TAM family of Receptor Protein Tyrosine Kinases

Downstream signaling pathway analysis

and protein expression in mouse brain

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Summary

Receptor Protein Tyrosine Kinases (RPTKs) are transmembrane proteins that relay extracellular signals to the intracellular environment. Tyro3, Axl and Mer, are structurally related RPTKs, and can be activated by their ligands, Gas6 or Protein S. TAM signaling has been shown to be important in the regulation of the immune response, maturation of spermatogonia and maintenance of the photoreceptor cells in the retina. The expression pattern of mRNA of the TAM receptors in brain has been investigated previously, however the expression of TAM receptor proteins has not been thoroughly studied. In the present study, an attempt was made to elucidate the downstream signaling pathway by engineering recombinant Gas6 and Protein S and expressing them in human embryonic kidney 293 (HEK293) cells. In addition to these recombinant ligands, constructs encoding wild type (WT) receptors or receptors carrying gain- or loss-of-function mutations at specific Tyrosine residues were engineered to be expressed in mouse embryonic fibroblasts (MEF) cells derived from mice that are triple knock out (TKO) for the TAM receptors. Furthermore, in order to characterize the expression of Tyro3 protein in brain, immunohistochemical procedures were applied to brain sections of WT mice. Characterization of Tyro3 expression in the brain revealed a pattern similar to what has been found for Tyro3 mRNA, with distinct Tyro3 protein in Purkinje cells in cerebellum, CA1 and CA3 regions of hippocampus, lateral intermediate septal nuclei and the basolateral amygdaloid nuclei and in cortex. Future research addressing the role of the TAMs in these areas could focus on analyzing specific neuronal functions, and will be aided by downstream signaling analysis and crossing mice that are single, double and triple KO for the TAM receptors and Gas6 or conditional KO for Protein S.

Abbreviations: bac: Basolateral Amygdaloid Nuclei; BSA: Bovine Serum Albumin; DG: Dentate Gyrus; DMEM: Dulbecco's Modified Eagle Medium; EGF: Epidermal Growth Factor; FBS: Fetal Bovine Serum; FGFR3: Fibroblast Growth Factor 3; Gla: γ-carboxyglutamic acid residues; HEK293: Human Embryonic Kidney 293; IP: immunoprecipitation; ITIM: Immunoreceptor Tyrosine-based Inhibitory; LG: Laminin G; LTD: Long Term Depression; LTP: Long Term Potentiation; MEF: Mouse Embryonic Fibroblast; NDS: Normal Donkey Serum; PCR: Polymerase Chain Reaction; Pfa: Paraformaldehyde; PVDF: Polyvinylidene fluoride; RPTK: Receptor Protein Tyrosine Kinase; SH2: Src Homology 2; SHBG: Sex Hormone-Binding Globulin; STAT1: Signal Transducer and Activator of Transcription 1; TKO: triple knock out; TLR: Toll Like Receptor; WT: Wild Type.

Introduction

Receptor Protein Tyrosine Kinases (RPTKs) are transmembrane proteins that relay extracellular signals to the intracellular environment (*1*). The TAM family of RPTKs consists of three structurally related RPTKs Tyro3, Axl and Mer. These three transmembrane receptors share a similar extracellular domain, composed of two N-terminal Immunoglobulin-like domains and two Fibronectin type III-like domains, a transmembrane domain and an intracellular kinase domain (*2, 3*). Binding of a ligand to the extracellular domain of a receptor induces dimerization of receptor molecules, resulting in activation of the intracellular tyrosine kinase domain. This activation of one monomer leads to phosphorylation of tyrosine residues on the next monomer, thereby increasing the catalytic efficiency of the kinase and possibly creating docking sites for signal transduction molecules (*4, 5*).

