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## Receptor protein tyrosine phosphatase $\sigma$ binds to neurons in the adult mouse brain

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### Abstract

The role of type IIA receptor protein tyrosine phosphatases (RPTPs), which includes LAR, RPTP $\sigma$  and RPTP $\delta$ , in the nervous system is becoming increasingly recognized. Evidence supports a significant role for these RPTPs during the development of the nervous system as well as after injury, and mutations in RPTPs are associated with human disease. However, a major open question is the nature of the ligands that interact with type IIA RPTPs in the adult brain. Candidates include several different proteins as well as the glycosaminoglycan chains of proteoglycans. In order to investigate this problem, we used a receptor affinity probe assay with RPTP $\sigma$ -AP fusion proteins on sections of adult mouse brain and to cultured neurons. Our results demonstrate that the major binding sites for RPTP $\sigma$  in adult mouse brain are on neurons and are not proteoglycan GAG chains, as RPTP $\sigma$  binding overlaps with the neuronal marker NeuN and was not significantly altered by treatments which eliminate chondroitin sulfate, heparan sulfate, or both. We also demonstrate no overlap of binding of RPTP $\sigma$  with perineuronal nets, and a unique modulation of RPTP $\sigma$  binding to brain by divalent cations. Our data therefore point to neuronal proteins, rather than CSPGs, as being the ligands for RPTP $\sigma$  in the adult, uninjured brain.

### Keywords

Protein Tyrosine Phosphatase; Receptor Affinity Probe Assay Chondroitin Sulfate; Heparan Sulfate Proteoglycan

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## Introduction

There is significant interest in the putative roles of the class II A (R2A) Receptor Protein Tyrosine Phosphatases (RPTPs), which includes the leukocyte common antigen receptor (LAR), as well as RPTP $\sigma$  and RPTP $\delta$ , in brain function. Evidence has accumulated supporting a role for these RPTPs in the control of neuronal migration, axonal targeting, synaptic formation and neuronal plasticity (Chagnon et al., 2004; Dunah et al., 2005; Stoker and Dutta, 1998; Takahashi and Craig, 2013). Most recently, they have been identified as playing a role in restricting regeneration after neuronal injury (Fry et al., 2010; Shen et al., 2009). Mutations in RPTPs are increasingly recognized as causes of human disease (Nikolaienko et al., 2012; Stewart et al., 2013; Xu and Fisher, 2012). Less understood is the role that they play in the normal adult nervous system.

Members of the R2A class of phosphatases share features of their extracellular domain (ECD) common to many cell adhesion molecules, i.e., immunoglobulin and fibronectin type III repeats, as well as a membrane-spanning domain and two phosphatase domains, one of which (D1) is active, while the other (D2) is thought to control interactions with other intracellular proteins (Chagnon et al., 2004). The ECD binds to several different neuronal transmembrane proteins, including members of the Trk family of kinases, and molecules involved in synaptogenesis, including slitrak family members, neuroligin-3, and interleukin 1 receptor related protein (Kwon et al., 2010; Takahashi et al., 2011; Woo et al., 2009; Yoshida et al., 2011). However, the most studied interaction has been with proteoglycan glycosaminoglycan (GAG) chains: all members of the R2A class bind both heparan and chondroitin sulfate proteoglycans (Aricescu et al., 2002; Fox and Zinn, 2005).

These studies on the interactions of R2A class members with proteoglycan GAG chains have concluded that LAR, RPTP $\sigma$ , and RPTP $\delta$  are receptors mediating the actions of proteoglycan GAG chains in the response to neuronal injury (Shen et al., 2009). Because these results were obtained through the use of knockout animals, these results may not generalize to the function of these receptors in the normal, uninjured brain, where proteoglycan levels are relatively low. To address this issue, we have used a receptor affinity probe assay to evaluate the location and cells in the adult mouse brain which bind RPTP $\sigma$ . Our results indicate that binding sites in the adult mouse brain for RPTP $\sigma$  are neuronal and do not depend upon the presence of either chondroitin or heparan sulfate GAG chains.

## Materials and methods

### Animals

All animal studies were approved by the Uniformed Services University of the Health Science (USUHS) Institutional Animal Care and Use Committee and were conducted in accordance with the NRC guide to the Care and Use of Laboratory Animals. A total of 8 mice were used for this study. Male mice (C57BL/6) of age 8-10 weeks (NCI, MD, USA) were kept under 12:12 light and dark cycle with access to food and water ad libitum. Mice were deeply anesthetized using ketamine/xylazine (100 mg/kg-ketamine/20 mg/kg-xylazine) and transcardially perfused with 4 % buffered formalin solution. Brains were carefully removed and post fixed in 4 % buffered formalin solution overnight at 4°C, then immersed

in 30 % sucrose solution until they sank. 30  $\mu$ m coronal sections were cut using a Leica SM2000 R sliding microtome with an attached freezing stage. Sections were kept in a PBS-based antifreeze solution containing ethylene glycol at  $-20^{\circ}\text{C}$  until used for receptor affinity probe (RAP) assays.

