Role of MAST3 kinase in PP2A regulation and neuronal activity in striatum

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INTRODUCTION

Drugs of abuse alter dopaminergic and glutamatergic neurotransmission in medium spiny neurons (MSN) of the striatum. Acting on complex signaling networks, drugs of abuse impair functional and structural neuroplasticity, resulting in transition to the addicted biological state and behavioral outcomes that typify addiction. Protein phosphorylation and dephosphorylation are fundamental mechanisms underlying synaptic plasticity that are deregulated by drugs of abuse. Increased understanding of the regulatory mechanisms by which protein kinases and phosphatases exert their effects during normal reward learning and the addiction process may lead to novel targets and pharmacotherapeutics. In our current studies, we identified a new regulatory pathway in striatum involving protein phosphatase 2A (PP2A) and the striatally-enriched phosphoprotein ARPP-16. Members of the ARPP-16 family are ARPP-19 and endosulfine, which are ubiquitously expressed, and ARPP-16, an alternatively spliced form that is highly enriched in MSNs in striatum. Two phosphorylation sites have been identified in ARPP-16, one at the C-terminus phosphorylated by PKA, and a second site, Ser46 (Ser62 in ARPP-19) located in the central region. We identified microtubule associate serine/threonine (MAST) kinase 3 as potential regulator of ARPP-16 in striatum following on from studies of the Greatwall (Gwl) kinase and ARPP-19/ENSA in Xenopus oocytes. In these studies, ARPP-19/ENSA were found to be phosphorylated by Gwl during the G2/M phase of the cell cycle, resulting in inhibition of PP2A (Mochida et al. 2010; Gharby-Ayachi et al. 2010). Our sequence analysis indicated a relationship between Gwl and MAST kinases. Based on our biochemical results that indicate MAST3 efficiently phosphorylates Ser46 of ARPP-16, and that MAST3, but not other related MAST kinase isoforms, is highly expressed in striatum, it is likely that MAST3 is responsible for regulation of ARPP-16 in striatum. Furthermore, we demonstrated that ARPP-16 is basally phosphorylated to a high level by MAST3 kinase in striatal neurons, leading to inhibition of PP2A towards selective substrates including Thr75 of DARPP-32. We also demonstrated that PKA plays a fundamental role in the MAST-mediated phosphorylation of ARPP-16 and regulation of PP2A activity: PKA-phosphorylation of ARPP-16 negatively acts in an intramolecular fashion on Ser46 phosphorylation. At the same time, MAST3 may also be suppressed by cAMP signaling as suggested by preliminary results. Finally, experiments are in progress to identify other specific MAST3 substrates in order to characterize and better understand the role of this kinase in the regulation of MSNs in striatum

METHODS

Cell line culture: HEK 293-T cells were grown on un-coated plates in DMEM (Invitrogen), supplemented with 10% FBS.

GST pull-downs: His-ARPP-16 was immobilized onto 80 µl/sample (of 50/50 slurry) Talon metal affinity resin (Clontech Laboratory Inc, Mountain View, CA). Increasing amounts of purified PP2A-A (0, 20 ng, 40 ng, 200 ng, or 400 ng) were added to the beads and samples were incubated for 1 hr at 4°C with rotation. As a control, 40 ng PP2A-A was added to beads with no His-ARPP-16 immobilized.

Plasmids and Transfection: MAST3-HA, MAST3T389A/D-HA, ARPP-16-HA, Balfa-FLAG, B56δ-FLAG and PR72-FLAG constructs were transfected in HEK293 cells with Lipofectamine 2000 (Invitrogen) in Opti-MEM media (Invitrogen) an. Protein expression was assayed at least 24 hr later.

