Striatin proteins within STRIPAK complex regulate the dephosphorylation of DARPP-32 as B subunits of PP2A

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INTRODUCTION

Dopaminergic signaling is predominantly mediated through altered downstream signaling mechanisms that control protein phosphorylation. Though the majority of studies in this field have focused on the activity of protein kinases, a significant body of research has also investigated the role of protein phosphatases. In particular, the serine/threonine protein phosphatase PP2A is known to dephosphorylate several targets within dopamine-regulated signaling pathways. One of these is DARPP-32, a mediator of psychostimulant action through regulation of its phosphorylation sites (Figure 1a) (Swenningson *et al.* 2005; Walaas *et al.* 2011).

PP2A is a heterotrimer composed of a catalytic C-subunit, structural A-subunit, and a regulatory B-subunit. The regulatory B-subunit allows for substrate selectivity, with at least four identified distinct sub-types of B-subunits (Lambrecht *et al.* 2013). The striatin family, comprised of striatin-1, striatin-3, and zinedin, are members of the B‘’’ family of B-subunits and are of interest due to their recently demonstrated capability in non-neuronal systems to organize large signaling complexes. These complexes have been implicated in a variety of cellular processes in non-mammalian model systems, including endocytosis, cell junction stability, and Golgi assembly, and are referred to as STRIPAK: striatin-interacting phosphatase and kinase complexes (Figure 1b) (Hwang and Pallas 2014). However, nothing is known of striatin or STRIPAK function within the striatum, despite the fact that striatin-1 is highly enriched within this brain region. Through the use of immunoprecipitation and affinity purification to interrogate the PP2A-striatin interactome and through *in vitro* assays measuring dephosphorylation of ^{32}P-DARPP-32, we have accumulated evidence identifying DARPP-32 as a substrate of PP2A-striatin. Due to the role of DARPP-32 as a mediator of the action of drugs of abuse, this data may also implicate striatin and the STRIPAK complex as potential targets of these drugs.

MATERIALS AND METHODS

Immunoprecipitations, Pulldowns, and Mass Spectrometry

Binding partners of striatin and PP2A were identified using two different approaches. Cortical and striatal homogenates were harvested from 2 month old C57BL/6 mice. Samples were taken through a standard immunoprecipitation protocol and incubated with striatin-1 antibody (Novus Biologicals NB110-74571) for 1 hour and Protein-G sepharose beads (GE Healthcare) for 2 hours. Samples were then analyzed by mass spectrometry at the Yale/NIDA Neuroproteomics Center to identify binding partners of striatin-1. To assess the binding partners of PP2A, the technique of microcystin pulldown was used. Microcystin is a polypeptide inhibitor that binds with high specificity to PP2A and can reliably pull down PP2A complexes. Cortical and striatal homogenates were incubated with microcystin-sepharose beads (Millipore) and similarly analyzed by mass spectrometry. This experiment was also repeated using primary neuronal cultures.

Primary Cultures

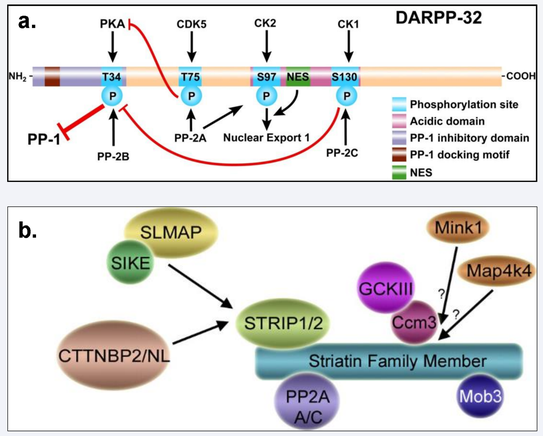
Following determination of striatal striatin-1 and PP2A binding partners through mass spectrometry, these results were verified using antibodies specific to identified binding partners in primary striatal cultures. Striatal cultures were harvested at E18 from pregnant Sprague-Dawley rats. These cultures were periodically lysed and probed with antibody to determine expression level of striatin-1 and PP2A binding partners over a period of 21 days.

