Receptor protein tyrosine phosphatases control Purkinje neuron firing

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Abstract (173/200 words):

Spinocerebellar ataxias (SCA) are a genetically heterogeneous family of cerebellar neurodegenerative diseases characterized by abnormal firing of Purkinje neurons and degeneration. We recently demonstrated the slowed firing rates seen in several SCAs share a common etiology of hyper-activation of the Src family of non-receptor tyrosine kinases (SFKs)[1]. However, because of the lack of effective neuroactive, clinically available SFK inhibitors, alternative mechanisms to modulate SFK activity are needed. Previous studies demonstrate that SFK activity can be enhanced by the removal of inhibitory phospho-marks by receptor-protein-tyrosine phosphatases (RPTPs)[2,3]. In this Extra View we show that MTSS1 inhibits SFK activity through the binding and inhibition of a subset of the RPTP family members. RPTP activity normally results in SFK activation in vitro, and lowering RPTP activity in cerebellar slices using recently described RPTP peptide inhibitors increases the suppressed Purkinje neuron basal firing rates seen in two different SCA models. Together these results identify RPTPs as novel effectors of cerebellar activity, extending the MTSS1/SFK regulatory circuit we previously described and expanding the therapeutic targets for SCA patients.

Introduction:

Cells organize kinase and phosphatase complexes within distinct micro-domains on their membranes to locally control responses to extrinsic signals. One organizer of these micro-domains is the I-BAR family of proteins who both sense, and induce, negative membrane curvature before assembling relevant regulatory complexes to control the kinase / phosphatase output. Mtss1, a founding member of the I-BAR family, acts as a docking site for receptor protein tyrosine phosphatases (RPTP) to locally control the activity of Src family non-receptor tyrosine kinases (SFKs) [2,4,5]. This local kinase regulation gives rise to a variety of cell biological phenotypes including local changes in the actin cytoskeleton[6], as well as receptor internalization and recycling[7]. In highly polarize cells, such as neurons, Mtss1 restraint of SFKs stabilizes dendritic spines[1,8].

The cerebellum is an evolutionarily conserved brain region with well-defined circuits that control movement and motor learning. A prominent feature of the cerebellum

is that several distinct neuronal inputs are integrated by Purkinje neurons, who are the sole output. Spinocerebellar ataxias are a genetically heterogeneous family of cerebellar wasting disorders characterized by motor control problems, abnormal eye movements, and impairments in motor learning [9]. Causative SCA loci are numbered in order of discovery and affect many compartments in the cell, ranging from ion channels at the cell membrane[10] to transcription factors[11] to proteolytic machinery[12] or ER calcium flux[13,14]. Defects in any of these diverse pathways lead to a common endophenotype: Purkinje neuron dysfunction and degeneration. While the logic of the diverse array of loci appeared opaque, we recently demonstrated that multiple SCA models, originating from possible defects in transcription (SCA1), translation (SCA2) and cytoskeleton (SCA5, Mtss1), converge on preventing hyperactivity of the Src family of non-receptor tyrosine kinases[1].

Mammals have 9 members of the Src family of non-receptor tyrosine kinases who have partially overlapping patterns of expression and activity, which may help mask identification of their individual functions (reviewed in [15]). SFKs play essential roles in cell polarity and cytoskeletal organization as well fundamental cell processes including protein translation[16,17]. In the nervous system SFKs underlie the induction of long term potentiation(LTP)[18], a synaptic correlate of learning, and are hyper-activated in a variety of neurodegenerative disorders associated with reduced synapse density including SCA[1] and Alzheimer disease[19,20]. This suggests the localization and control of tyrosine kinase activity, within neurons, can help drive neurodegenerative disorders and represents a intriguing new therapeutic target. While we have shown that small molecular SFK inhibitors can ameliorate the clinical severity of the ataxia phenotype, clinically available drugs lack favorable CNS biodistribution[21], and cause myeloproliferative defects preventing further clinical development to treat SCA.

By contrast, RPTPs that control SFK activation remain attractive therapeutic targets. The RPTP super family is divided into 8 subtypes, where type IIa members LAR, PTPRS and PTPRD are strongly implicated in nervous system function, including synaptic density[22], LTP[23], spatial memory[24] and neurofibrillary tangle formation for a variety of dementias[25]. Type IIa RPTPs are characterized by dual catalytic domains where the membrane proximal D1 domain removes phosphates from substrates while the membrane-distal D2 domain has little activity alone, rather it controls dimerization and D1 catalytic function[26,27]. Exogenous expression of the PTPRS wedge domain that

links the D1 and D2 catalytic sites is sufficient to reduce PTPRS function[28], establishing a new class of specific RPTP inhibitor.

