fong *et al.*, 2002), probably involving dynamic interactions of CaMKII with multiple binding partners in the PSD (Colbran, 2004a). Thus, mechanisms of CaMKII targeting to PSDs in striatum and the role of dopamine modulation warrant further investigation.

Mechanisms for increased Thr^{286} phosphorylation following dopamine depletion

Thr^{286} autophosphorylation of CaMKII is acutely induced by Ca^{2+} influx via voltage-gated calcium channels or NMDA receptors or by the release of intracellular Ca^{2+} stores (Hanson & Schulman, 1992). These effects of Ca^{2+} mobilization are opposed and reversed by multiple protein phosphatases (Colbran, 2004b). For example, PSD-associated CaMKII is selectively dephosphorylated by PP1, presumably the $PP1\gamma_1$ isoform that appears to be selectively targeted to dendritic spines by binding to spinophilin and / or neurabin (MacMillan *et al.*, 1999; Strack *et al.*, 1999; Terry-Lorenzo *et al.*, 2002; Carmody *et al.*,

2004; Bordelon *et al.*, 2005). Thus, increased Thr^{286} phosphorylation following dopamine depletion might be due to increased Ca^{2+} mobilization resulting from lack of modulation of corticostriatal excitatory inputs to MSNs, or due to inhibition of protein phosphatases acting on CaMKII.

Many acute effects of dopamine in the striatum are mediated by modulation of protein phosphatases, especially via the modulation of PP1 by DARPP-32 and spinophilin (Svenningsson *et al.*, 2004). However, we found no evidence for changes in the total levels of multiple phosphatase catalytic subunits or of PP1 regulatory proteins following dopamine depletion, in either whole dorsolateral striatal extracts or PSD-enriched subcellular fractions. DARPP-32 is phosphorylated at Thr^{34} by PKA following acute D1-like dopamine receptor activation (presumably decreasing PP1 activity) and dephosphorylated by PP2B in response to activation of D2-like dopamine receptors, as well as the NMDA-, AMPA- and mGluR5-type glutamate receptors (Svenningsson *et al.*, 2004; Nishi *et al.*, 2005). Surprisingly, but consistent with prior reports (Picconi *et al.*, 2003; Chergui *et al.*, 2004), there were no significant changes in Thr^{34} phosphorylation at any time point following 6-OHDA lesion. Thus, additional cellular mechanisms may normalize DARPP-32 phosphorylation at Thr^{34} after chronic dopamine depletion.

In contrast to the lack of changes in Thr^{34} phosphorylation, DARPP-32 phosphorylation at Thr^{75} was significantly elevated by dopamine depletion. Moreover, L-DOPA injections rescued this increase. Thr^{75} is phosphorylated by cyclin-dependent kinase 5 (cdk5) and dephosphorylated by PP2A. PP2A activity may be reduced following dopamine depletion due to the loss of a D1-like receptor-activated PKA-dependent activation of PP2A (Nishi *et al.*, 2000), or due to CaMKII-dependent inhibition of PP2A (Fukunaga *et al.*, 2000). It is possible that increased glutamatergic signalling following dopamine depletion also activates cdk5, contributing to the enhanced phosphorylation of DARPP-32 Thr^{75}.

In combination, our studies provide little evidence for alterations in protein phosphatases following dopamine depletion, although the data cannot rule out alterations in specific subtypes. Further studies will be required to determine the contributions of PP2A and cdk5 to the elevated phosphorylation of DARPP-32 following dopamine depletion, and whether these changes play a role in enhancing Thr^{286} phosphorylation of CaMKII. Alternatively, increased Thr^{286} phosphorylation of CaMKII following dopamine depletion may be due to stimulation of dendritic Ca^{2+} signalling, perhaps due to enhanced corticostriatal glutamatergic signalling. Additional studies will be required to resolve these issues.

Consequences of ageing and chronic dopamine depletion

Increased Thr^{286} phosphorylation of CaMKIIα is evident within 3 weeks of 6-OHDA lesion surgery and is maintained for up to 18 months. However, increased phosphorylation of a well-established CaMKII substrate, Ser^{831} in the AMPA-type glutamate receptor GluR1 subunit, did not become evident until 9–11 months and was maintained for up to 18 months. Thus, prolonged dopamine depletion can have biochemical consequences beyond those seen in the shorter-term studies that are typically performed.