### Production of RPTP $\sigma$ -AP

The extracellular domain of mouse RPTP $\sigma$  (accession number: NM\_001252453) was expressed as an alkaline phosphatase fusion protein. cDNA corresponding to the RPTP $\sigma$  ECD (amino acids #1-847) was subcloned into the NheI/HindIII sites in pAPTAG5 (GeneHunter) which contains a sequence coding for alkaline phosphatase followed by a Myc epitope and a (His)<sub>6</sub> tag at its C-terminus. Putative GAG binding residues (Lys68, Lys69, Lys71, Lys72) were mutated to Ala with site-directed mutagenesis. Stable transfectants were established in BHK cells under zeocin selection. Secreted fusion proteins (RPTP $\sigma$ -AP, RPTP $\sigma$ -Lys-AP or AP only) were purified by immobilized metal affinity chromatography (Profinity IMAC Ni-Charged Resin, Bio-Rad) via their (His)<sub>6</sub> tags and their purity was determined by gel electrophoresis. Purified recombinant proteins were separated on a 10% SDS-PAGE and immunoblot analysis was performed with an anti-(His)<sub>6</sub> antibody (Wang et al., 2008) (Supplementary Fig. 1).

### In vitro binding of PTP $\sigma$ -AP

PTP $\sigma$ -AP binding to immobilized GAGs was examined in an ELISA format (Dickendesher et al., 2012). GAG chains were biotinylated according to the method described by Briani et al. (1998). ELISA plates (Immulon4, NUNC) precoated with streptavidin (Invitrogen, 5  $\mu$ g/ml) and blocked with 5% BSA were incubated with biotinylated GAGs for 15 min and washed with BS (10 mM epe, 150 mM NaCl, p .3). Three nM of PTP $\sigma$ -AP was incubated at 4  $\square$  C for 2 h. Bound AP activity was measured with BluePhos (KPL) at 37 $^{\circ}$ C under kinetics mode. The data were expressed as mOD/min.

### Receptor affinity probe assay

Sections were incubated overnight at 4 C with RPTP $\sigma$ -AP, RPTP $\sigma$ -Lys-AP or AP alone. The next day, sections were washed and bound AP was visualized with HistoMark RED (KPL). To evaluate the dependency of binding on divalent cations, incubation of RPTP $\sigma$ -AP was done in Tris-buffered saline (pH 7.4, TBS) with the addition of either 5 mM Ca<sup>2+</sup>, 5mM Mg<sup>2+</sup>, 5mM EDTA, or 5mM EGTA. To verify whether RPTP $\sigma$  binds to brain GAGs, sections were digested with either chondroitinase ACII (Seikagaku) and/or heparatinase III (Sigma) for 3 h at 3 C prior to incubation with RPTP $\sigma$ -AP (Mizumoto and Sugahara, 2012). Parallel sections were stained with mouse monoclonal antibodies to native chondroitin-4-sulfate (2H6, Seikagaku), native heparan sulfate (HepSS-1, Seikagaku), cleaved chondroitin-4-sulfate (MAB2030, Millipore) or cleaved heparan sulfate (3G10, Seikagaku), followed by HRP-conjugated secondary antibodies. Widefield images were obtained on a Nikon Eclipse microscope equipped with a Zeiss Axiocam. Low magnification images spanning entire sections were taken using a 4 $\times$  objective, while higher magnification images were obtained using a 20 $\times$  objective. Montages of entire sections were created using Photoshop.

## Immunofluorescence and confocal microscopy

For fluorescence staining for RPTP $\sigma$ , the C-terminal (His)<sub>6</sub> tag was used to detect bound RPTP $\sigma$  on the sections. Free-floating sections were first incubated with RPTP $\sigma$ -AP. After washing in PBS, the sections were blocked for 1 hour with 10% goat serum (Invitrogen) in PBS containing 0.1% Triton X-100 (Sigma) followed by incubation with an anti-(His)<sub>6</sub> antibody (Abcam) and different antibodies: rabbit anti-GFAP (DAKO), NeuN (Millipore) or biotinylated sheep anti-Neurocan (R&D Systems), CS-56 (Sigma), 2B6 (Seikagaku) or anti-HS (HepSS-1), or biotinylated Wisteria Floribunda Agglutinin (WFA, Sigma) in the same solution. Following washes, sections were incubated with appropriate secondary antibodies: Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 568 anti-rabbit IgG (Life Technologies); FITC-conjugated goat F(ab')<sub>2</sub> anti-mouse IgM chain (Abcam); Alexa Fluor 488-streptavidin (Jackson Labs); Alexa Fluor 568-streptavidin (Life Technologies). Sections were mounted on silanated slides (KD Medical) and coverslipped using Fluoromount™ (Sigma) with or without DAPI. Confocal images were taken using a Zeiss LSM 510 UV microscope. 3-D co-localization analysis of confocal z-stacks was performed using routines incorporated in Imaris (Bitplane).

## Results

To determine the binding sites for RPTP $\sigma$  within the adult mouse brain, a fusion protein was constructed consisting of the extracellular domain of RPTP $\sigma$  attached to alkaline phosphatase (RPTP $\sigma$ -AP). After incubation of this fusion protein with frozen sections of mouse brain, bound AP was visualized on the sections, indicating the sites of RPTP $\sigma$  binding. There was significant AP activity above background to brain sections (Fig. 1A). The binding was not uniform across the brain, with the highest binding found in the piriform cortex, hippocampus, deep cerebellar nuclei and Purkinje cell layer of the cerebellum. However, in virtually all regions of the brain there were neurons that bound RPTP $\sigma$ , albeit at lower levels. No reactivity was observed when brain sections were incubated with AP alone (Fig. 1A).

