Protein phosphatase 1 modulation of neostriatal AMPA channels: regulation by DARPP-32 and spinophilin

Zhen Yan, Linda Hsieh-Wilson, Jian Feng, Kazuhito Tomizawa, Patrick B. Allen, Allen A. Fienberg, Angus C. Nairn and Paul Greengard

Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, 1230 York Avenue, New York, New York 10021, USA
Correspondence should be addressed to P.G. (greengd@ rockvax.rockefeller.edu)

Modulation of AMPA-type glutamate channels is important for synaptic plasticity. Here we provide physiological evidence that the activity of AMPA channels is regulated by protein phosphatase 1 (PP-1) in neostriatal neurons and identify two distinct molecular mechanisms of this regulation. One mechanism involves control of PP-1 catalytic activity by DARPP-32, a dopamine- and cAMP-regulated phosphoprotein highly enriched in neostriatum. The other involves binding of PP-1 to spinophilin, a protein that colocalizes PP-1 with AMPA receptors in postsynaptic densities. The results suggest that regulation of anchored PP-1 is important for AMPA-receptor-mediated synaptic transmission and plasticity.

The neostriatum is critically involved in the control of movement. The activity of neostriatal neurons is regulated by two major inputs: a glutamatergic input from the cortex and a dopaminergic input from the substantia nigra. Ionotropic glutamate receptors mediate synaptic transmission and plasticity in the neostriatum through intrinsic ligand-gated ion channels. In contrast, dopamine receptors modulate the excitability of neostriatal neurons by coupling to G-protein pathways. An understanding of the functional interactions between glutamate and dopamine systems in the neostriatum would have considerable clinical significance, because imbalances in dopaminergic and glutamatergic synaptic transmission have been implicated in several neurological disorders, including Parkinson's disease, Huntington's disease and schizophrenia[1–3].

Ionotropic glutamate receptors are classified as AMPA receptors, kainate receptors or NMDA receptors, based on their physiological and pharmacological properties. Each of these ligand-gated channels is an oligomeric complex composed of different subunits[4,5]. Functional AMPA receptors and NMDA receptors have been found in basal ganglion neurons[6]. Stimulation of corticostriatal fibers can induce AMPA-receptor-mediated long-term depression (LTD) in neostriatum[7,8]. Changes in postsynaptic AMPA receptors have been implicated in synaptic plasticity[9–11], but the underlying molecular mechanisms for the regulation of AMPA channels are unclear. One potential mechanism is alteration of the phosphorylation state of AMPA receptors. Protein kinase A (PKA) and calcium/calmodulin-dependent kinase II (CaMKII) potentiate AMPA receptor currents in hippocampal neurons[12–14], but the specific protein phosphatase responsible for the modulation of AMPA channels has not been identified[13].

High levels of dopamine receptors are found in neostriatal neurons[15,16]. Dopamine receptors are grouped into two classes: D_1-class (D_1, D_5) and D_2-class (D_2, D_3, D_4), based on their distinct pharmacological properties and signaling cascades. Activation of different dopamine receptors modulates a variety of voltage-dependent and ligand-gated channels in neostriatum[17–19]. Glutamatergic transmisson is regulated by dopamine in neostriatal neurons[20]. However, the signaling pathways mediating dopaminergic modulation of glutamate channels remain to be elucidated. In neostriatum, activation of D_1 receptors elevates PKA activity and increases the phosphorylation of DARPP-32, a dopamine- and cAMP-regulated phosphoprotein highly enriched in all dopamine-innervated neurons[21]. DARPP-32, in its phosphorylated but not dephosphorylated form, acts as a potent inhibitor of protein phosphatase 1 (PP-1)[22]. PP-1 is localized to postsynaptic densities[23], presumably through association with spinophilin, a novel PP-1-binding protein, which is highly enriched in dendritic spines[24].