GluR1 phosphorylation depends not only on CaMKII activity but also on the activity of opposing phosphatases, such as PP1 / PP2A and PP2B (Lee *et al.*, 2000; Snyder *et al.*, 2000; Vinade & Dosemeci, 2000). Total PP1 / PP2A and PP2B activities increase during ageing even though PP2A and PP2B protein levels remain constant (Norris *et al.*, 1998; Foster *et al.*, 2001; Foster *et al.*, 2003; see also supplementary Fig. S1, C). We found that, while total levels of the PP1γ_1 isoform increased significantly with ageing, the levels of spinophilin, neurabin and DARPP-32 decreased (Fig. 4). Thus, we hypothesize that in young rats, PSD-targeted PP1γ1 prevents the accumulation of Ser^{831}-phosphorylated GluR1 following dopamine depletion; reduced levels of PSD-associated CaMKIIα may also contribute to the lack of increase in Ser^{831}-phosphorylated GluR1. However, as levels of spinophilin and neurabin decrease with normal ageing, levels of PSD-targeted PP1 may decrease (despite the overall increase in PP1γ_1 levels), allowing accumulation of Ser^{831}-phosphorylated GluR1 in response to increased levels of Thr^{286}-phosphorylated CaMKII following dopamine depletion. Further studies are clearly warranted to understand the interplay between changes in signalling pathways induced by dopamine depletion and by normal ageing.

Relationship of observed changes to synaptic plasticity

Dopamine acutely regulates both CaMKII (Gu & Yan, 2004; Picconi *et al.*, 2004) and AMPA receptors (Snyder *et al.*, 2000; Chao *et al.*, 2002), and CaMKII regulates the unitary conductance and trafficking of AMPA-type glutamate receptors (Malinow & Malenka, 2002; Malinow, 2003; Allen, 2004). Moreover, normal synaptic plasticity requires phosphorylation of CaMKII at Thr^{286} (Lisman *et al.*, 2002; Colbran & Brown, 2004) and of AMPA receptor GluR1 subunits (Lee *et al.*, 2003). Although roles of CaMKII and GluR1 phosphorylation in the striatum are poorly understood, short-term striatal dopamine depletion disrupts multiple forms of striatal synaptic plasticity (Centonze *et al.*, 1999; Partridge *et al.*, 2000; Norman *et al.*, 2005). Recently, CaMKII inhibitors were shown to rescue a defect in striatal synaptic plasticity following short-term dopamine depletion (Picconi *et al.*, 2004). However, our data suggest that long-term dopamine depletion may enhance glutamate receptor-mediated transmission by increasing the levels of Ser^{831}-phosphorylated GluR1. It will be interesting to determine whether prolonged periods of dopamine depletion induce additional changes in striatal synaptic plasticity and whether CaMKII inhibitors are similarly effective after GluR1 phosphorylation has increased.

Relationship of observed changes to morphological alterations in MSNs

Dendritic morphology of cortical and hippocampal neurons is sensitive to changes in expression of many proteins, including CaMKIIα, PSD-95, neurabin and spinophilin (Feng *et al.*, 2000; Oliver *et al.*, 2002; Jourdain *et al.*, 2003; Lee *et al.*, 2004; Li *et al.*, 2004; Tang *et al.*, 2004). Given the morphological changes associated with striatal dopamine depletion in Parkinson's disease and in animal models of parkinsonism (Ingham *et al.*, 1998; Arbuthnott *et al.*, 2000; Meshul *et al.*, 2000; Zaja-Milatovic *et al.*, 2005), it is somewhat surprising that total striatal levels of all proteins analysed here were unchanged, even after prolonged dopamine depletion. However, our observations confirm and extend previous studies demonstrating a lack of changes in GluR2 / 3, α-actinin-2 and PSD-95 following 6-OHDA lesion (Dunah *et al.*, 2000), although some studies report changes in levels of other glutamate receptor subunits (Porter *et al.*, 1994; Oh *et al.*, 1999; Dunah *et al.*, 2000; Betarbet

et al., 2004; Picconi *et al.*, 2004). In contrast to the modest effects of dopamine depletion, decreased spinophilin levels (Fig. 4) may be associated with age-related losses of dendritic spines (Ingham *et al.*, 1989; Ou *et al.*, 1997). Thus, morphological changes which occur following dopamine depletion and during normal ageing are probably caused by different cellular mechanisms.

Relevance to Parkinson's disease

L-DOPA administration completely reversed increases in the phosphorylation of CaMKII and DARPP-32 at Thr^{286} and Thr^{75}, respectively. In our studies tissue was harvested 16 h following the final L-DOPA injection and the half-life of L-DOPA is ≈90 min. Thus, little (if any) L-DOPA would be present when animals were killed, consistent with the fact that L-DOPA did not affect phosphorylation of CaMKII or DARPP-32 in the intact (normal) striatum (Figs 1C and 3C). These data suggest that dopamine depletion induces significant sustained alterations in striatal signalling mechanisms that can be reversed by L-DOPA within a few weeks of dopamine depletion. This mechanism may contribute to the sustained therapeutic benefits of L-DOPA administration during initial phases of Parkinson's disease (Thanvi & Lo, 2004). Additional studies that examine signalling in normal and dopamine-depleted striatum at various times after 6-OHDA lesion surgery may provide useful insights into the evolving responses to dopamine replacement therapy in Parkinson's disease.