The TAM receptors are widely expressed in many adult tissues. The importance of regulatory TAM receptor signaling becomes apparent in at least three well described phenotypes of mice that are single or triple KO for the TAM receptors. In TKO mice testes are smaller than in WT mice, the cellular organization of the seminiferous tubules is severely disrupted and progressive death of differentiating germ cells leads to infertility (*6*). TAM receptors are expressed in the testis in Sertoli cells. TAM signaling might function in regulating the physical and trophic support that Sertoli cells provide for the developing spermatogonia, although this has not been studies in great detail. TAM receptor signaling also provides homeostatic regulation of the immune system, as TKO mice develop a severe lymphoproliferative disorder and broad spectrum auto-immunity. TAM receptors are expressed by macrophages and dendritic cells and serve as a self-extinguishing regulatory mechanism that limits the severity and duration of inflammatory immune responses (*7*). Furthermore, TAM receptor signaling plays and important function retina homeostasis. The photoreceptor cells continuously synthesize outer segment membrane, which is cleared by retinal pigment epithelium cells. This specialized phagocytosis is dependent on Mer, and the importance of this Mer dependent phagocytosis is underscored by the finding that in Mer KO mice postnatal degeneration of photoreceptors leads to blindness (*8*). In humans, mutations in the *MER* orthologue, *MERTK*, are associated with retinitis pigmentosa as revealed by a screening of this gene in several hundred individuals with various retinal dystrophies (*9*).

The TAM receptors are also expressed in distinct and highly regionalized patterns in the adult brain. In adult brain, Axl and Mer mRNAs were found markedly in Purkinje cells in the cerebellum. Mer mRNA was also reported in the molecular layer of the Dentate Gyrus (*10*). During prenatal development Tyro3 mRNA expression is already present at high levels; postnatally Tyro3 was found in the granule cell layer in the cerebellum and in the Purkinje cells. Western blot analysis showed that postnatal increase in Tyro3 mRNA in whole brain is paralleled by an increase in protein expression (*10, 11*). Currently, no studies have addressed the cellular expression profile of the Tyro3 receptor in the CNS. Establishing the expression pattern of TAM receptors by measuring protein will give additional indications to functional expression. The ideal negative control for the immunohistochemistry will be the analysis of mice that are TKO. Because TKO mice develop autoimmunity, neuronal phenotypes in these mice could be the result of both cell autonomous, or non-cell autonomous as a result of the autoimmunity. With respect to this issue, analyses in the single and double KO, where autoimmunity is less pronounced, could prove more valuable.

Two ligands for the TAM receptors have been described: Gas6, the product of Growth Arrest Specific 6 gene, and Protein S (*12*). These two proteins share about 44% overall amino acid identity and contain a similar domain distribution: an N-terminal region that contains eleven γ-carboxyglutamic acid residues (Gla), a loop region, four epidermal growth factor (EGF)-like repeats and a C-terminal sex hormone–binding globulin (SHBG)-like structure, composed of two globular laminin G (LG)-like domains. The Gla-domain can be γ-carboxylated, a process that requires Vitamin K as a cofactor and is needed for Gas6 to bind to membrane outer segment in retinal pigment epithelium (*13*). A systematic *in vitro* approach revealed that human Tyro3 and Axl are activated by human Gas6 but not by human Protein S; murine Tyro3 however was activated by human Protein S (*14*). This species specificity for activation by Protein S is caused by critical residues that are distributed throughout the N-terminal LG-like domain of the SHBG domain (*15*). Still, the status of Protein S as a bona fide ligand for the TAM receptors remains controversial, and except for the study in RPE cells (*8*), no further studies have definitively determined this status for Protein S. However, preliminary results from this lab (*16*) (Conducted in the lab of Dr. Greg Lemke Molecular Neurobiology Laboratory, Salk Institute for Biological Studies, La Jolla, CA, USA) indicate that full length mouse Protein S activates the mouse Tyro3 receptor in HEK293 (HEK293) cells.

In adult rat, Gas6 mRNA was found to be widely expressed in part of the hippocampus, the amygdala, the striatum, the optic tract, the substantia nigra, the oculomotor nuclei, the raphe nucleus and the medial geniculate nucleus. It was pointed out that there are many overlapping regions of Gas6 mRNA and Tyro3 mRNA expression, such as the CA1 and CA3 region in the hippocampus and in the Purkinje cells in the cerebellum (*17*). *In situ* hybridization and immunohistochemistry on rabbit brain suggested expression of Protein S in the pyramidal neurons in the cortical internal pyramidal cell layer and in the hippocampus (*18*). However, rat Protein S mRNA was only detected in the locus coeruleus and choroid plexus (*17*). In the same report Gas6 was found in Purkinje cell bodies and dendrites by immunohistochemistry (*17*). In order to elucidate possible functions for the TAM receptor system, establishing more thoroughly the protein expression patterns of the TAM receptor ligands, Gas6 and Protein S, and identify co localization with TAM receptors will aid in determining the functions of TAM receptor signaling in the nervous system.