Here we provide proof of concept that RPTP inhibitors control cerebellar synaptic stability and circuit activity, leading to the possibility of neuron survival, and amelioration of the SCA phenotype.

Materials and Methods:

Cell culture: MB55 mouse medulloblastoma cells[29] were maintained in floating spheres in DMEM:F12 + B27 supplement. For ISP treatment cells were dissociated with accutase and plated on 1% matrigel. For ISP treatment cells were incubated in increasing concentrations overnight. Cos7 cells were maintained in DMEM+10% FBS and transfected with Fugene 6.

Co-IPs: GST-fusions encoding the intracellular D2 phosphatase domains of human CD45 (NM_002838 bp 2887-3831), LAR (AB177857 bp 5228-6014), PTPRS (NM_002850.3 bp 5290-6012), PTPRD (AB211400 bp 5667-6450), PTRG (L09247 bp 3614-4300), PTPRN (BC070053 bp 2081-2926), were cloned into pGEX expression vectors and purified from bacteria using glutathion-sepharose beads then incubated with cos7-cell lysate expressing recombinant human MTSS1. Protein complexes bound to beads were washed 3 times in PBS+0.5% NP40 before denaturing and western blot.

TAT-ISP peptide: TAT-ISP was synthesized by Genscript. Sequence: GRKKRRQRRRCDMAEHELADHIERLKANDNLKFSQEYE-amidation

Purkinje cell recordings from cerebellar slices: All experimental procedures were approved by Stanford University IACUC. Purkinje cell recordings from cerebellar slices was performed as in our previous publication[1] with the following changes for TAT-ISP: Mice were injected with 50μg TAT-ISP the night before harvest, then cerebellar slices were bathed in ACSF supplemented with 2μM TAT-ISP during cutting and recording.

Western Blots: Cells or tissues were lysed in RIPA buffer supplemented with Roche complete-mini protease and Pierce phosphatase inhibitors. Samples were normalized with BCA assay (Pierce) and 30μg total protein was run on Novex 4-12% gels. Active SFK-Y416 (CST 6943), β‑Actin (Sigma A1978) primary antibodies were detected by IR-dye conjugated secondaries and imaged with a LiCor scanner.

Results:

MTSS1 binds specific RPTP family members

Because current SFK inhibitors lack favorable CNS biodistribution[21] we explored the possibility that RPTP peptide inhibitors would demonstrate efficacy in our model cerebellar system. We have previously shown the reciprocal interaction between MTSS1 and Type IIa-family member RPTPD controls membrane localization[4] as well as the ability to remove inhibitory phospho-marks from SFKs[2]. To determine whether MTSS1 specifically binds individual RPTPs expressed in the cerebellum, we performed co-precipitation experiments. We purified GST-fusion constructs of the intracellular domain of 5 of the 8 RPTP subtypes[30]: CD45 (type 1/6), PTPRF (IIa), PTPRS (IIa), PTPRD (IIa), PTPRA (type 4), PTPRG (type 5), PTPRN (type 8), then incubated with recombinant MTSS1 from cos7 cell-lysate. We observed specific interactions between MTSS1 and type IIa family members PTPRS and PTPRD with weak interaction with PTPRF (LAR) (Figure 1A). Interestingly, these type II RPTP family members are also highly expressed in the cerebellum[31-34] and associated with synapse formation in other brain regions[22,23].

To determine whether RPTP inhibition reduced activation of SFKs, we took advantage of the recently described RPTP wedge inhibitor for type IIa fused to the cell permeant TAT peptide for greater tissue distribution (TAT-ISP). We treated mouse medulloblastoma cells[29], that express type IIa RPTPs (PTPRD,PTPRF,PTPRS), with TAT-ISP inhibitory peptide and assayed SFK activation through the abundance of the active phospho-mark SFK-Y416. We found increasing levels of ISP reduced SFK-Y416 suggesting RPTP inhibition is sufficient to reduce SFK activity (Figure 1B).

We recently demonstrated that Purkinje neuron basal firing rates are highly dependent on SFK activity, and that reducing SFK activity is sufficient to boost the suppressed firing rates and improve behavior seen in many spinocerebellar ataxias[1]. To determine whether RPTP inhibition impacts basal firing rates we treated cerebellar slices from both MIM^{EX15} and ATXN2^{Q127} mice with TAT-ISP. We found 2µM TAT-ISP treatment boosted firing rates in both models to a level comparable to the small molecule SFK inhibitor dasatinib (Figure 2), documenting the efficacy of the TAT-ISP approach.