Phosphorylation Assays

We assessed the ability of the striatin-PP2A complex to dephosphorylate DARPP-32 *in vitro* using a radioactive dephosphorylation assay. HEK293T cells were transfected with FLAG-tagged striatin-1, striatin-3, and/or STRIPAK adaptor protein STRIP1 cDNA using Lipofectamine 2000 (Invitrogen) in OptiMem (Invitrogen). A known B-subunit capable of dephosphorylating DARPP-32, B56δ, was used as a positive control. Cell cultures were lysed after 24 hours and the lysates were immunoprecipitated using FLAG antibody (Sigma F3165) to pull down striatin binding proteins. The immunoprecipitation product was then incubated with ^{32}P-DARPP-32, and scintillation counting was used to measure the amount of radioactive phosphate released through dephosphorylation. The experiments were also carried out with 50uM of okadaic acid for 10 minutes to confirm the assay was specifically measuring PP2A activity.

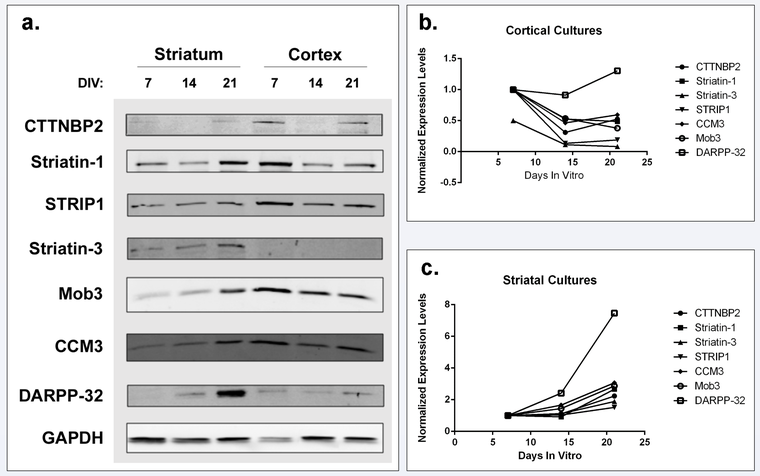
RESULTS

Figure 1: DARPP-32 and the structure of the STRIPAK complex.



a. A schematic of the structure of DARPP-32 and phosphorylation sites relevant to dopaminergic signaling. PP2A dephosphorylates DARPP-32 at Thr75 and Ser97; a known PP2A B subunit that targets DARPP-32 is B56δ (figure from Walaas *et al.* 2011); **b.** A schematic of the STRIPAK complex. The core STRIPAK complex consists of a striatin family member, the scaffolding and catalytic subunits of PP2A, the adaptor proteins Mob3 (Phocein), CCM3 (PDCD10), and STRIP1, as well as a member of the GCKIII kinases, including MST4, STK24, and STK25. Proteins in the STRIPAK complex are highly conserved across species and have been studied in relation to yeast cell cycle arrest, fungi sexual development, and *Drosophila* Erk signaling (figure from Huang *et al.* 2014).

Figure 3: Primary striatal cultures exhibit an increasing level of STRIPAK protein expression over time.



a. The results of the mass spectrometric analysis were confirmed using immunoblot analysis of primary striatal and cortical cultures. Cultures were lysed every 7 days for 21 days and analyzed by immunoblot; membranes were incubated with antibodies for identified STRIPAK proteins (Figure 1b, 2). DARPP-32 expression was evaluated as a positive control for striatal protein expression. **b.** Primary cortical cultures appear to exhibit a general decrease over time, n=2 **c.** Primary striatal cultures demonstrated an increase in STRIPAK protein levels over the course of 21 days, n=2. Immunoblots were normalized to GAPDH.

Figure 2: Proteomic analysis identifies STRIPAK proteins as PP2A binding partners within mammalian striatum.

a. Striatal Homogenates (Striatin-1 IP)

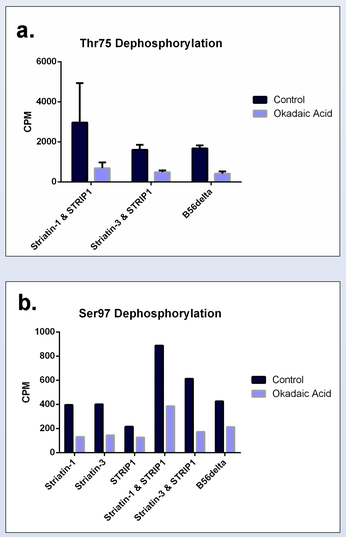
Score	Expectation	Protein ID	Protein Name	MW	% Coverage
988	2.9E-95	STRN_MOUSE	Striatin-1	85913	44.6
602	1.1E-56	CTTB2_MOUSE	Cortactin-binding protein 2	178662	13
546	3.9E051	STRN4_MOUSE	Striatin-4 (Zinedin)	81595	27.1
433	8.5E-40	STRN3_MOUSE	Striatin-3	87096	24.6
338	2.6E-30	2AAA_MOUSE	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform (PP2A-A)	65281	27.8
222	1E-18	PHOCN_MOUSE	MOB-like protein phocein (MOB3)	26016	56.9
173	8.1E-14	STRP1_MOUSE	Striatin-interacting protein 1 (STRIP1)	95524	10
90	0.000019	PP2AA_MOUSE	Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform (PP2A-C)	35585	10.4
20	150	PDC10_MOUSE	Programmed cell death protein 10 (CCM3)	24700	11.3