ARPP-16 in vitro phosphorylation: Recombinant ARPP-16 fused to 6xHis tag was expressed in Escherichia coli (BL21) and purified using Ni-NTA Agarose (Qiagen). Purified 6xHis-ARPP-16 (1 µM) has been resuspended in 100 µl of phosphorylation buffer (50 mM Hepes pH 7.4, 10 mM MgCl2) in the presence of 200 µM ATP (Sigma) or 1 mM thio-γ-ATP (Roche) and incubated at 37°C for different time with immunoprecipitated MAST3 kinase

DARPP-32 in vitro phosphorylation and PP2A activity assay: recombinant purified DARPP-32 (200 ug) was phosphorylated by CDK1 at 30°C for 1 h, in buffer containing 50 mM Tris-HCl, pH 7.1, 150 mM KCl, 10 mM magnesium acetate and 200 µM [γ-^{32}P] ATP. Proteins were precipitated in 100% trichloroacetic acid (TCA) and after extensive washing the pellet was resuspended and dialyzed in 20 mM Tris-HCl pH 7.6, 5 mM β-mercapto-ethanol. For the PP2A assay, different PP2A heterotrimers were incubated with or without ARPP-16 or thio-γ-phosphorylated ARPP-16 (200 nM) in the presence of 75 µg of [^{32}P]-DARPP-32 for 10 min at 30 °C. Free [^{32}P] level was measured by scintillation counting after precipitation of [^{32}P]-phosphoproteins with cold TCA.

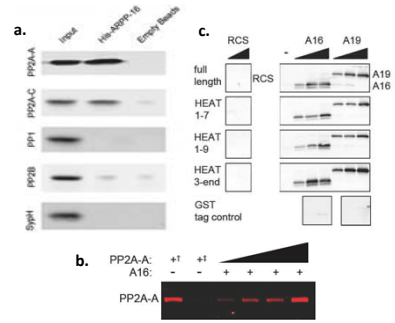
Cell line treatments: transfected cells were treated with the cAMP activator forskolin (10 µM) for 30 minutes then lysed with 25mM Tris-HCl, 150 mM NaCl, 0.1%tryton in presence of proteases and phosphatases inhibitors. The levels of the different proteins were analyzed by immunoblotting with specific antibodies after SDS-PAGE. Blots were analyzed and quantified using an Odyssey Infrared Immaging System (LI-COR Bioscience).

CONCLUSIONS

- **ARPP-16 interacts with phosphatase PP2A by direct binding with the A subunit (Fig.1),**
- **ARPP-16 is phosphorylated at S46 by MAST3 kinase (Fig.2),**
- **Phospho-S46-ARPP-16 inhibits PP2A's ability to dephosphorylate specific substrates in striatum – eg DARPP-32 phosphorylated at Thr75 (Fig.3),**
- **Three heterotrimers of PP2A are inhibited by p-Ser46-ARPP16, but to varying extent, phosphorylation on Ser88-ARPP-16 does not affect PP2A activity (Fig.3),**
- **Phosphorylations of ARPP-16 by PKA (on S88) or MAST3 (on S46) mutually attenuate each others ability to phosphorylate ARPP-16 *in vitro* (Fig.4),**
- **PKA phosphorylates MAST3 and decreases its activity *in vitro* (Fig.5),**
- **MAST3 plays a key role, headed cAMP, in the regulation of phosphorylation on S46-ARPP16 (Fig.6).**

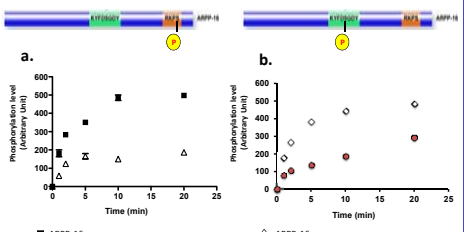
RESULTS

Figure 1: Identification of binding partners of ARPP-16 in rodent striatum, ARPP-16 interacts with the A subunit of PP2A.



a. Striatal lysate fraction S2 was incubated with His-ARRP-16 (100 ug) or beads alone (negative control). Eluted proteins were separated by SDS-PAGE and immunoblotted with antibodies against PP2A-A, PP2A-C, PP1, PP2B or synaptophysin. The lysate input (1%) is showed in lane 1, while lane 3 contains the elution from the negative control. Recombinant RCS (100 nM or 1000 nM) was incubated with beads to determine non-specific binding. Eluted samples were analyzed by SDS-PAGE and immunoblotting for ARPP-16 or ARPP-19. lane 1, while lane 3 contains the elution from the negative control. **b.** Increasing amounts of recombinant, SEC-purified PP2A-A were incubated with immobilized His-ARPP-16 and bound protein was analyzed by SDS-PAGE and immunoblotting (lane 1 PP2A input, lane 2 eluate from beads with no His-ARPP-16 that were incubated with PP2A-A). **C.** Full-length GST-PP2A-A, GST-tagged truncation mutants of PP2A-A that included HEAT repeats HEAT1-7, HEAT1-9, or HEAT3-END, or a GST tag control were incubated with increasing amounts of recombinant, His-ARPP-16 (A16, 10 nM, 100 nM, or 1000 nM) or His-ARPP-19 (A19, 10 nM

Figure 4: PKA/Ser88-ARPP-16 phosphorylation attenuates MAST3 ability to phosphorylate Ser46-ARPP-16 and vice versa.