Discussion:

We previously demonstrated SFK hyper-activation suppresses Purkinje neuron basal firing rates and leads to SCA by 1 month of age. Here we demonstrate that inhibition of RPTP family members known to interact with MTSS1 and control SFK activity also rescues SCA-dependent suppression of firing rates. These data suggest that aberrant regulation of RPTPs underlie the earliest SCA phenotype: altered Purkinje neuron firing

rates. RPTPs have large extracellular domains sufficient for dimerization and activation either through contact with other cells[35] or with the extracellular matrix (ECM) [36,37]. The ability of RPTPs to respond to extracellular signals suggest some aspects of SCA disease progression may be driven in a non-cell autonomous manner, possibly by altered interaction with the ECM. Supporting the idea that ECM contributes to neurologic disease, conditionally removing HSPGs a major component of the extracellular matrix leads to neurologic deficits ranging from cerebellar agenesis[38] to socio-communicative deficits[39], while ablating CSPGs, an alternative ECM polysaccharide family, improves re-innervation after spinal injury[40].

While the majority of studies using PTPRS and PTPRD mutant mice have focused on the hippocampus, the observed phenotypes contrast the cerebellar phenotypes seen in many SCA models. For example, PTPRS and PTPRD mutant mice have been shown to have increased hippocampal dendritic spine density[22], and enhanced synaptic transmission associated with learning defects[23], contrasting the reduced Purkinje neuron spine density[1,41,42] and attenuated synaptic strength[43] seen in some SCA models SFKs and RPTPs work through a variety of mechanisms to regulate synapse stability, however additional studies are required to elucidate which events result in the reduced synapse stability seen in SCA. In the hippocampus, SFK activity enhances LTP[18,44] by controlling glutamate receptor presentation and activity[45], and helps control the formation and stability dendritic spines[46] possibly through regulating the activity of the actin bundling protein Cortactin[47]. In the cerebellum, SFKs and protein tyrosine phosphatases have been shown to modulate mGluR1 activity at the Purkinje neuron/ parallel fiber synapse to control LTD[48]. Additionally, SFKs are a key link between RPTPs and TrkB to potentiate BDNF signaling and increase synaptic function[49]. These findings suggest that one function for RPTPs in the nervous system is local control of SFK activity to stabilize the synapse, likely in response to multiple signaling pathways.

Our data expands the MTSS1/SFK regulatory circuit and identifies RPTPs as novel effectors of Purkinje neuron firing. Given the pleiotropic effects of SFK inhibition, RPTPs may prove a more ideal candidate for SCA treatment.

Declaration of interests: The authors declare no competing interests.

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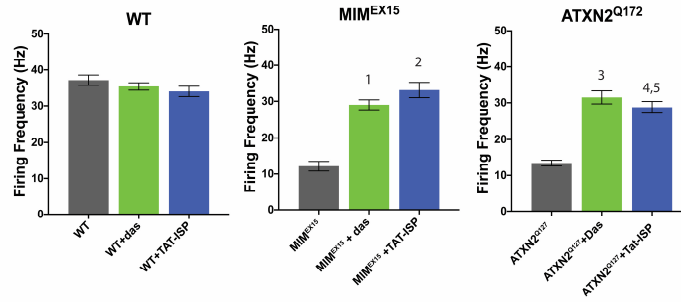
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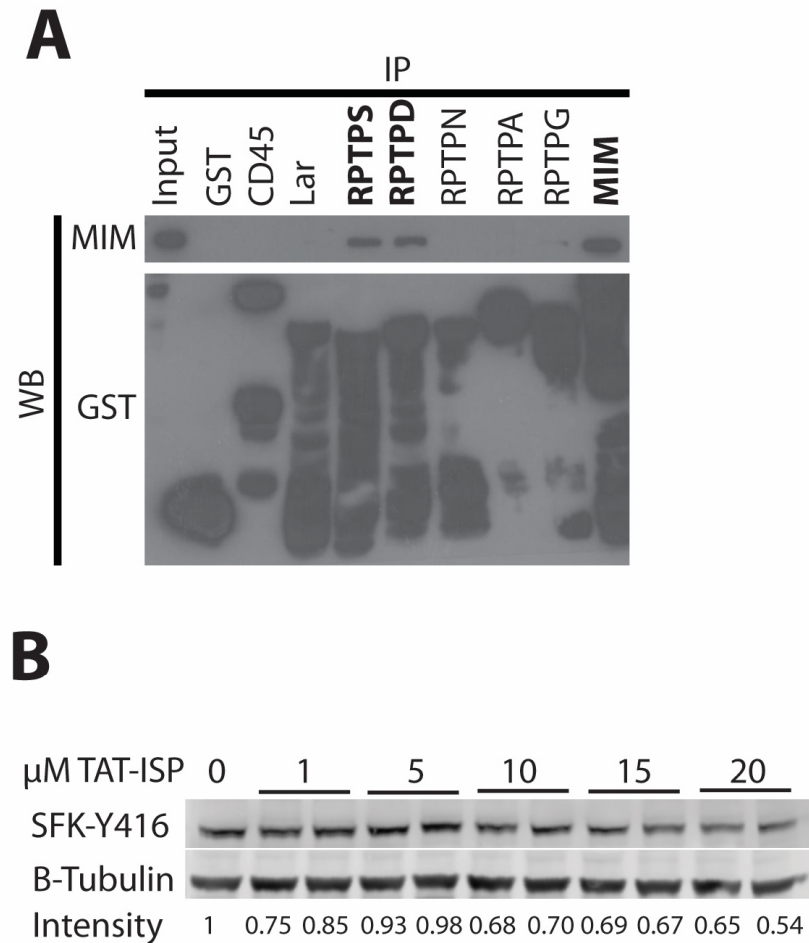
A. We purified gst-fusions of the intracellular domain of CD45 (type 1), LAR (2a), PTPRS (2a), PTPRD (2a), PTPRN (type 8), PTPRA (type 4), PTPRG (type 5), which represent 5 of the 8 RPTP subtypes, then incubated with cos7-cell lysate with recombinant MTSS1. We observed specific interactions between MTSS1 and type IIa family members PTPRS and PTPRD with weak interaction with PTPRF (LAR). **B.** we treated mouse medulloblastoma cells[29], that express both PTPRS and PTPRD, with inhibitory peptide and assayed SFK activation through the abundance of the active phospho-mark SFK-Y416. We found increasing levels of ISP reduced SFK-Y416 suggesting RPTP inhibition is sufficient to reduce SFK activity.