Higher magnification images of stained sections demonstrate that binding of RPTP $\sigma$ -AP was confined to neuronal somata with a wide range of intensities. RPTP $\sigma$ -AP bound to neurons within the superficial layers of the cerebral cortex (Fig. 1B) and more intensely to Purkinje neurons in the cerebellum (Fig. 1C). Further confirmation of the neuronal binding of RPTP $\sigma$  was obtained using co-localization of RPTP $\sigma$ -AP with cell-type specific markers by immunofluorescence. RPTP $\sigma$ -AP co-localized with the neuronal marker NeuN (Fig. 1D), but not with the astrocytic marker GFAP (Fig. 1E). RPTP $\sigma$ -AP was reported to bind to dorsal root ganglion neurons in culture (Coles et al., 2011) and we found that RPTP $\sigma$ -AP also bound to cultured cerebellar granule neurons (Fig. 1F). Thus, the major binding partners for RPTP $\sigma$  are neuronal.

Considerable attention has been paid to the role of RPTP $\sigma$  and other R2A subfamily members as putative receptors for proteoglycans during development and after injury to the mammalian CNS. We used double-labeling techniques to evaluate the co-localization of RPTP $\sigma$  binding sites with both chondroitin and heparan sulfate proteoglycans. In the normal, uninjured, brain, the level of chondroitin sulfate proteoglycans in brain parenchyma

is relatively low (Yi et al., 2012). When brain sections of cerebral cortex are stained with antibody CS-56, which recognizes CS GAG chains, the staining is very diffuse and patchy, and does not co-localize with cells (Fig. 2A). Moreover, there is no overlap with CS-56 staining and RPTP $\sigma$  binding. While HSPGs are found to be associated with neuronal cell bodies as indicated by HepSS-1 antibody staining which recognizes HS GAG chains, many HepSS-1 positive neurons showed no PTP $\sigma$ -AP bound (Fig. 2B). Moreover, in double-labeled neurons, the staining patterns for RPTP $\sigma$  and HEPSS-1 were overall not the same. As a further test of binding, sections were incubated with RPTP $\sigma$ -AP in the presence of CS-56 or HepSS-1 antibodies (Fig. 2C). Co-incubation of RPTP $\sigma$ -AP with these anti-GAG chain antibodies (Fig. 2A, B) did not cause a reduction in binding of the recombinant protein. Thus, it would appear that the binding sites for RPTP $\sigma$  in the normal mouse brain are not proteoglycan GAG chains.

In the uninjured brain, the most intense CSPG staining is observed in perineuronal nets, which surround certain classes of neurons in the cerebral cortex (Celio et al., 1998). In a naive mouse brain, antiserum raised against the CSPG neurocan intensely labels these perineuronal nets. Double-labeling of these brain sections with anti-neurocan antibody together with RPTP $\sigma$ -AP shows no overlap of the RPTP $\sigma$  binding sites with neurocan immunoreactivity, but instead RPTP $\sigma$ -AP binds to the neurons surrounded by the perineuronal nets (Fig. 3A, B). Perineuronal nets can also be labeled with the wisteria floribunda lectin (WFA), which binds to the GAG chains of chondroitin sulfate proteoglycans (Hartig et al., 1992). Double-label experiments with WFA and RPTP $\sigma$  again demonstrate that RPTP $\sigma$  binds to neuronal somata, but not the perineuronal nets that surround them (Fig. 3D, E). The lack of overlap in each case was confirmed with 3D-colocalization analysis (Fig. 3C, F).

GAG chains can be removed from both heparan and chondroitin sulfate proteoglycans through the use of appropriate enzymes: chondroitinases remove GAG chains from CSPGs, while heparatinase removes GAG chains from HSPGs, with no reported cross-reactivity. To further examine whether RPTP $\sigma$  was binding to any proteoglycan GAG chains in brain sections, we treated brain sections with either chondroitinase ACII, heparatinase, or a combination of both, and then evaluated the binding of RPTP $\sigma$ -AP. Disappearance of staining with antibodies which react with native GAG chains and the concomitant appearance of immunoreactivity with antibodies to cleaved chains demonstrated that the enzymes were efficacious (Fig. 4). However, no change in binding was observed when these treated brain sections were incubated with RPTP $\sigma$ -AP. Thus, RPTP $\sigma$  binds to brain sections even after ablation of both HS and CS GAG chains.

We have previously used ELISA assays to characterize the binding of RPTP $\sigma$  to proteoglycan GAG chains in vitro (Dickendeshner et al., 2012). The RPTP $\sigma$ -AP fusion protein used here has the same binding specificity (Supplementary Fig. 2): RPTP $\sigma$ -AP binds to highly sulfated GAGs, including heparin and heparan sulfate as well as the oversulfated chondroitin GAGs, CS-D and CS-E, but not the monosulfated GAGs, CS-A or CS-C. To determine if the sites on RPTP $\sigma$  necessary for neuronal binding overlap with the binding sites for GAG chains, RPTP $\sigma$ -AP was incubated with brain sections in the presence or absence of increasing concentrations of GAGs. Increasing concentrations of heparin or CS-E

reduce the binding of RPTP $\sigma$  to brain sections, suggesting that the binding sites in RPTP $\sigma$  are capable of binding to both proteoglycan GAG chains and binding sites on brain sections (Fig. 5).