b. Primary Striatal Culture (MC Pulldown)

Score	Expectation	Protein ID	Protein Name	MW	% Coverage
2206	0	STRN_RAT	Striatin-1	85913	50.5
759	9.2E-73	STRN3_RAT	Striatin-3	87096	26.8
715	2.4E-68	2AAB_RAT	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform (PP2A-A)	65964	21.3
653	3.6E-62	2ABA_RAT	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform (PP2A-B)	51645	38.7
452	4.5E-42	2ABD_RAT	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform (PP2A-B)	51950	27.2
388	1.4E-35	PP2AB_RAT	Serine/threonine-protein phosphatase 2A catalytic subunit beta isoform (PP2A-C)	35552	25.2

a. To determine if the components of the STRIPAK complex were physiological binding partners of striatin family proteins and PP2A, mass spectrometry was used to analyze proteins isolated by pulldown experiments. Co-immunoprecipitation using striatin-1 antibody pulled down the majority of complex proteins, including Mob3 (phocein), STRIP1, PP2A, and CCM3, as well as the striatins, confirming that STRIPAK components were assembled within the striatum. **b.** A second experiment was performed using microcystin pulldown, using both rat primary culture and mouse striatal tissue (data not shown). The pulldown only isolated PP2A subunits and the striatins, possibly due to PP2A’s more peripheral position within the complex. Interestingly, the striatin proteins were highly abundant, comparable to the ubiquitous PP2A subunit, Bα.

Figure 4: Striatin family proteins are capable of targeting DARPP-32 for dephosphorylation *in vitro*.



a, b. DARPP-32 is a known target for PP2A through dephosphorylation of Thr75 and Ser97 (Figure 1a) and a possible target for the striatin/PP2A complex. HEK293T cells transfected with FLAG-tagged cDNA for various proteins were lysed and immunoprecipitated with FLAG antibody. Resulting samples were incubated with DARPP-32 phosphorylated with radioactive ^{32}P by CDK1 and CK2 respectively. Using a scintillation counter to measure the quantity of released phosphate, the ability of striatin family protein complexes to dephosphorylate DARPP-32 was compared to the positive control B56δ. Efficacy of dephosphorylation appeared to be comparable. Additionally, cotransfection with STRIPAK adaptor protein, STRIP1, increased dephosphorylation activity. Incubation with okadaic acid, a PP2A inhibitor, drastically reduced dephosphorylation activity, indicating that observed dephosphorylation primarily results from PP2A isolated by immunoprecipitation. A: n=2, B: n=1

CONCLUSIONS AND FUTURE DIRECTIONS

- **The STRIPAK complex is a physiological complex existing within mammalian striatum and includes adaptor proteins CCM3, Mob3/phocein, STRIP1, and the scaffolding and catalytic subunits of PP2A (Figure 2).**
- **Primary striatal cultures demonstrate an increasing level of STRIPAK protein expression over time, while cortical cultures do not, suggesting a potential role of this complex throughout striatal development (Figure 3).**
- ***In vitro* experimentation shows that the striatin/PP2A complex is capable of dephosphorylating DARPP-32 to an equal or greater extent than previously identified B subunits. Cotransfection of STRIPAK adaptor proteins increases the dephosphorylation of DARPP-32 (Figure 4).**
- **Future experiments will assess DARPP-32 phosphorylation in a more physiological context. A HEK293 cell line stably expressing DARPP-32 has been developed and phosphorylation status will be assessed following transfection of STRIPAK proteins.**
- **Adenoviruses packaging shRNA targeting STRIPAK proteins for knockdown have been developed and will be used in primary striatal cultures and live animals to assess the effects of lowered STRIPAK protein expression on DARPP-32 phosphorylation and dopaminergic signaling.**

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