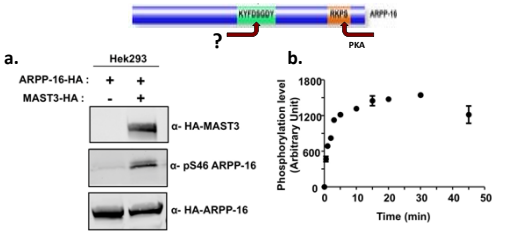


	Vm ³²p. incorporation/minute	Km nM
ARPP-16	1398±27.21	90.01±5.16
pSer88-ARPP-16	866.9±59.53	158.1±26.35

	Vm ³²p. incorporation/minute	Km nM
ARPP-16	6999±127.21	1685±51.16
pSer46-ARPP-16	1027±39.8	870.4±47.4

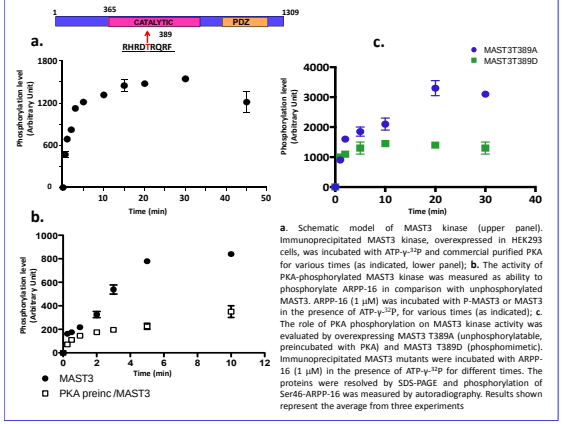
a. Recombinant purified ARPP-16 or P-S88-ARPP-16 (1 µM) were incubated with ATP-γ-^{32}P and immunoprecipitated MAST3 Kinase overexpressed in HEK293 cells, for various times (as indicated); the proteins were separated by SDS-PAGE and phosphorylation of Ser46 was measured by autoradiography . **b.** Recombinant purified ARPP-16 (1 µM) or P-S46-ARPP-16 were incubated with ATP-γ-^{32}P and commercial purified PKA, for various times (as indicated); the proteins were separated by SDS-PAGE and phosphorylation of Ser46-ARPP-16 was measured by autoradiography. Results shown represent the average from three experiments. Kinetic analysis is summarized in the lower tables.

Figure 2: ARPP-16 is phosphorylated at Ser46 in intact cells and *in vitro* by MAST3 Kinase



a. HEK293 were transfected with HA-ARPP-16 without or with HA-MAST3 kinase and the phosphorylation at Ser46 of ARPP-16 was analyzed by immunoblotting using a phospho-specific antibody**. b.** Recombinant purified ARPP-16 (1 µM) was incubated with immunoprecipitated MAST3 Kinase for various times (as indicated), and phosphorylation of Ser46 was measured by immunoblotting. Ser-46 phosphorylation was normalized to total ARPP-16 levels, and to the zero time value in each experiment. Results shown represent the average from three experiments.

Figure 5: MAST3 is phosphorylated by PKA *in vitro* and this inhibits MAST3 activity.



a. Schematic model of MAST3 kinase (upper panel). Immunoprecipitated MAST3 kinase, overexpressed in HEK293 cells, was incubated with ATP-γ-^{32}P and commercial purified PKA for various times (as indicated, lower panel); **b.** The activity of PKA-phosphorylated MAST3 kinase was measured as ability to phosphorylate ARPP-16 in comparison with unphosphorylated MAST3. ARPP-16 (1 µM) was incubated with P-MAST3 or MAST3 in the presence of ATP-γ-^{32}P, for various times (as indicated); **c**. The role of PKA phosphorylation on MAST3 kinase activity was evaluated by overexpressing MAST3 T389A (unphosphorylatable, preincubated with PKA) and MAST3 T389D (phosphomimetic). Immunoprecipitated MAST3 mutants were incubated with ARPP-16 (1 µM) in the presence of ATP-γ-^{32}P for different times. The proteins were resolved by SDS-PAGE and phosphorylation of Ser46-ARPP-16 was measured by autoradiography. Results shown represent the average from three experiments