Idiopathic Parkinson's disease is associated with ageing. It will be important to determine whether changes in phosphorylation of CaMKII, DARPP-32 and GluR1 occur in Parkinson's disease, although such studies may be complicated by dephosphorylation of these proteins in *post mortem* human tissue. Nevertheless, to the best of our knowledge, the slow development of increased GluR1 phosphorylation at Ser^{831} following dopamine depletion represents the first report of unique biochemical effects of long-term (9–20 months) dopamine depletion in rodents. These data may be explained by a novel interaction between ageing and dopamine depletion, indicating that short-term dopamine depletion in animal models of parkinsonism may not fully recapitulate the human disease. Moreover, the evolving responses of signalling proteins following dopamine depletion may play a role in the progression of symptoms during Parkinson's disease, as well as in the changing efficacy and debilitating side-effects associated with dopamine replacement therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

6-OHDA	6-hydroxydopamine

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CaMKII	calcium–calmodulin-dependent protein kinase II
cdk5	cyclin-dependent kinase 5
DARPP-32	dopamine- and cAMP-regulated phospho-protein of 32 kDa
L-DOPA	levodopa
MSN	medium spiny neuron
NMDA	*N*-methyl-D-aspartate
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PSD	postsynaptic density
TH	tyrosine hydroxylase

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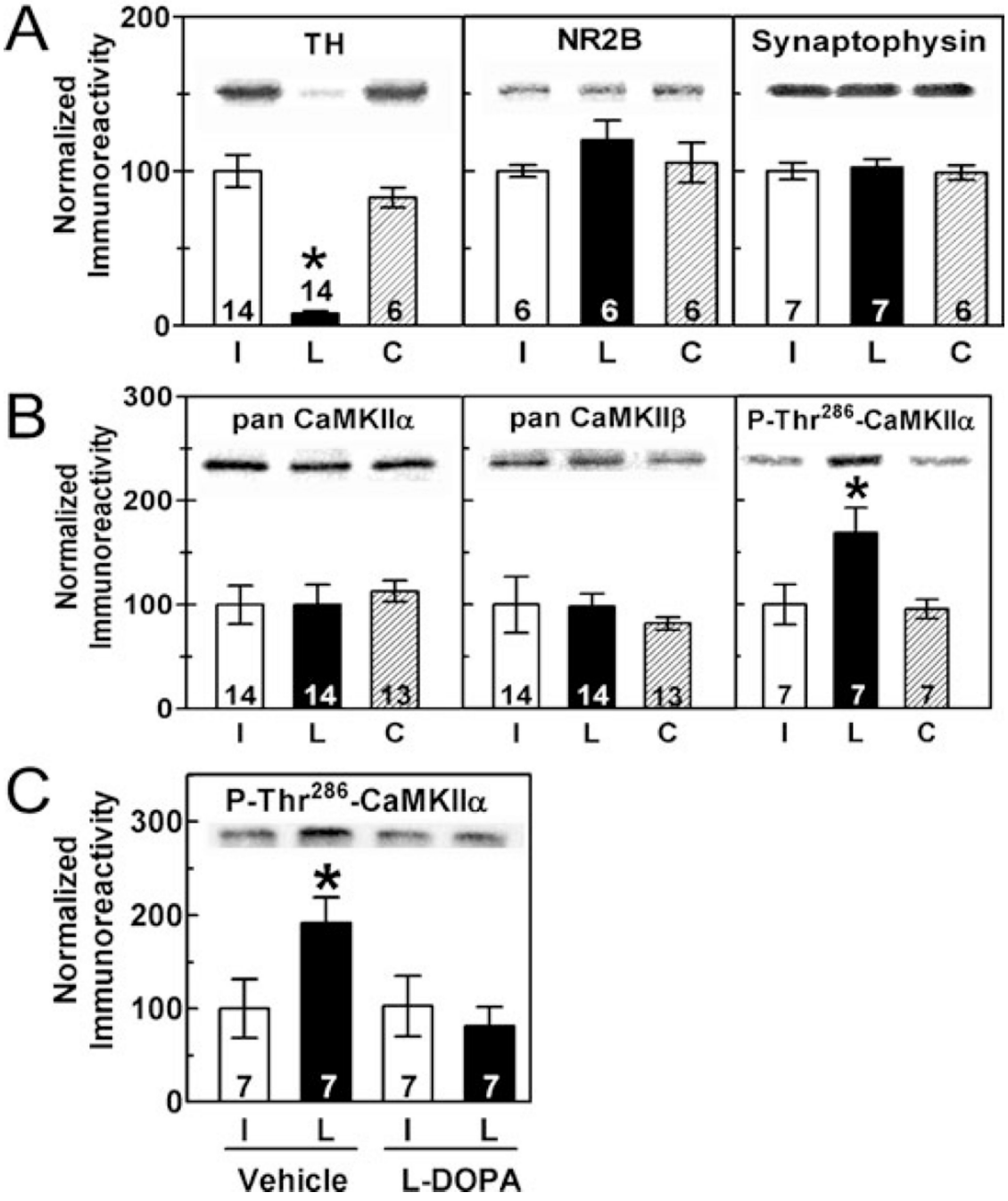