Dendritic cells of the immune system express Toll-Like receptors (TLRs). TLRs are transmembrane receptor proteins that are activated upon recognition of pathogen associated molecular patterns (PAMPs). TLRs trigger intracellular cascades of signaling events that ultimately up regulate genes encoding pro inflammatory cytokines (*19, 20*). Gas6 induced TAM receptor activation inhibits TLR mediated cytokine expression, and preliminary data from this laboratory revealed that this inhibition is dependent on transcription and translation, indicating that TAM receptor signaling results in increased expression of negative regulators of TLR signaling. In agreement with this hypothesis, it was found that TAM receptor activation leads to phosphorylation, i.e. activation, of Signal Transducer and Activator of Transcription 1 (STAT1) (*21*). Activated STAT1 dimerizes and translocates to the nucleus where it binds to DNA and regulates gene transcription (*22*). Another RPTK, Fibroblast Growth Factor 3 (FGFR3), was reported to activate STAT1 through phosphorylation of a critical and conserved tyrosine residue in the intracellular domain of FGFR3 (Y^{724})(*23*). This tyrosine together with the flanking motif, C-x-x-x-x-**pY**-x-x-M, is conserved in the TAM receptors (Figure 1), hinting towards a role for this tyrosine in TAM receptor mediated STAT1 activation.

Furthermore, all three TAM receptors share an immunoreceptor tyrosine-based inhibitory (ITIM) motif (I/V/L-x-**pY**-x-x-I/V/L) and Axl and Mer share an additional ITIM-like motif (E-I/M-Y-D/N-pY-L) (*7*) (Figure 1). Instead of the central

tyrosine present in the Axl and Mer ITIM-like motif, Tyro3 contains an Alanine in an otherwise identical ITIM motif. ITIM and ITIM-like motifs are binding sites for the highly conserved Src homology 2 (SH2) domains (*24*). Two ubiquitously expressed phosphatases, SHP1 and SHP2, share highly similar sequences and contain two adjacent SH2 domains at the N-terminus, and a tyrosine phosphatase domain at the C-terminus (*24, 25*). SHP1 has been reported to play a role in dephosphorylation of STAT1 (*26*), implying that SHP1 functions in switching off gene transcription by STAT1. This way, SHP1 could alleviate the inhibition on TLR mediated cytokine production. However, contrary to this hypothesis, it was reported that defective SHP1 can lead to severe autoimmunity and that low levels of functional SHP1 correlated with excessively tyrosine-phosphorylated STAT1 in patients suffering from multiple sclerosis, an immune mediated brain disease (*26*). This indicates that SHP1 might function in another signaling pathway too, that, when shutdown, results in autoimmunity. Both STAT1 and SHP1 are present in brain; STAT1 is expressed in rat brain at the day of birth, with increasing levels towards adulthood and is up regulated upon brain infarction, in a time course coincidental with strong glial reaction (*27*), and SHP1 has been shown to be necessary for the normal differentiation and distribution of glia within mouse brain (*28*). As these proteins have been shown to be working in at least partly overlapping signaling pathways in cells of the immune system, STAT1 and SHP1 could function in similar pathways in brain.

Consistent with the presence of ITIM motifs in the Axl and Mer receptors, preliminary data from this laboratory (*21*) indicate that SHP1 associates with Axl and Mer in bone marrow derived dendritic cells. Possibly, TAM receptor mediated inhibition of TLR induced cytokine expression requires SHP1 to get activated by associating with the ITIM or ITIM-like motif contained by TAM receptors. Currently no studies on the role of the TAM receptors in STAT1-SHP1 or STAT1-SHP2 signaling have been reported. In this study, an attempt was made to unravel signaling pathways downstream of the TAM receptors. To this end, different constructs encoding recombinant Gas6 and Protein S proteins that lack the Gla domain and contain an N-terminal or C-terminal histidine tag for purification were engineered. These recombinant ligands are essential for studying the activation of TAM receptors, as they enable the specific activation of only TAM RPTKs. Furthermore, obtaining the ligands without the Gla domain, could increase their bioactivity, as the need for γ-carboxylation is abolished. The recombinant proteins were to be expressed in HEK293 cells and purified from the medium by affinity chromatography.

In order to assess the biological activity of the recombinant ligands, MEF (MEFs) derived from mice that are triply KO for the receptors were transfected with either WT Tyro3, Axl or Mer. Subsequently, these cells were stimulated with individual purified recombinant proteins to confirm phosphorylation of the intracellular domain of the receptor.