OUR MODEL

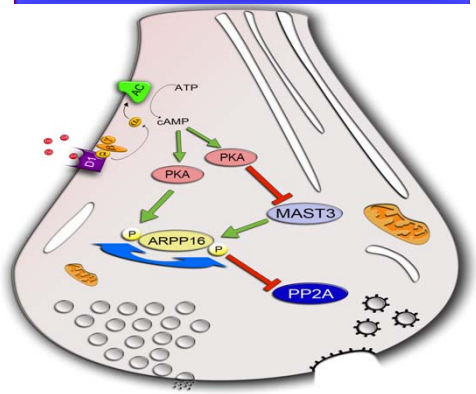
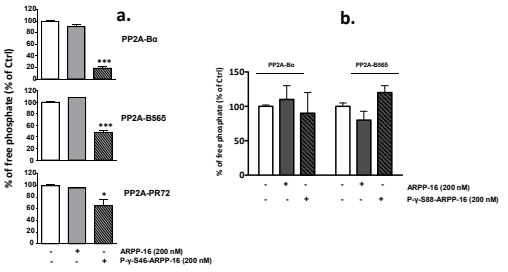
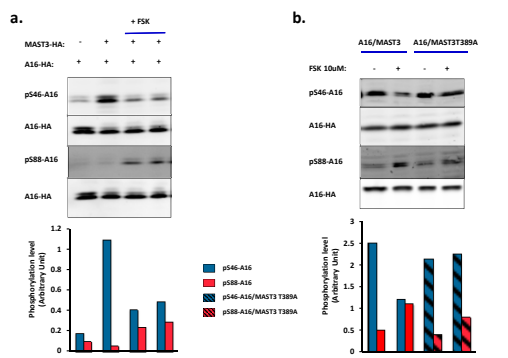


Figure 3: P-S46-ARPP-16 but not P-S88-ARPP-16 inhibits PP2A heterotrimer ability to dephosphorylate P-T75-DARPP-32 *in vitro*.



a. & **b.** Recombinant Flag-Bα, Flag-B56δ and Flag-PR72 PP2A trimers over-expressed in HEK293 cells and isolated by immunoprecipitation with anti-Flag Ab, were incubated with 200 nM of ARPP-16, P-γ-Ser46-ARPP-16 (**a**) or P-γ-Ser88-ARPP-16 (**b**) for 10 min at 37°C with P^{32}-P-T75-DARPP-32 as substrate. The ability of different PP2A trimers to dephosphorylate DARPP-32 was measured by scintillation counting. Results are expressed as percent changes with respect to PP2A alone (white bar). *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$, Newman-Keuls multiple comparison test.

Figure 6: cAMP-dependent signaling regulates ARPP-16 phosphorylation via MAST3/PKA in intact cells



a. The effect of cyclic AMP (cAMP) signaling as mutual regulator of ARPP16 phosphorylation (S46 and S88) in intact cells was investigated. Hek293T cells were transfected with ARPP16-HA (A16-HA) alone or plus MAST3-HA. After 24 hours cells were treated with 10 µM Forskolin (FSK). Levels of S46 and S88 A16-HA were measured by immunoblotting with phospho-specific antibodies on SDS-PAGE-resolved cell lysates (upper panel). **b**. To address the role of PKA in the regulation of MAST3 activity in living cells A16-HA was co-transfected with wild type MAST3 or MAST3 T389A (unphosphorylatable) in Hek293T cells. Effect of FSK on the transfected cells was measured by immunoblotting with phospho-specific A16 antibodies on cell lysates (upper panel). Phosphorylation levels were plotted after normalization to the total amount of transfected A16-HA (**a.** & **b.** lower panels).

ACKNOWLEDGMENTS

This research was supported by NIH, DA10044
Proteomic analysis was supported by the Yale/NIDA Neuroproteomics Center, DA018343

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