The present study provides physiological and biochemical evidence that AMPA channels are modulated by PP-1 and that this modulation occurs through at least two distinct mechanisms. One mechanism involves the dopamine-induced phosphorylation of DARPP-32, inhibition of PP-1 activity and an increase in the phosphorylation state of AMPA channels. The second mechanism involves spinophilin-mediated anchoring of PP-1 to postsynaptic densities and facilitated dephosphorylation of AMPA channels.

Results

Whole-cell AMPA currents were recorded in acutely dissociated neostriatal medium spiny projection neurons and cholinergic interneurons. Kainate was selected as the ligand to elicit AMPA currents in voltage-clamped neurons. Kainate activates both AMPA and kainate receptors, but in mature neurons the large nondesensitizing component of the kainate-induced current is due to activation of AMPA receptors, whereas only a small transient component is due to activation of kainate receptors[25]. In neostriatal neurons, application of kainate (200 μM) evoked an inward current that was completely blocked by the non-NMDA receptor antagonist CNQX (10 μM, $n = 3$), confirming that the kainate-induced current was mediated mainly by AMPA receptors. Under control conditions, the amplitude of AMPA currents gradually declined over 10 minutes of recording to 72.5 ± 6.5% (mean ± standard deviation, $n = 18$) of the initial value (**Fig. 1a**). This 'rundown' (loss of activity) of glutamate-activated ion channels has been used as a bioassay to exam-

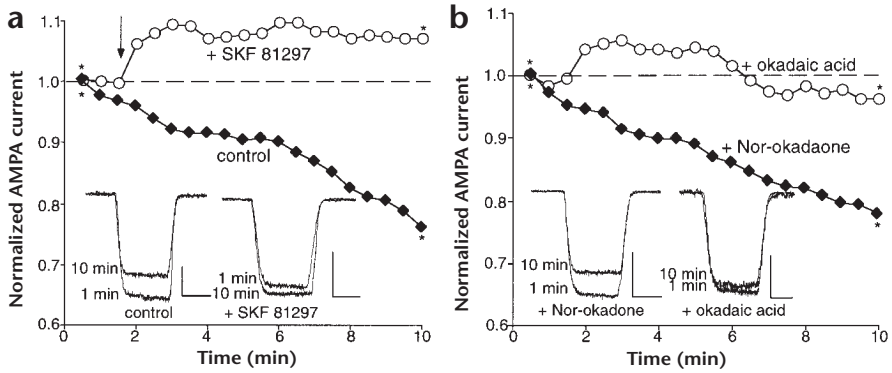

Fig. 1. Regulation of AMPA currents by a D_1-class dopamine receptor agonist and by a protein phosphatase inhibitor. **(a)** Time course of AMPA current amplitude in the absence (control) or presence of D_1-class agonist SKF 81297 (10 μM). The starting point for continuous application of SKF 81297 is marked by the arrow. SKF 81297 enhanced and stabilized AMPA currents. **(b)** Time course of AMPA current amplitude with okadaic acid (1 μM) or Nor-okadaone (1 μM) in the recording pipette. Dialysis with the PP-1/2A inhibitor okadaic acid, but not its inactive analog Nor-okadaone, blocked the gradual decrease in channel activity. Insets in (a) and (b), superimposed current traces at 1 and 10 min after the start of whole-cell recordings, as indicated by the asterisks. Scale bars, 0.5 nA and 1 s.

ine the molecular basis of channel regulation[13,26]. Bath application of the D_1-class dopamine receptor agonist SKF81297 (10 μM) increased (110.7 ± 6.1% at 3 min, $n = 15$) and stabilized (103.7 ± 5.6% at 10 min, $n = 5$; $p < 0.005$ compared to control at 10 min) AMPA currents (**Fig. 1a**).