The binding of RPTP $\sigma$  to GAG chains depends upon at least four lysine residues in the first immunoglobulin repeat of RPTP $\sigma$  (Aricescu et al., 2002). To assay if this site was involved in binding to brain sections, we mutated all four lysines to alanine, and conducted RAP assays with the RPTPs- Lys-AP. Binding of RPTP $\sigma$ - Lys-AP to mouse brain was significantly reduced as compared to binding of wild-type RPTP $\sigma$ -AP (Fig. 6A), suggesting that a major portion of binding to mouse brain was dependent upon these charged amino acids.

The dependence of RPTP $\sigma$  binding upon the positively charged amino acids in the first Ig domain suggests that the binding of RPTP $\sigma$  to its ligand(s) is through an electrostatic interaction. To test this hypothesis, we incubated brain sections with RPTP $\sigma$ -AP in the presence of increasing concentrations of NaCl. Indeed, RPTP $\sigma$  binding to brain sections was displaced as ionic strength was increased from the normal 150 mM NaCl in TBS, with complete elimination of binding at 500 mM NaCl (Fig. 6B). Thus, the binding of RPTP $\sigma$  to its ligand(s) is through electrostatic interactions.

To characterize further the binding of RPTP $\sigma$ -AP to mouse brain sections, we tested the effects of varying the concentration of divalent cations. Addition of 5 mM Ca<sup>2+</sup> or 5 mM Mg<sup>2+</sup>, or a combination of both to TBS, reduced RPTP $\sigma$ -AP binding to brain sections, suggesting that divalent cations can inhibit RPTP $\sigma$ -ligand interaction (Fig. 7A). Surprisingly, addition of either 5 mM EDTA or 5 mM EGTA, chelating agents for divalent cations, also reduced binding. These changes in binding were only observed when RPTP $\sigma$ -AP was incubated with brain sections in the presence of altered divalent cation concentration. When sections were pre-incubated with either divalent cations or chelating agents, but the RAP assay was conducted after a return to TBS, binding was restored to control levels (Fig. 7B). Further, decreasing free Ca<sup>2+</sup> concentration by incrementally increasing the concentration of 5 mM EGTA in the presence of 5 mM Ca<sup>2+</sup> progressively restores RPTP $\sigma$ -AP binding to brain sections (Figure 7C), suggesting that an optimal concentration of free divalent cations is necessary for RPTP $\sigma$ -AP binding. Thus, our results indicate a complex relationship between the concentration of divalent cations and RPTP $\sigma$  binding.

## Discussion

R2A subfamily protein tyrosine phosphatases have been implicated in several different developmental processes related to axonal outgrowth and synaptic formation and stabilization as well as the response to central nervous system injury. Because much of the current data has been obtained using genetic tools to modify the levels of R2A subfamily members during development or after injury to the central nervous system, we sought to evaluate the binding of the extracellular domain of RPTP $\sigma$  to normal adult brain using the receptor affinity probe assay. We found that RPTP $\sigma$  bound exclusively to neurons in the brain, and that RPTP $\sigma$  binding did not co-localize with proteoglycans, either in the brain



parenchyma or in perineuronal nets. Enzymatic removal of CS/HS GAGs from brain sections did not impact RPTP $\sigma$  binding, nor was binding to brain sections inhibited by addition of antibodies to CS-GAG or HS-GAG chains. Thus, these data indicate that the binding partners for RPTP $\sigma$  in the normal adult mouse brain are produced by neurons and are not proteoglycan GAG chains. A similar result was obtained with LAR binding to syndecan-2 in the *Drosophila* nervous system: elimination of syndecan-2 abolished LAR binding to glia, with no change in neuronal binding (Fox and Zinn, 2005).

Solid phase assays using the extracellular domain of RPTP $\sigma$  have demonstrated high-affinity binding to the chondroitin sulfate neurocan (Shen et al., 2009) as well as the heparan sulfate proteoglycans syndecan-2 (Coles et al., 2011), agrin and collagen XVIII (Aricescu et al., 2002). We also used solid phase assays to confirm that RPTP $\sigma$  binds to S and CS GAG chains. In these earlier studies, binding in solid-phase assays was significantly reduced or eliminated by treatment with enzymes that remove GAG chains. In contrast, ECD binding to brain sections was not altered by enzymatic treatment. There are potential explanations for the difference between solid phase binding to GAG chains and binding to tissue. The first is that the level of GAG chains in normal brain is very low. A second possibility is that the affinity of RPTP $\sigma$ -ECD constructs to GAG chains depends upon the sulfation composition of the GAG chains: RPTP $\sigma$  binds with high affinity to HS and the highly sulfated CS-D and CS-E units, while there is low or no affinity for the singly-sulfated CS-A or CS-C (Dickendeshler et al., 2012). CS-A and CS-C are the predominant species in the normal, uninjured mouse brain, while there is very little CS-D or CS-E (Maeda, 2010). Thus, RPTP $\sigma$  would not have significant binding to CS GAG chains in the normal mouse brain. The third is that there exists some ligand(s) in normal brain that inhibit binding of the RPTP $\sigma$ -ECD to GAG chains. These reasons may also account for the finding by Shen, et al. (2009) that RPTP $\sigma$ -ECD-Fc did not bind to GAG chains in uninjured spinal cord.