Fig. 1.

Short-term 6-OHDA lesion increased CaMKIIα phosphorylation at Thr^{286}. (A and B) Representative blots and summary graphs quantitating total striatal protein levels or phosphorylation (mean ± SEM) in samples harvested 3–12 weeks postoperatively. The number of rats analysed is indicated within or above each bar. ‘L’ (lesion) and ‘I’ (intact) indicate samples ipsilateral and contralateral to the lesion, respectively. ‘C’ indicates tissue from sham-lesioned rats. Only the decrease in TH ($F_{2,49}$ = 37.66, P < 0.0001) and the increase of P-Thr^{286}-CaMKIIα ($F_{2,21}$ = 11.98, P = 0.0003) were significantly altered in

dopamine-depleted striatum. (C) The increase in Thr^{286} phosphorylation of CaMKIIα was reversed by L-DOPA administration ($F_{1,27} = 5.61$, $P = 0.026$).

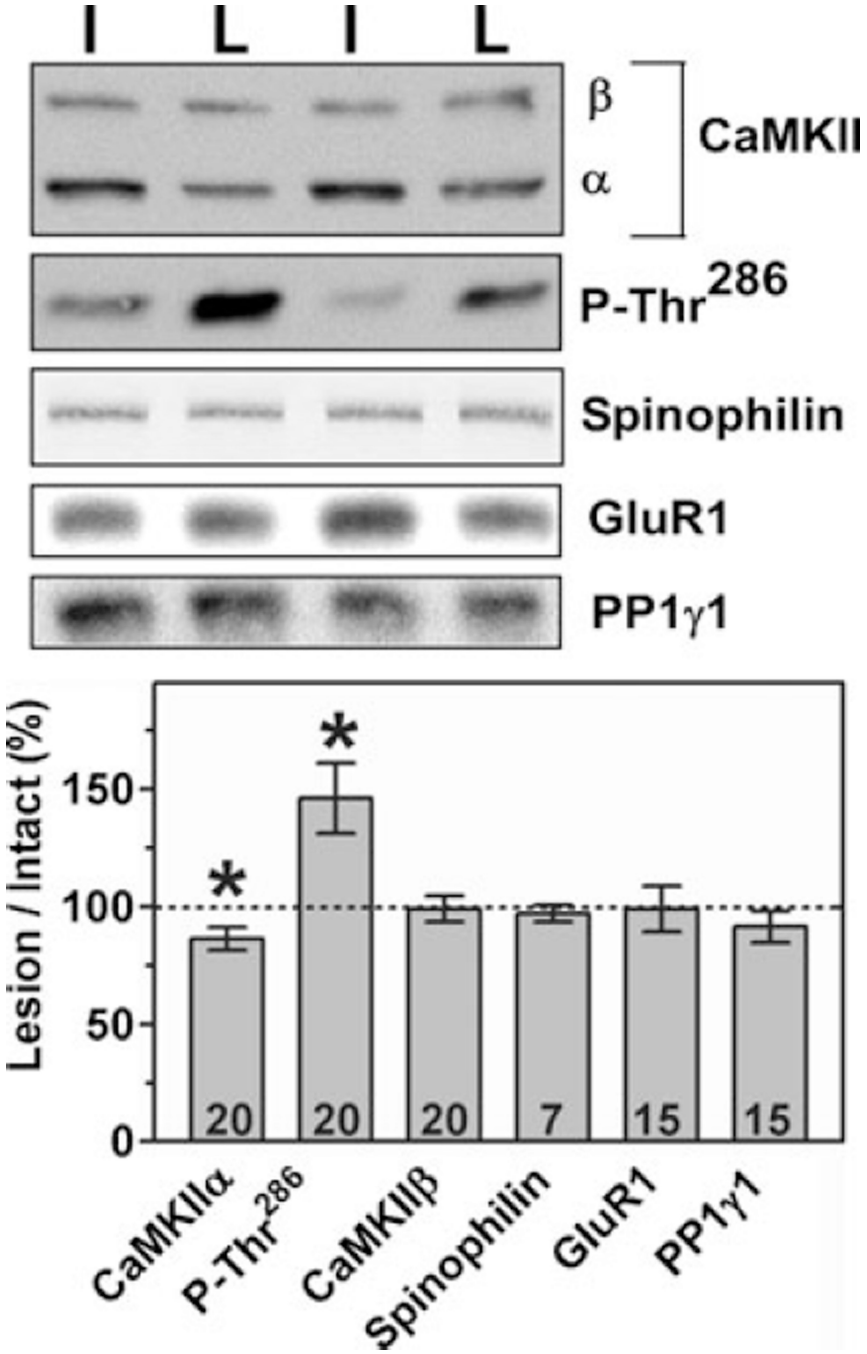

Fig. 2.

6-OHDA lesions changed PSD-associated CaMKIIα. PSD-enriched P2 fractions taken from rats 3 weeks after 6-OHDA lesion surgery were analysed by immunoblotting. The top panel shows representative blots from contralateral (intact) and lesioned hemispheres of two representative animals. The levels in samples from 6-OHDA-lesioned striatum are expressed as a percentage of levels in samples from the contralateral (intact) striatum (mean ± SEM). Total CaMKIIα was significantly decreased ($t_{18} = 2.92$, $P = 0.009$) and Thr^{286}-

phosphorylated CaMKIIα was elevated (t_{19} = 0.33, P = 0.006) following dopamine depletion, as determined by ANOVA followed by *post hoc t*-test.