We next investigated possible mechanisms by which activation of D_1-class dopamine receptors might regulate AMPA channel activity in neostriatal neurons, and in particular, whether the D_1/DARPP-32/PP-1 signaling cascade is involved in the modulation of AMPA channels in neostriatal neurons. Rundown of AMPA currents was prevented by intracellular application of the protein phosphatase (PP) 1/2A inhibitor okadaic acid (1 μM, 101.2 ± 10.6%, $n = 10$; $p < 0.001$ compared to control at 10 min), but not by its inactive analog Nor-okadaone (1 μM, 74.3 ± 8.4%, $n = 8$; **Fig. 1b**), indicating that endogenous protein phosphatase activity modulates the AMPA channel current. In contrast, application of the potent and selective calcineurin (PP2B) inhibitor cyclosporin A, in the presence of intracellular free calcium (20 nM), had no effect on AMPA channel rundown (data not shown).

The phospho-DARPP-32 peptide p-D32[6–38] (2 μM), derived from the PP-1 interaction region[27], also prevented the rundown of AMPA currents (98.6 ± 4.1%, $n = 6$; $p < 0.001$ compared to control at 10 min). In contrast, the dephospho-DARPP-32 peptide D32[6–38] (2 μM) was ineffective (78.5 ± 4.2%, $n = 4$; **Fig. 2a**). Biochemical analysis showed that the phospho-DARPP-32 peptide p-D32[6–38] potently inhibited PP-1 catalytic activity with an IC_{50} of 5 nM, whereas the dephospho-DARPP-32 peptide D32[6–38] was much less effective, with an IC_{50} of 250 μM (ref. 27; present study). Comparing the effects of the DARPP-32 peptides on PP-1 catalytic activity and on AMPA channels indicates that constitutively active PP-1 downregulates AMPA channels, and that this

