Cell-type specific regulation of DARPP-32 phosphorylation by psychostimulant and antipsychotic drugs

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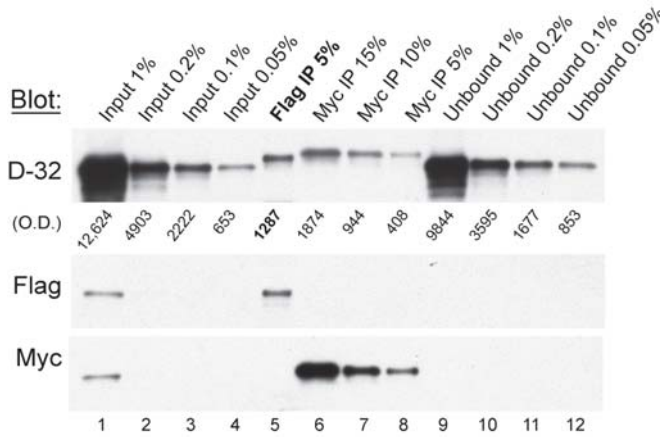
Supplementary Figure 1



Quantification of tagged DARPP-32 versus endogenous DARPP-32.

Striatal homogenates from D1R/D2R-DARPP-32 mice were incubated with Flag and Myc IP antibodies. To generate quantifiable data within the linear range of western blotting, we loaded a fixed percentage of Flag IP eluate (5%, lane 5) next to dilutions of the IP input, Myc IP, and unbound fractions, and blotted with DARPP-32, Flag and Myc antibodies. Note that there is no Flag or Myc tagged protein detectable in the unbound fractions; therefore the unbound signal in the DARPP-32 blot (top panel) corresponds to endogenous untagged DARPP-32.

We quantified the amount of DARPP-32 in 5% of the Flag IP (lane 5) compared to 1%, 0.2%, 0.1%, 0.05% dilutions of the unbound fraction (lanes 9-12). Quantification and comparison of optical density (O.D.) values revealed that 5% of the Flag IP'd DARPP-32 was equivalent to 0.075% of the unbound DARPP-32. This analysis was repeated with 4 different mice and yielded a similar value in all samples.

Based on this value, we calculated that there was 66.67 fold (= 5%/0.075%) more DARPP-32 in the unbound fraction compared to the Flag IP. After correction for an IP efficiency of 50% (= 66.67/2), we estimate that there was 33.3 times more endogenous DARPP-32 than Flag tagged DARPP-32 in these cells. In other words, the Flag-tagged DARPP-32 represented approximately 3% of the endogenous DARPP-32 protein. We also determined that 5% of the Flag IP'd DARPP-32 was equal to approximately 12% of the Myc IP'd DARPP-32, indicating about a 2.5 fold difference in expression between the Flag and Myc-tagged proteins. Comparison of Myc IP'd DARPP-32 and unbound DARPP-32 revealed a 90 fold difference in expression. Therefore, the Myc-tagged DARPP-32 represented approximately 1.10% of endogenous DARPP-32 protein.

Supplementary Methods

Genotyping primers for D1R-DARPP-32/Flag and D2R-DARPP-32/Myc mice.

Litters were screened for positive transgenics by PCR genotyping from tail DNA using the following primers:

D1R-DARPP-32/Flag mice:	Forward-AGGTCCTGAAAGGCAGCAG
	Reverse-CTTATCGTCGTCGTCCTTGTAGTC
D2R-DARPP-32/Myc mice:	Forward-GAGATGAAGAGGAGGACGA
	Reverse-CAGAAATCAATTTTTGTTCAGAGG

Striatal slice preparation.

Brains were rapidly removed and placed in ice-cold, oxygenated Krebs–HCO_3^- buffer (124 mM NaCl, 4 mM KCl, 26 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1.25 mM KH_2PO_4, 1.5 mM $MgSO_4$ and 10 mM d-glucose, pH 7.4). Coronal slices (350 μm) were prepared using a vibrating blade microtome, VT1000S (Leica Microsystems). Striata were dissected from the slices in ice-cold Krebs–HCO_3^- buffer. Each slice was placed in a polypropylene incubation tube with 2 ml fresh Krebs–HCO_3^- buffer containing adenosine deaminase (10 μg/ml). The slices were preincubated at 30 °C under constant oxygenation with 95% O_2/5% CO_2 for 60 min. The buffer was replaced with fresh Krebs–HCO_3^- buffer after 30 min of preincubation. Slices were treated with drugs as specified in each experiment (all from Sigma). After the drug treatments, slices were transferred to Eppendorf tubes, frozen on dry ice, and stored at −80°C until used in the Flag and Myc immunoprecipitation protocol described below.

Flag and Myc Immunoprecipitations.

Bilateral striata from one mouse (*in vivo* experiments) or six striatal slices (slice experiments) were sonicated in 500μL IP lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, Complete Mini protease inhibitors (Calbiochem), and Halt phosphatase inhibitors (Pierce)). Homogenates were spun down for 20 minutes at 4^{o}C at 13,000 rpm. Supernatant was removed and 50μL/IP of washed EZView Red anti-Flag M2 affinity gel (Sigma) was added along with 45μL/IP of anti-Myc antibody (Novus) coupled to magnetic beads (Dynabeads M-280 Tosylactivated, Invitrogen). The homogenate/antibody mixture was gently rotated overnight at 4˚C. The Myc antibody was coupled to the Dynabeads following the protocol in the product manual; 3μg of Myc antibody was added for every 5μL of magnetic beads.

Following the overnight incubation, the homogenate/antibody mixture was placed on a magnetic particle concentrator (“MPC”, Invitrogen) for 2 minutes to separate the Myc magnetic beads from the Flag affinity gel. The supernatant containing the unbound homogenate and Flag affinity gel was removed to a new tube and kept on ice. The Myc magnetic beads were washed

three times in 1x PBS using the MPC to separate the beads each time. After the final wash, 30µL of non-reducing sample buffer (Pierce) was added and the beads were boiled for two minutes. Eluted supernatants were removed from the beads and samples were reduced with B-mercaptoethanol. Samples were stored at –80˚C until used for immunoblotting.

Flag IP/homogenate mixtures were spun down for 30 seconds at 13,000 rpm and unbound supernatant was removed to a fresh tube. This represented the total striatum sample. 1µL of this unbound supernatant was used in a BCA protein assay (Pierce) to determine protein concentration. Flag affinity gel was washed three times in 1x PBS and applied to the MPC to remove any residual magnetic beads. Flag IP's were eluted and stored at –80˚C as described above.

In addition to the anti-Myc-coupled Dynabeads, anti-Myc-coupled agarose (Novus) was also used for the Myc IP's with identical results. For this protocol, Myc-agarose was incubated with the striatal homogenate for 6 hours at 4˚C. The unbound supernatant was subsequently incubated with Flag-affinity gel overnight at 4˚C. Beads were washed and eluted as described above.