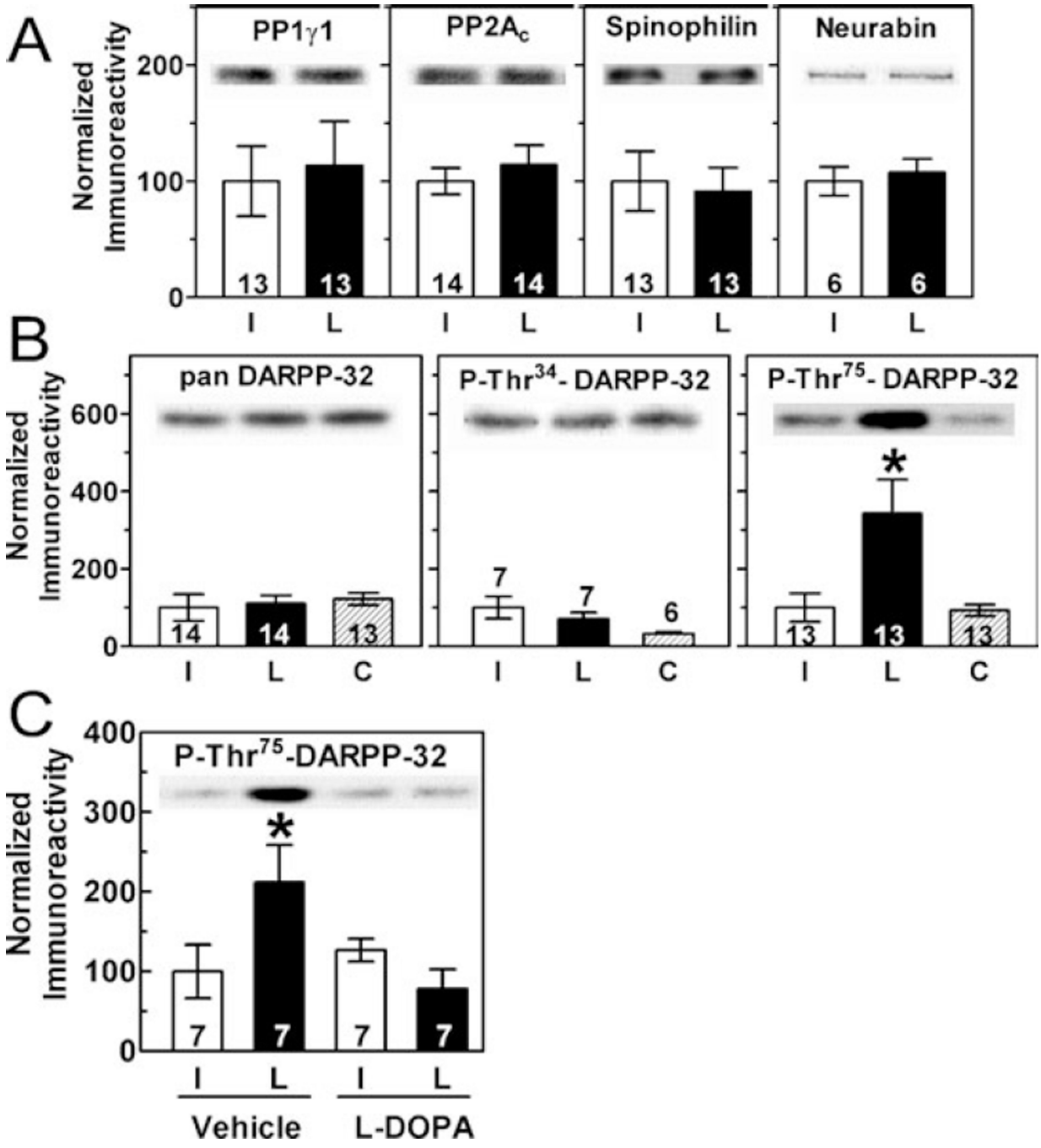

Fig. 3.

Effects of 6-OHDA lesion on striatal protein phosphatases. (A and B) Representative immunoblots and summary graphs quantifying total striatal levels of protein phosphatase catalytic subunits and PP1 targeting and regulatory proteins. Phosphorylation of