The binding of RPTP $\sigma$  and other R2A subfamily members to both heparin and chondroitin GAG chains has been localized to the first immunoglobulin domain of the protein (Lee et al., 2007). In solid phase assays, binding of receptor body constructs to GAGs is competed by either heparin or chondroitin sulfate GAG chains. In addition, mutation of four lysine residues in this domain causes a significant reduction in binding to GAGs (Aricescu et al., 2002). Our data indicate that this same region of RPTP $\sigma$  is important for binding to neurons in mouse brain, as binding was reduced by addition of soluble heparin and chondroitin sulfate GAGs and the mutation of these lysines in the RPTP $\sigma$ -Lys-AP fusion protein.

Because the elimination of the basic lysine residues drastically reduced RPTP $\sigma$  binding to brain sections, we hypothesized that the interaction between RPTP $\sigma$  and its binding partner(s) was electrostatic. Indeed, increasing the concentration of NaCl in the incubation medium reduced binding. A similar reduction in binding of RPTP $\sigma$  to heparin has been reported (Aricescu et al., 2002). On the other hand, we found that binding to brain sections was critically dependent upon the concentration of free divalent cations, as binding was reduced with addition of Ca<sup>2+</sup> or Mg<sup>2+</sup> or EGTA or EDTA. This is unusual for cell adhesion molecules where the binding site resides in the Ig repeats, and the mechanisms of binding of RPTP $\sigma$  to brain sections remain to be elucidated.

RPTP $\sigma$  has been suggested to mediate the inhibitory response to CSPGs after spinal cord injury, because recovery is enhanced in both RPTP $\sigma$  and LAR knockout animals as compared to wild type animals (Fry et al., 2010; Shen et al., 2009). RPTP $\sigma$  levels increase after nerve injury (Haworth et al., 1998). That the inhibitory response is actually mediated by CSPGs binding to RPTP $\sigma$  has not been directly demonstrated. Peptides directed against the wedge domain of LAR alter intracellular signaling (Xie et al., 2006) and neurite outgrowth (Fisher et al., 2011), but the dependence of signaling upon receptor occupancy was not measured in these experiments. Additionally, deletion of LAR results in a change in EphA2 phosphorylation and cell migration (Lee and Bennett, 2013). However, the confirmation of the R2A subfamily members as signal transduction molecules depends upon directly showing a change in intracellular signaling following ligand binding, rather than by genetic disruption of RPTP levels. Moreover, the major increase in CSPG core proteins following injury is accompanied by a parallel increase in 4-sulfated GAG chains (Wang et al., 2008), which does not bind RPTP $\sigma$  (Dickendesher et al., 2012).

Our data suggest that RPTP $\sigma$  binding sites in the adult brain are not CSPGs; the question then arises as to what the binding partner(s) and functions of RPTP $\sigma$  might be in the adult animal. Several different reports have supported a role for members of the R2A subfamily in synaptogenesis. Thus, RPTP $\sigma$  has been implicated in modulation of synaptic plasticity by interactions with both TrkB and TrkC (Kurihara and Yamashita, 2012; Takahashi et al., 2011). Most recently, a specific role for RPTP $\sigma$  in promoting excitatory and RPTP $\delta$  in promoting inhibitory synaptic development by interacting with specific slitraks has been reported (Takahashi et al., 2011; Yim et al., 2013). The interleukin-1-receptor accessory protein like 1 (IL1RAP1) has also been implicated in synapse formation by interacting with RPTP $\delta$  (Valnegri et al., 2011; Yoshida et al., 2011). Furthermore, the modulation of BDNF signal transduction by CSPGs was altered in RPTP $\sigma^{-/-}$  animals, supporting a role for CSPGs and RPTP $\sigma$  in plasticity (Kurihara and Yamashita, 2012). Because each of the ligands for the R2A subfamily continues to be expressed at high levels in the adult CNS, their role in the adult may be in maintaining synaptic function and plasticity in addition to their developmental roles in establishing synaptic contacts. Because activation of RPTP $\sigma$  is involved in several negative processes, such as inhibition of NGF signaling (Faux et al., 2007), it would make sense that the CNS would evolve a mechanism to avoid binding of RPTP $\sigma$  to the CSPGs of the perineuronal nets to circumvent competition with these other protein ligands.

In summary, we have demonstrated that the binding sites for RPTP $\sigma$  in adult brain are on neurons, and do not depend upon chondroitin or heparan sulfate proteoglycans. Because of the redundancies in binding and actions of the R2A family of protein tyrosine phosphatases, their binding partners and function in the adult brain is still a matter of active investigation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.