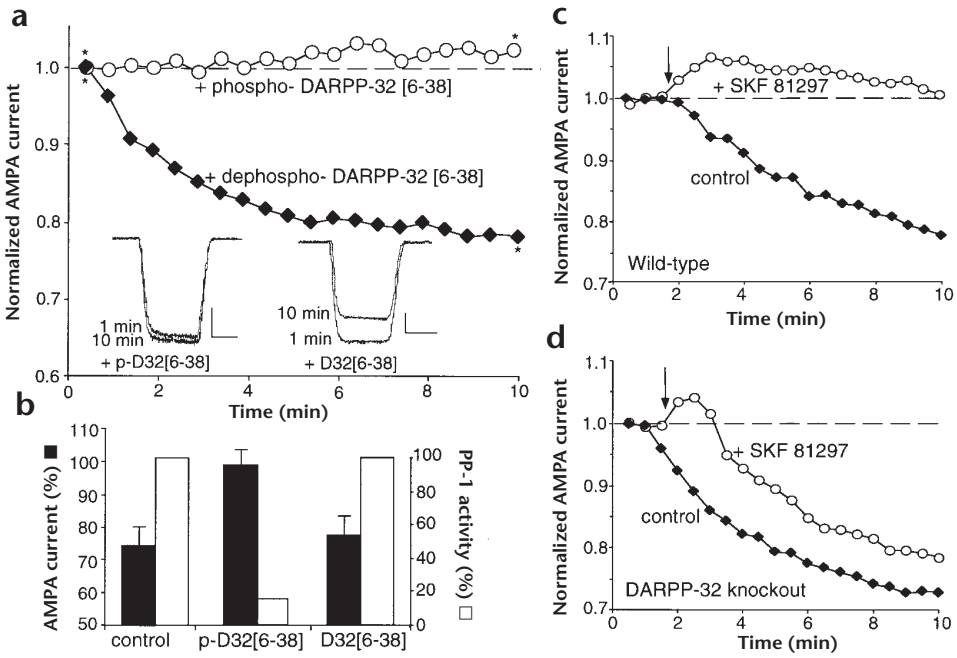

Fig. 2. Regulation of AMPA currents and PP-1 activity by DARPP-32. **(a)** Time course of AMPA current amplitude with phospho- or dephospho-DARPP-32 peptide [6–38] (2 μM) in the recording pipette. Dialysis with the phospho-DARPP-32 peptide, but not the dephospho-DARPP-32 peptide, blocked the gradual decline of channel activity. Insets, superimposed current traces at 1 and 10 min after the start of whole-cell recordings, as indicated by the asterisks. Scale bars, 0.5 nA and 1 s. **(b)** Double histogram showing the effect of phospho- and dephospho-DARPP-32 peptide [6–38] (2 μM) on AMPA current amplitude after 10 min of recording (filled bars) and on PP-1 catalytic activity (open bars). **(c, d)** Time course of AMPA current amplitude in the absence (control) or presence of SKF 81297 (10 μM) in wild-type (c) or DARPP-32-deficient (d) neostriatal neurons. The starting points for continuous application of SKF 81297 are marked by the arrows. SKF 81297 was able to stabilize AMPA currents in neurons from wild-type mice, but not from DARPP-32 knockout mice.