DARPP-32 at Thr^{75} was significantly elevated in dopamine-depleted striatal samples ($F_{2,45} = 3.5$, $P = 0.038$). (C) The increase in Thr^{75} phosphorylation of DARPP-32 was reversed by L-DOPA administration ($F_{1,27} = 22.13$, $P = 0.0005$).

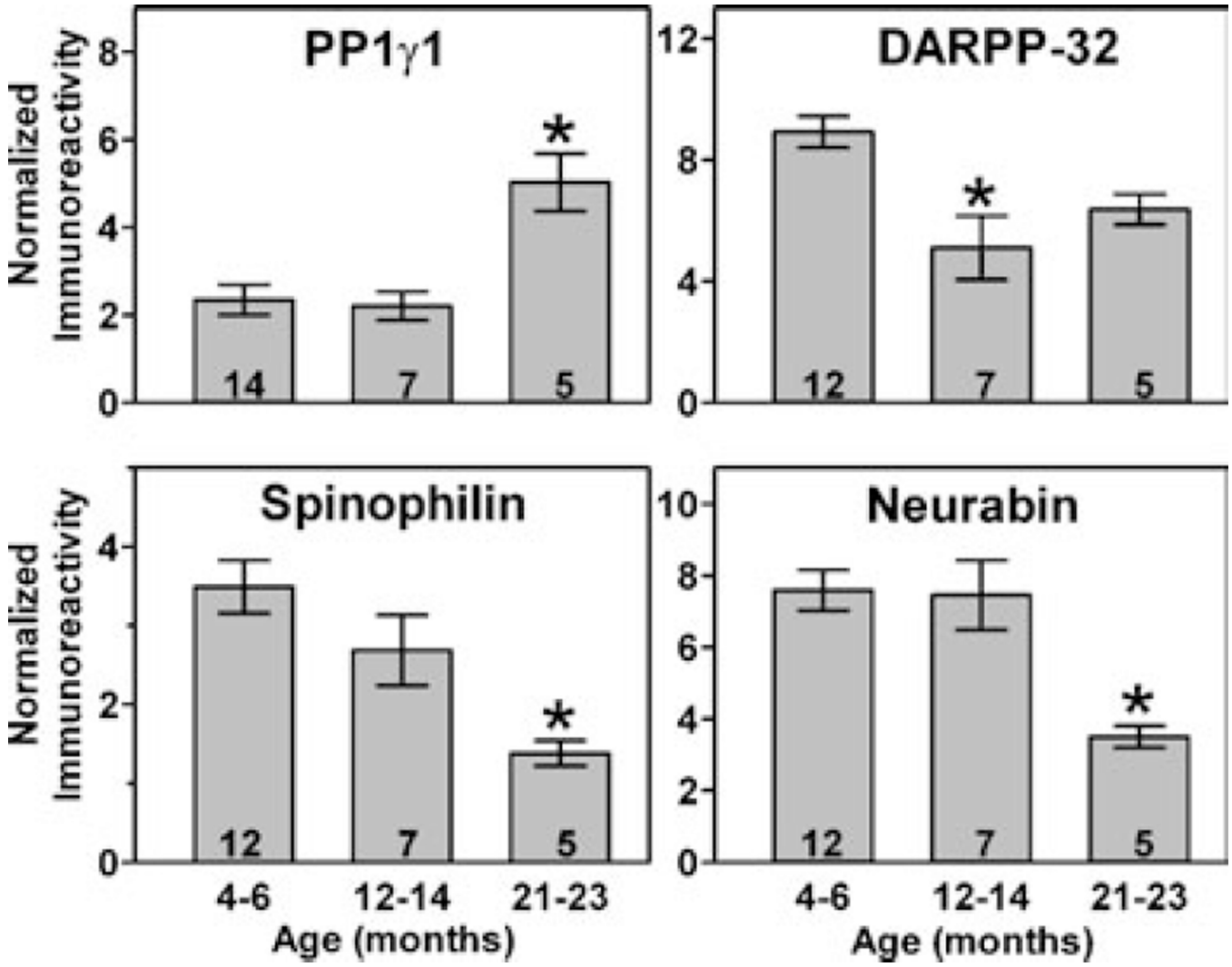

Fig. 4.