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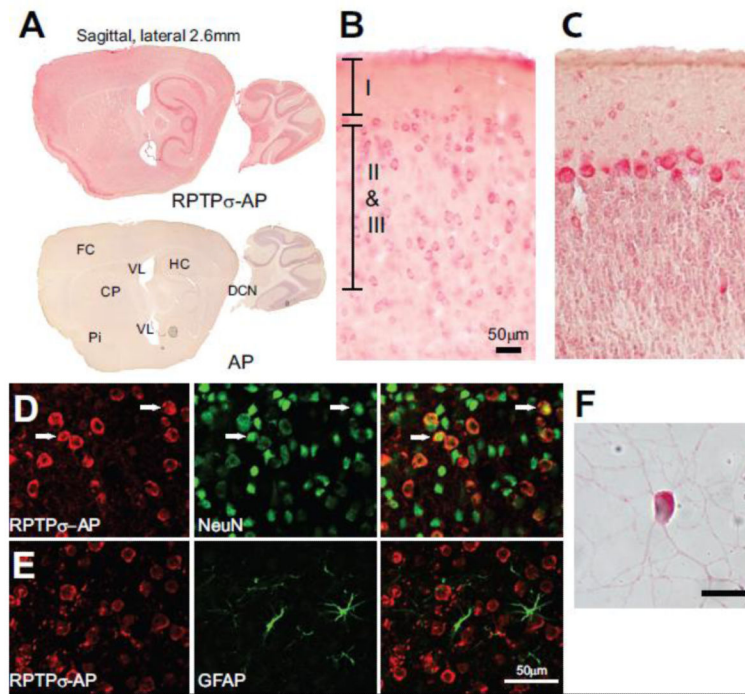
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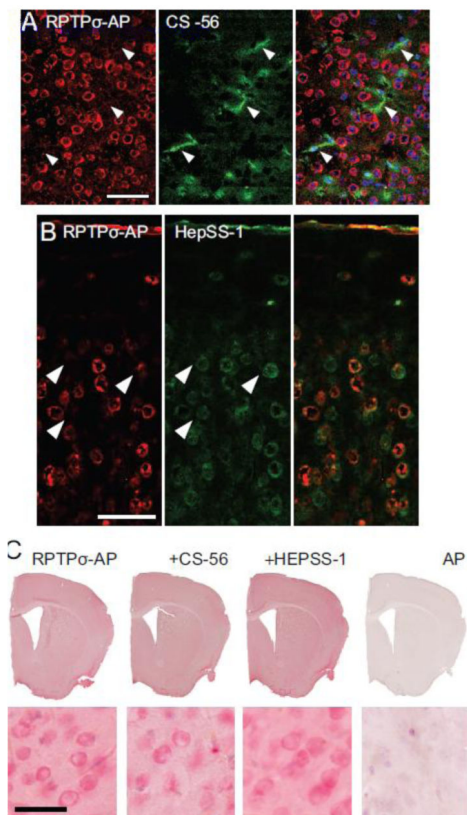
### Highlights

- A receptor affinity probe assay shows that RPTP $\sigma$  binds to neurons in sections of adult mouse brain
- RPTP $\sigma$  binding does not overlap with CSPG staining in adult mouse brain cerebral cortex
- Binding of RPTP $\sigma$  remains when heparan sulfate or chondroitin sulfate are removed enzymatically
- Binding of RPTP $\sigma$  is displaced by incubation with CS-E and heparin but not CS-A or CS-C
- Binding of RPTP $\sigma$  is altered by changes in ionic strength



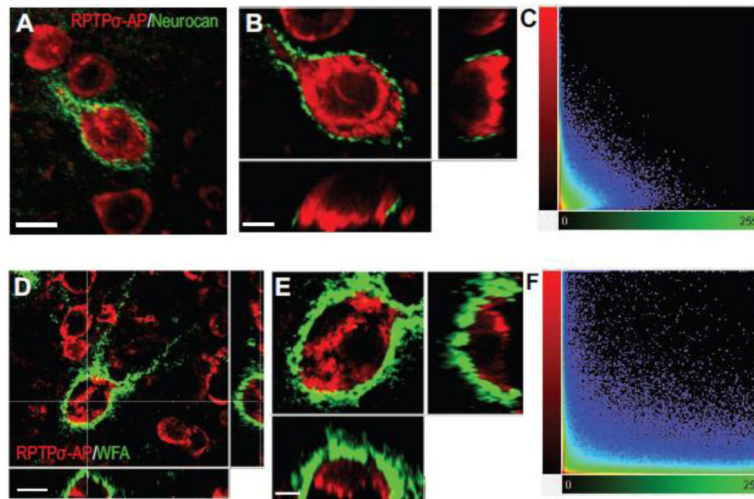
**Fig. 1.**

RPTP $\sigma$  binds to neurons in adult mouse brain sections. (A) Montage of sagittal brain sections incubated with RPTP $\sigma$ -AP or AP shows intense AP activity in the piriform cortex (Pi), hippocampus (HC), deep cerebellar nuclei (DCN), and frontal cortex (FC) of sections incubated with RPTP $\sigma$ -AP as compared to AP alone. (B) Higher magnification view of the frontal cortex shows many neurons labeled with RPTP $\sigma$ -AP. (C) Section through cerebellar cortex showing strong RPTP $\sigma$ -AP labeling of Purkinje neurons and weaker labeling of basket and stellate cells in the molecular layer. (D) RPTP $\sigma$ -AP binds to NeuN positive cells in the cerebral cortex (arrows). (E) RPTP $\sigma$ -AP does not colocalize with GFAP in the cerebral cortex. (F) RPTP $\sigma$ -AP binds to cerebellar granule neurons in culture. Scale Bars: B-E 50  $\mu$ m, F 10  $\mu$ m.