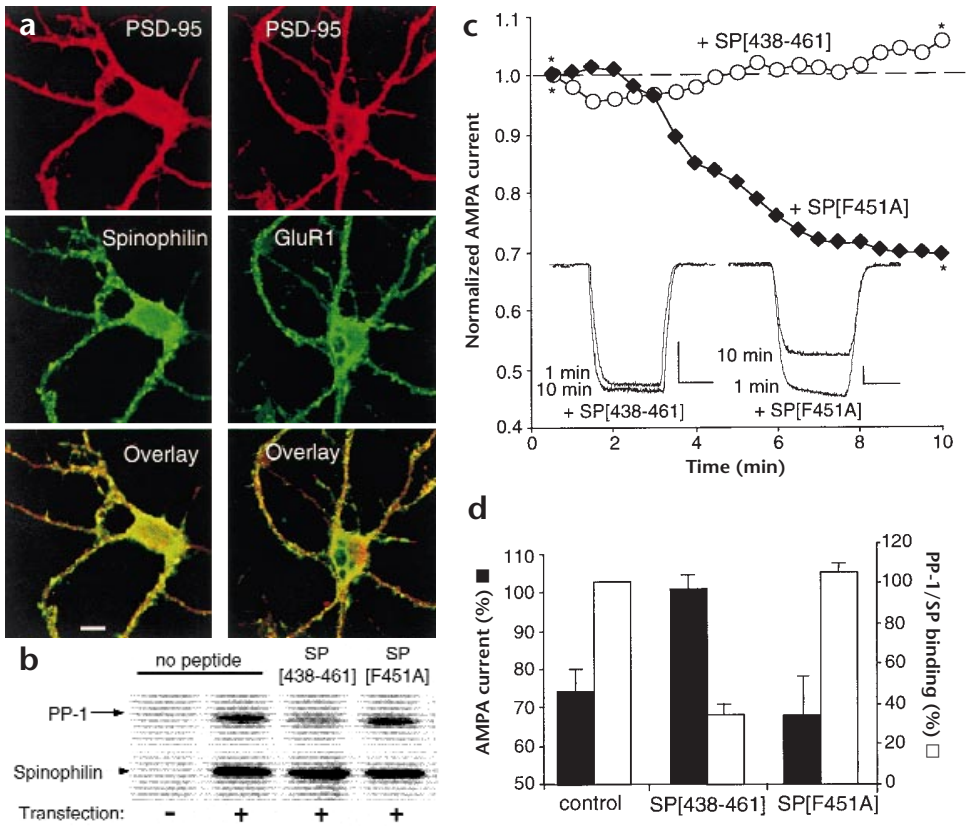

Fig. 3. Regulation of AMPA currents and of PP-1 anchoring by the spinophilin peptide. **(a)** Confocal images of double immunostaining with antibodies against PSD-95 (red) and spinophilin (green) or with antibodies against PSD-95 (red) and GluR1 (green) in cultured neostriatal neurons. Spinophilin (left) and GluR1 (right) colocalized with PSD-95 (a marker for postsynaptic densities), as indicated by the yellow puncta throughout the dendritic tree. Scale bar, 10 μm. **(b)** Effect of spinophilin peptides on the interaction of PP-1 with spinophilin. A lysate of spinophilin-transfected HEK293 cells was incubated in the absence or presence of spinophilin peptide SP[438–461] or control peptide SP[F451A] (100 μM), followed by immunoprecipitation with anti-spinophilin antibody and western blot analysis for PP-1 and spinophilin. SP[438–461], but not SP[F451A], disrupted the binding of PP-1 to spinophilin. **(c)** Time course of AMPA current amplitude with SP[438–461] or SP[F451A] (50 μM) in the recording pipette. Dialysis with SP[438–461], but not with SP[F451A], blocked the gradual decline of channel activity. Insets, superimposed current traces at 1 and 10 min after the start of whole-cell recordings, as indicated by the asterisks. Scale bars, 0.5 nA and 1 s. **(d)** Double histogram showing the effect of spinophilin peptides on AMPA current amplitude after 10 min of recording (filled bars) and on PP-1 binding to spinophilin (open bars).

modulatory effect can be blocked by inhibiting PP-1 activity with phosphorylated DARPP-32 (**Fig. 2b**).

To further test the involvement of DARPP-32 in the dopaminergic modulation of AMPA currents, neurons from wild-type mice and mutant mice with a targeted disruption of the DARPP-32 gene[28] were analyzed. Baseline amplitudes of AMPA currents in DARPP-32-deficient and wild-type neurons were similar. Under control conditions, rundown of AMPA channels in neostriatal neurons from both wild-type mice (75.8 ± 4.9%, $n = 8$; **Fig. 2c**) and DARPP-32 knockout mice (74.0 ± 5.8%, $n = 5$; **Fig. 2d**) resembled that found in rat cells (**Fig. 1a**). SKF81297 (10 μM) increased (110.6 ± 4.4% at 3 min, $n = 8$) and stabilized (101.2 ± 6.6% at 10 min, $n = 7$; $p < 0.005$ compared to control at 10 min) AMPA currents in wild-type mouse neurons (**Fig. 2c**) similar to its effects in rat (**Fig. 1a**). In contrast, in DARPP-32-deficient neurons, SKF81297 induced a transient increase (107.4 ± 3.6% at 3 min, $n = 8$) in AMPA currents, but failed to stabilize them (77.9 ± 4.7% at 10 min, $n = 7$; $p < 0.001$ compared to wild-type neurons with SKF81297; **Fig. 2d**). This transient increase in response to a D_1 agonist is consistent with evidence that AMPA channel activity is enhanced by direct PKA phosphorylation[12,29]. Furthermore, our results indicate that stabilization of AMPA channel activity involves the PKA/DARPP-32/PP-1 pathway.

In addition to its regulation by DARPP-32 and other small cytosolic inhibitory phosphoproteins, PP-1 is regulated by a variety of membrane-associated targeting proteins that serve to localize the enzyme to specific subcellular compartments and determine its substrate specificity[30]. PP-1 is highly enriched in dendritic spines[23], presumably via a targeting protein, spinophilin, which is concentrated in dendritic spines of neurons[24]. Immunocytochemical results revealed that spinophilin and the AMPA receptor subunit GluR1 colocalize with PSD-95 (a postsynaptic density marker), and therefore with each other, in cultured neostriatal neurons (**Fig. 3a**).

We tested the possibility that spinophilin, by forming a complex with the PP-1 catalytic subunit (PP-1c), anchors PP-1 in the vicinity of AMPA receptors, therefore facilitating the dephosphorylation of AMPA channels by this phosphatase. Binding experiments indicated that the region of spinophilin from residues 438 to 461 bound strongly to PP-1 (data not shown). This region of spinophilin contains a phenylalanine residue (F-451) important for the interaction of PP1c with PP1 binding proteins[31]. Therefore we tested peptide SP[438–461], derived from this region of spinophilin, for its ability to compete with spinophilin for the binding of PP-1 and for its possible effect on AMPA channel activity. For comparison, we used a control peptide, SP[F451A], which had a single point mutation at residue F-451.