A

WT

Firing Frequency (Hz)

WT | WT+das | WT+TAT-ISP

MIM^EX15

Firing Frequency (Hz)

MIM^EX15 | MIM^EX15 + das [1] | MIM^EX15 +TAT-ISP [2]

ATXN2^Q172

Firing Frequency (Hz)

ATXN2^Q127 | ATXN2^Q172+Das [3] | ATXN2^Q127+Tat-ISP [4,5]

B

Genotype	WT	WT	WT	MIM^EX15	MIM^EX15	MIM^EX15	ATXN2^Q127	ATXN2^Q127	ATXN2^Q127
Treatment	DMSO	Dasatinib	TAT-ISP	DMSO	Dasatinib	TAT-ISP	DMSO	Dasatinib	TAT-ISP
n Animals	2	2	2	2	2	2	2	2	2
n Cells	48	79	87	55	62	81	89	72	58
Mean Firing (Hz)	37.12	35.36	33.99	12.15	29.02	33.12	13.28	31.50	28.74
sem	1.43	1.02	1.43	1.34	1.34	2.9	0.79	1.82	1.62

C **WT** **MIM^EX15 ATXN2^Q127** **TAT-ISP**
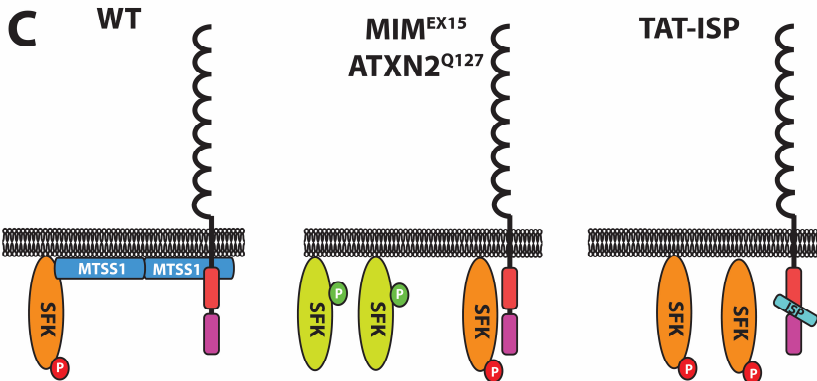
Figure 2: RPTP inhibition rescues firing defects from multiple ataxias

A. We found 2μM TAT-ISP treatment boosted firing rates in both models to a level comparable to the small molecule SFK inhibitor dasatinib. [1]p <0.0001, [2]p <0.0001, [3]p <0.0001, [4]p =<0.0001, [5]p = 0.0095 ATXN2^Q127+Das vs ATXN2^Q127+TAT-ISP. 1-way ANOVA with Tukey post-hoc. Error bars s.e.m. **B.** Summary table of firing data shown in **A**. **C.** Model where MTSS1 interacts with RPTPs and SFKs to restrain activity. When MTSS1 is lost (center) RPTPs remove inhibitory phospho marks from SFKs allowing activation. TAT-ISP blocks RPTP activity preventing the activation of SFKs.