**Fig. 2.**

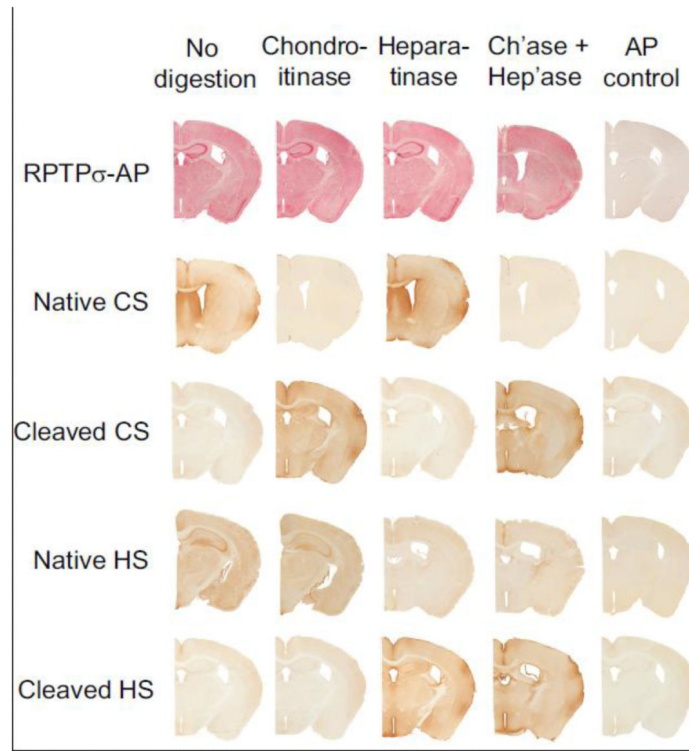
RPTP $\sigma$  does not bind to proteoglycans in the mouse brain. (A) RPTP $\sigma$ -AP and anti-CS (CS-56) double labeling in the mouse cerebral cortex show no overlap. Arrowheads show areas of higher CSPG content in the cortex stained positively for anti-CS that are not binding RPTP $\sigma$ -AP. (B) PTP $\sigma$ -AP binds to mouse cerebral cortical neurons which also demonstrate immunoreactivity with anti-HS antibody HepSS-1, but no overlap in reactivity is observed. (C) RPTP $\sigma$ -AP binding is not diminished by co-incubation with CS-56 or HepSS-1. Scale Bars: A-B 50  $\mu$ m, C - E 25  $\mu$ m.



**Fig. 3.**

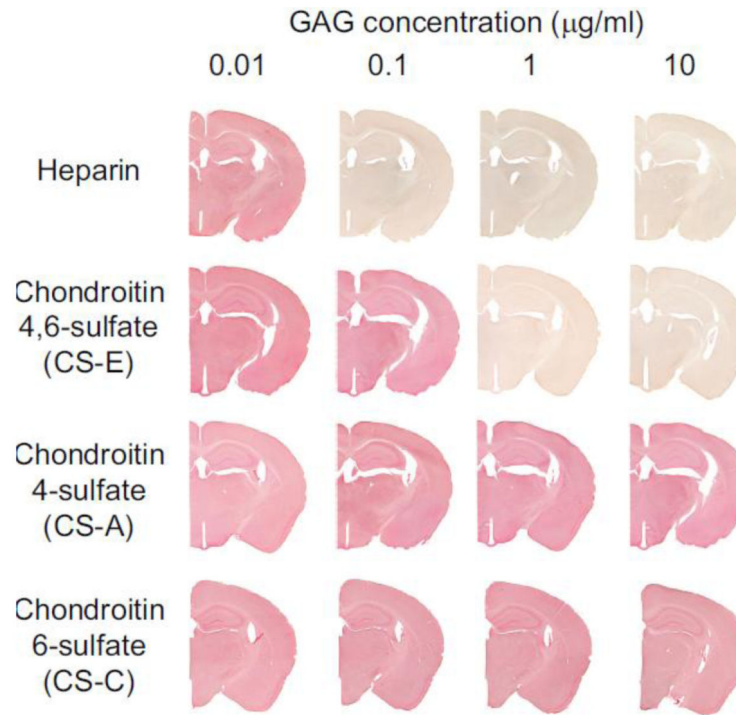
RPTP $\sigma$ -AP does not bind to perineuronal nets in mouse brain. (A-B) Double labeling with RPTP $\sigma$ -AP and neurocan. A) Labeling of neurons by RPTP $\sigma$ -AP and perineuronal nets by anti-neurocan. B) Higher magnification Z-stack images of RPTP $\sigma$ -AP and neurocan double labeling shows no overlap. C) 2D fluorogram showing no co-localization of RPTP $\sigma$ -AP and neurocan. Correlation coefficient of -0.07. Perfect correlation = 1, No correlation = 0, Inverse correlation = -1. D-E) Double labeling with RPTP $\sigma$ -AP and WFA. D) Labeling of neurons by RPTP $\sigma$ -AP and perineuronal nets by WFA. E) Higher magnification Z-stack images of RPTP $\sigma$ -AP and WFA double labeling shows no overlap. F) 2D fluorogram showing no co-localization of RPTP $\sigma$ -AP and WFA. Correlation coefficient of 0.00. Scale Bars: A, D 10  $\mu$ m, B, E 5  $\mu$ m.