To test whether spinophilin peptides can disrupt the binding of PP-1 to spinophilin, lysates of HEK293 cells that had been transfected with spinophilin were incubated in the absence or presence of spinophilin peptide SP[438–461] or control peptide SP[F451A], followed by analysis of PP-1 co-immunoprecipitated with spinophilin. The peptide SP[438–461], but not the control peptide SP[F451A], reduced the amount of PP-1 that was co-immunoprecipited with spinophilin (**Fig. 3b**). Quantitation of five experiments indicated that peptide SP[438–461] reduced the PP-1 that bound

to spinophilin by 65 ± 5%. In contrast to its ability to disrupt PP-1 binding to spinophilin, SP[438–461] (up to 1 mM) had no inhibitory effect on PP-1 catalytic activity (data not shown).

Rundown of AMPA currents was blocked by spinophilin peptide SP[438–461] (**Fig. 3c**; 50 μM, 99.0 ± 5.6%, $n = 11$; $p < 0.001$ compared to control at 10 min). In contrast, the control peptide SP[F451A] had no effect on the rundown of AMPA currents (68.0 ± 12.5%, $n = 8$), indicating that the action of spinophilin peptide SP[438–461] on AMPA channels was specific. Comparing the effects of the spinophilin peptides on the interaction of spinophilin with PP-1 and on AMPA channels (**Fig. 3d**) suggests that peptide SP[438-461], but not the control peptide SP[F451A], interferes with the dephosphorylation of AMPA channels by displacing PP-1c from the vicinity of these channels.

Discussion

Previous studies of transfected cell lines and hippocampal neurons found that activation of PKA potentiates AMPA currents[12,13,29], presumably by controlling the phosphorylation state of the AMPA channel subunit itself[29], or of a regulatory protein. In neostriatal neurons, the AMPA receptor GluR1 subunit is also phosphorylated by PKA (Gretchen Snyder and Paul Greengard, unpublished results), suggesting that neostriatal AMPA channel activity is regulated by phosphorylation and dephosphorylation. Based on the present study, we propose a hypothetical model involving two distinct mechanisms by which PP-1 may regulate AMPA channels. Constitutively active PP-1, anchored in the vicinity of AMPA receptors by spinophilin, keeps the channel in the dephosphorylated ('low activity') state. Activation of the D_1/PKA/DARPP-32 cascade converts AMPA channels to the phosphorylated ('high activity') state, through a synergistic action involving a direct phosphorylation of the channel and a DARPP-32/PP-1-mediated inhibition of dephosphorylation of the channel. On the other hand, dissociation of PP-1 from spinophilin and its removal from the vicinity of AMPA channels allows conversion of AMPA channels to the phosphorylated (high activity) state.

The dopaminergic regulation of AMPA channels is particularly relevant to the physiology of the neostriatum because it reveals a mechanism through which dopamine regulates the efficacy of glutamatergic neurotransmission. Activation of D_1-class dopamine receptors potentiated and stabilized AMPA currents. We attribute the effect of dopamine to increased phosphorylation and decreased dephosphorylation of AMPA channels. The results from the DARPP-32 knockout mice suggest that maintenance of AMPA currents requires the inhibition of PP-1 activity by phosphorylated DARPP-32. The D_1/DARPP-32/PP-1 cascade provides a new signaling pathway for regulating AMPA channel activity through phosphorylation. In addition, because PP-1 and AMPA channels are widely expressed throughout the brain, our results suggest that a variety of neurotransmitters may use signal transduction pathways, analogous to those used by dopamine, to modulate glutamatergic transmission in other brain regions.