Changes in PP1γ1 and PP1-regulatory proteins during normal ageing. Quantification of protein levels in dorsolateral striatal homogenates from normal rats at 4–6, 12–14 or 21–23 months of age. PP1γ1 was elevated only at 21–23 months ($F_{2,47}$ = 12.02, P < 0.0001), DARPP-32 was decreased only at 12–14 months ($F_{2,45}$ = 7.98, P = 0.001), while both spinophilin ($F_{2,45}$ = 7.77, P = 0.001) and neurabin ($F_{2,43}$ = 10.46, P = 0.0002) were decreased at 21–23 months.

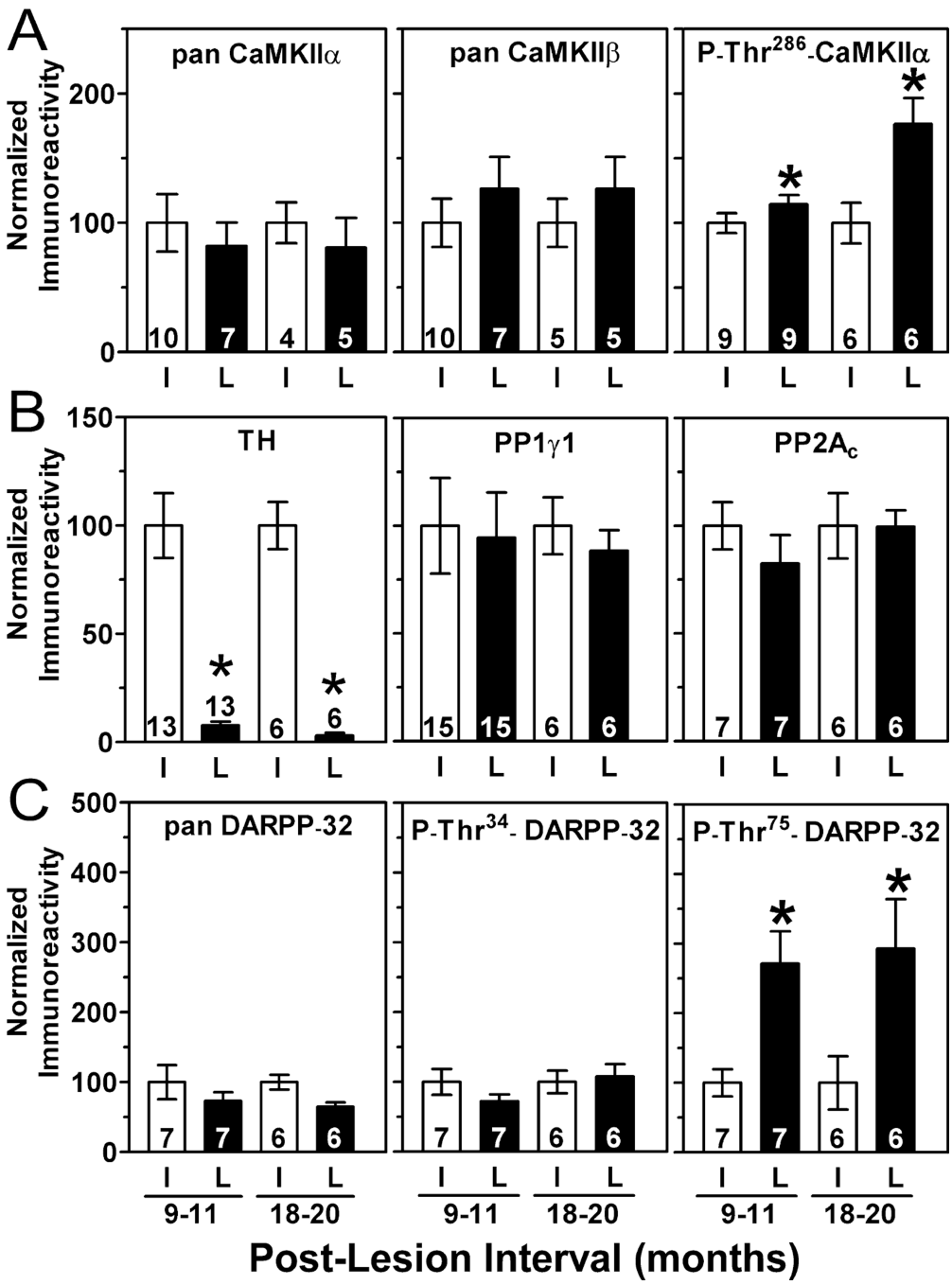

Fig. 5.