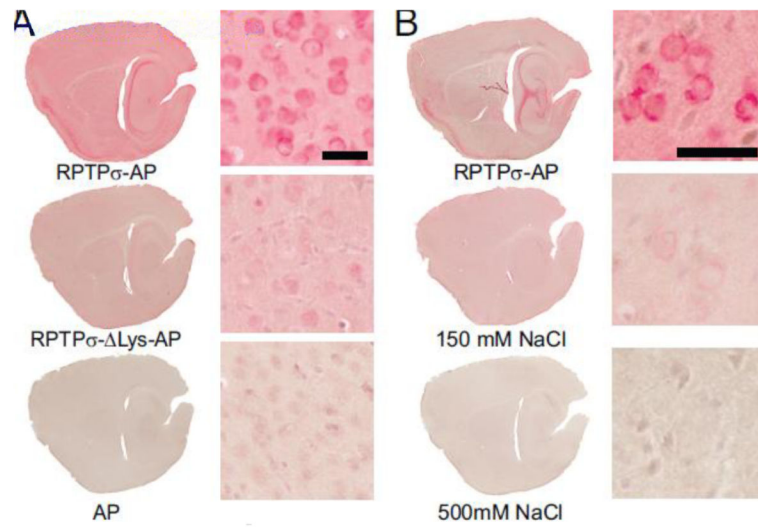
**Fig. 4.**

Enzymatic removal of proteoglycan GAGs does not reduce RPTP $\sigma$  binding. Predigestion of brain sections with chondroitinase or heparitinase or both removed native GAGs and created stub immunoreactivity, but did not affect the binding of RPTP $\sigma$ -AP to the brain sections. Native chondroitin-4-sulfate (2H6); native heparan sulfate (HepSS1); cleaved chondroitin-4-sulfate (MAB2030); cleaved heparan sulfate (3G10).



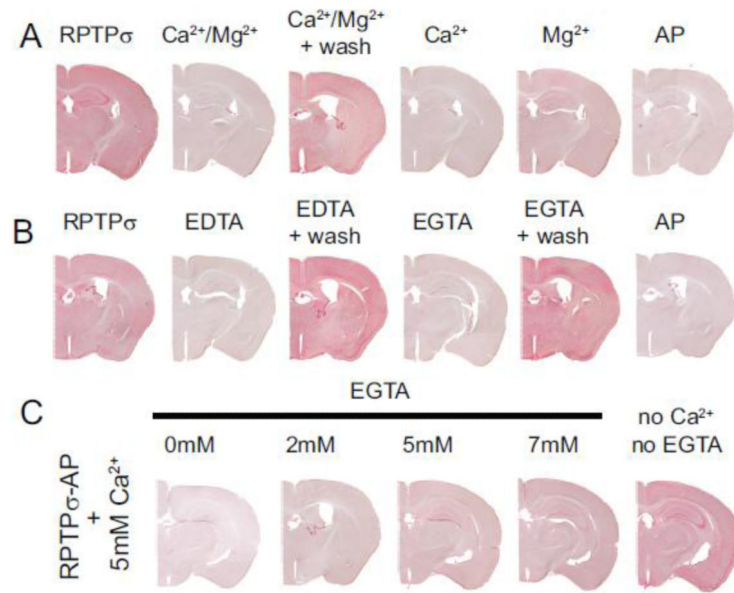
**Fig. 5.**

RPTP $\sigma$  binding to brain sections is effectively blocked by highly sulfated GAG chains. RPTP $\sigma$ -AP was incubated with brain sections with increasing concentrations of GAGs as indicated.



**Fig. 6.**

RPTP $\sigma$  binding to mouse brain is dependent on electrostatic interactions. (A) Mutation of lysines to alanines in the first immunoglobulin domain of RPTP $\sigma$  reduces binding to mouse brain sections. (B) Binding of RPTP $\sigma$ -AP to neurons in brain sections is reduced at 150 mM NaCl and eliminated at 500 mM NaCl. Scale bar = 20  $\mu$ m.



**Fig. 7.**

Binding of RPTP $\sigma$ -AP to mouse brain is altered by divalent cations. Brain sections were incubated with RPTP $\sigma$ -AP in (A) TBS, TBS supplemented with 5mM Ca<sup>2+</sup> or 5 mM Mg<sup>2+</sup> or both, or TBS after an exposure to high Ca<sup>2+</sup> and Mg<sup>2+</sup> (Ca<sup>2+</sup>/Mg<sup>2+</sup> + wash); (B) the presence of either EDTA or EGTA or TBS after exposure to EDTA or EGTA; (C) the presence of 5 mM Ca<sup>2+</sup> without or with increasing concentrations of EGTA.