Most protein kinases and protein phosphatases have relatively broad substrate specificity *in vitro*. It has become increasingly apparent that targeting/anchoring proteins endow signal-transduction pathways with specificity by locating enzymes in the vicinity of their substrates[32]. A well known example is the A-kinase anchoring protein (AKAP). The submembrane targeting of PKA by AKAP enhances cAMP-stimulated phosphorylation of AMPA/kainate channels and L-type calcium channels[33,34]. Likewise, each of the three major types of serine/threonine protein phosphatases (PP-1, 2A, 2B) is regulated by targeting subunits[32]. The study of PP-1 and PP-1 targeting subunits has been largely restricted to glycogen metabolism in skeletal muscle. G subunits anchor PP-1 to subcellular compartments and adapt the phosphatase activity to favor the dephosphorylation of certain substrates, thereby controlling glycogen synthesis[30]. Although high levels of PP-1 are present in the nervous system, the physiological functions of PP-1 and PP-1 anchoring proteins in neurons have remained elusive.

Our results indicate that targeting PP-1 to postsynaptic densities is crucial for the regulation of AMPA channel activity in neostriatal neurons. Spinophilin is highly enriched in dendritic spines[24], where AMPA receptors are concentrated[35,36], and it binds PP-1 with high affinity[24]. The ability of the spinophilin peptide SP[438–461] to disrupt the interaction between PP-1 and spinophilin and to stabilize AMPA currents supports the idea that spinophilin is responsible, at least in part, for the enrichment of PP-1 in spines, where it regulates the state of phosphorylation and activity of AMPA channels. Previous work showed that spinophilin inhibits PP-1 catalytic activity *in vitro*[24], whereas we show here that spinophilin, by targeting PP-1 to the vicinity of AMPA channels, facilitates the ability of PP-1 to dephosphorylate these channels. This contradiction is more apparent than real, and is common to all PP-1 targeting proteins. This paradox can be explained as follows. *In vivo* the PP-1/ spinophilin complex exists in a dynamic equilibrium. Spinophilin functions as a signaling scaffold to concentrate PP-1 in the proximity of AMPA channels. The transient dissociation of PP-1c from spinophilin allows the enzyme to dephosphorylate AMPA channels.

Methods

WHOLE-CELL RECORDINGS. Neostriatal neurons from 3-to-4-week-old rats or mice were acutely dissociated using procedures similar to those described[17,37]. Whole-cell current recordings used standard voltage-clamp techniques[38,18]. In each experiment, the cell type used for recording (neostriatal medium spiny neurons or cholinergic interneurons) was determined by its morphological appearance. The internal solution consisted of 180 mM N-methyl-d-glucamine (NMG), 40 mM HEPES, 4 mM $MgCl_2$, 5 mM 1,2 bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (BAPTA), 12 mM phosphocreatine, 2 mM Na_2ATP, 0.2 mM Na_3GTP and 0.1 mM leupeptin, pH 7.2–7.3 with H_2SO_4, 265–270 mosm/L. The external solution consisted of 135 mM NaCl, 20 mM CsCl, 1 mM $MgCl_2$, 10 mM HEPES, 0.001 mM TTX, 2.5 mM $BaCl_2$ and 10 mM glucose, pH 7.3 with NaOH, 300–305 mosm/L. Recordings were obtained with an Axon Instruments 200B patch clamp amplifier that was controlled and monitored with an IBM PC running pCLAMP (v. 6.0) with a DigiData 1200 series interface (Axon instruments, Foster City, California). Electrode resistances were typically 2–4 MΩ in the bath. After seal rupture, series resistance (4–10 MΩ) was compensated (70–90%) and periodically monitored. The cell membrane potential was held at –60 mV. Kainate (200 μM) was applied briefly (2–4 s) every 30 s. Drugs were applied with a gravity-fed 'sewer pipe' system. The array of application capillaries was positioned 250 μm from the cell under study. Solution changes were effected by altering the position of the array with a DC drive system controlled by a microprocessor-based controller (Newport-Klinger, Inc., Irvine, California). The time constant of the change in solutions was approximately 450 ms.

The amino-acid sequence for DARPP-32[6–38] peptide was RKKIQFSVPAPPSQLDPRQVEMIRRRRPTPAML. The phospho-DARPP-32[6–38] peptide, which was thio-phosphorylated at Thr-34, was prepared as described[27]. The amino-acid sequence for spinophilin peptide SP[438–461] was SEEEDPAPSRKIHFSTAPIQVFST. The spinophilin control peptide SP[F451A] had the same amino-acid sequence, except that Phe-451 was changed to Ala. DARPP-32 knockout mice were generated as described[28].