Effects of chronic dopamine depletion on striatal proteins. (A–C) Long-term dopamine depletion caused an enduring decrease in TH at 9–11 months (t_{12} = 6.82, P = 0.0001) and at 18–20 months (t_5 = 7.99, P = 0.0005). This was paralleled by an enduring increase in phosphorylation of both CaMKIIα at Thr^{286} at 9–11 months (t_{11} = 2.28, P = 0.043) and 18–20 months (t_5 = 3.50, P = 0.017) and of DARPP-32 at Thr^{75} at 9–11 months (t_6 = 5.03, P = 0.0024) and at 18–20 months (t_5 = 3.46, P = 0.018).

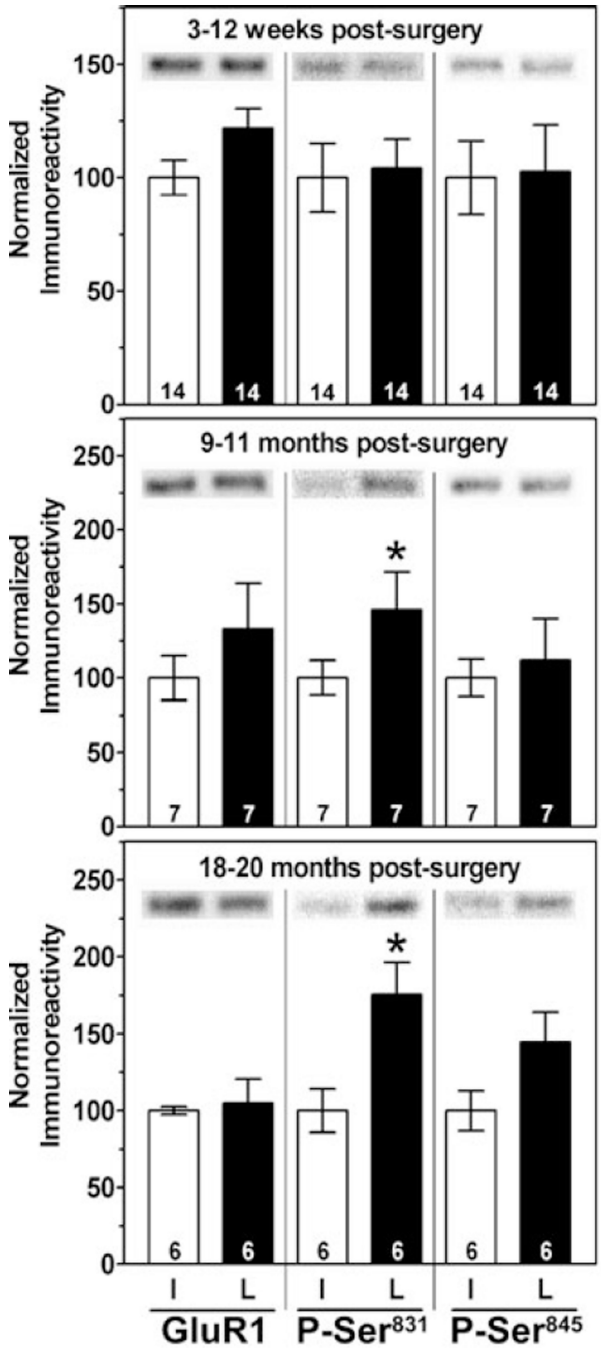

Fig. 6.

Chronic dopamine depletion selectively increased GluR1 phosphorylation at Ser^{831}. Total GluR1 levels and levels of GluR1 phosphorylated at Ser^{831} or Ser^{845} at 3–12 weeks, 9–11 months or 18–20 months following 6-OHDA lesion surgery. There was a significant effect of age ($F_{2,48}$ = 4.10, P = 0.0322 for Ser^{831}); *post hoc* tests revealed that Ser^{831} phosphorylation was increased at both 9–11 months (t_6 = 2.495, P = 0.047) and at 18–20 months (t_4 = 4.738,

$P = 0.009$) following dopamine depletion. In contrast, there was a trend for increased phosphorylation at Ser^{845} only 18–20 months following surgery ($t_5 = 2.490$, $P = 0.068$).