Data were analyzed with AxoGraph (Axon Instruments Inc., version 3.0), Kaleidagraph (Albeck Software, version 3.0.4) and Statview (Abacus Concepts, Inc., version 4.5). In all experiments, the amplitude of AMPA currents was compared to the initial value at 1 min. For statistical analysis, paired *t*-tests were used to compare groups.

PP-1 ACTIVITY ASSAY. To test the inhibitory potency of phospho- and dephospho-DARPP-32 peptides and spinophilin peptide SP[438–461] on PP-1 catalytic activity, purified PP-1c was assayed using [^{32}P]phosphorylase a as

substrate[39]. The reaction mixture contained 50 mM Tris.HCl, 0.15 mM EGTA, 15 mM 2-mercaptoethanol, 0.01% (wt/wt) Brij 35, 0.3 mg/ml BSA, 5 mM caffeine, 10 μM [^{32}P]phosphorylase a, various peptides and PP-1c. All components except PP-1c were preincubated at 30°C for 5 min. Dephosphorylation reactions were initiated by the addition of 10 μl of PP-1c, and reactions were carried out for 10 min at 30°C.

PP-1/SPINOPHILIN BINDING ASSAY. To test the capability of spinophilin peptide SP[438–461] and control peptide SP[F451A] to disrupt the binding of PP-1 to spinophilin, HEK293 cells were transiently transfected with 5 μg of spinophilin expression construct as described[24]. Sixty hours after transfection, cells were lysed in 0.5% Nonidet P-40, 0.1 mM EDTA, 50 mM Tris-HCl, 125 mM NaCl, 0.1 mM Na_3VO_4, 50 mM NaF and 1 mM phenylmethylsulfonyl fluoride (PMSF) on ice for 30 min. Cell lysates were centrifuged, and the supernatant fractions were incubated in the absence or presence of peptide SP[438–461] or peptide SP[F451A] (100 μM final concentration) for one hour at room temperature, followed by incubation with a polyclonal antibody against spinophilin[24] for one hour at 4°C. After incubation with protein A-sepharose, the immunoprecipitates were washed three times in lysis buffer. Proteins were separated by SDS/PAGE and analyzed by western blotting with anti-PP1α and anti-spinophilin antibodies, successively.

IMMUNOCYTOCHEMISTRY. Rat neostriatal cultures were prepared by modification of described methods[40]. Briefly, neostriatum was dissected from 20-day rat embryos, and cells were dissociated using trypsin and trituration through a Pasteur pipette. The neurons were plated on coverslips coated with poly-L-lysine in Dulbecco's Modified Eagle medium (DMEM) with 10% fetal calf serum at a density of 3000 cells/cm^2. After 4 hours, when neurons had attached to the coverslip, they were transferred to a dish containing a glial monolayer and maintained for up to three weeks in serum-free DMEM with B27 supplements.

For immunocytochemical staining, neurons were fixed at 18–21 days in culture in 4% paraformaldehyde and 4% sucrose in PBS for 20 min and were permeabilized with 0.3% Triton X-100 for 5 min. Following 1 h incubation with 10% bovine serum albumin (BSA) at 37°C to block nonspecific staining, the cultures were incubated with the primary antibodies in 1% BSA at 4°C overnight. Primary antibodies used included rabbit anti-GluR1 (1:1000), mouse anti-PSD-95 (5 μg/ml) (Upstate Biotechnology, Lake Placid, New York) and rabbit anti-spinophilin affinity-purified antibody[24] (1:2000). After primary antibodies were washed off, the cultures were incubated with rhodamine- or fluorescein-conjugated secondary antibodies (Sigma, 1:200) for 1 hour at 37°C. Fluorescent images were obtained using a Zeiss confocal microscope with a 100× oil lens.

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