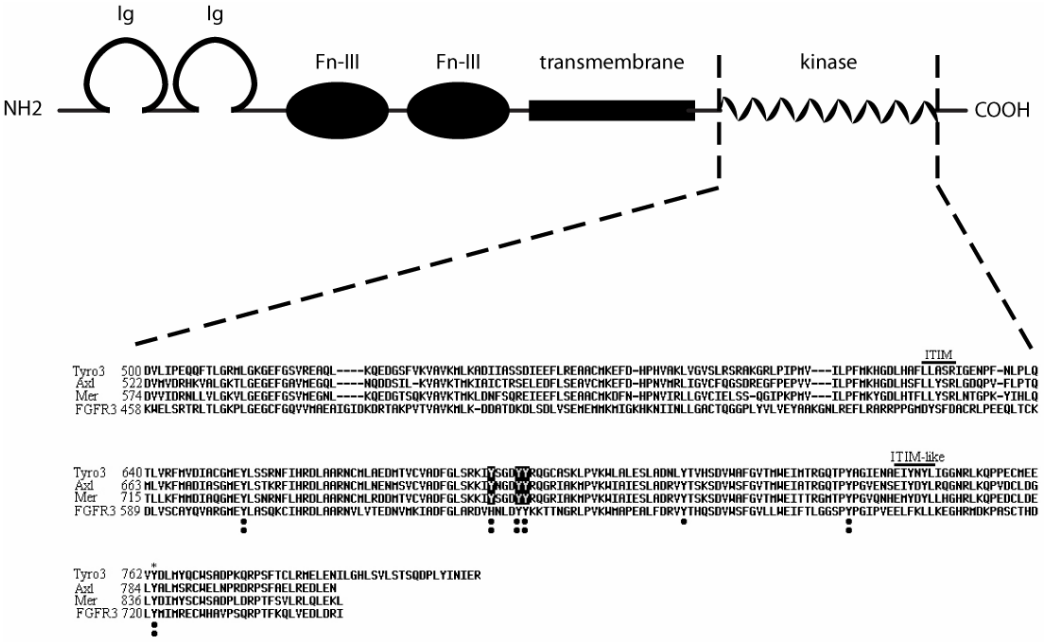


Figure 1 – *Schematic representation of the general TAM domain distribution and alignment (29) of the catalytic domain of the TAM receptors and FGFR3. The ITIM and ITIM-like motifs are indicated by a black bar and the FGFR3 STAT1-activating tyrosine by an asterisk. Blackened amino acids represent the major autophosphorylation sites of Mer (30). Twin dots indicate tyrosine residues that are conserved throughout subclasses of receptor protein tyrosine kinases (31) and the single dot indicates a tyrosine that is conserved in the TAM receptors.*

Essential prerequisites for understanding the importance of conserved tyrosine residues within the ITIM, ITIM-like and STAT1 activating motifs in the intracellular domain of the TAMs, are constructs encoding point mutations in the coding sequences for the TAM receptors so that the translated proteins for Tyro3, Axl or Mer carry gain-or loss-of-function mutations at specific tyrosine residues. In the present study, these constructs were designed and engineered, and these receptors were to be individually expressed in MEFs derived from triple KO mice. Introducing these mutations should only abolish their ability to associate with STAT1 or SHP1, and not interfere with the kinase activity of the mutated receptors. Therefore, kinase

activity of the receptors was to be achieved by assessing their level of phosphorylation upon stimulation of the cells with the recombinant ligands. Next, the ability of the mutated receptors to bind and/or phosphorylate STAT1 and SHP1 was to be examined by immunoprecipitation assays.

To address functional relevance to the downstream TAM signaling pathways in adult brain, establishing the expression patterns of the involved proteins at the cellular level by immunohistochemistry is essential. Initial studies have focused mainly on TAM receptor mRNA expression analysis in brain and found that Tyro3 is the most highly expressed and widely distributed of the TAM receptors in adult brain (*10*). However, currently no detailed studies have been performed on the expression of the TAM receptor by measuring protein in brain. To this end immunohistochemical procedures were performed with anti-Calbindin or anti-Tyro3 antibodies on WT mouse brain sections. Tyro3 protein was found in Purkinje cells in cerebellum, CA1 and CA3 region of hippocampus, lateral intermediate septal nuclei and the basolateral amygdaloid nuclei and in cortex.

Materials and Methods

DNA-constructs

In order to obtain recombinant proteins Gas6 and Protein S, both lacking the Gla-domain and carrying a HIS tag consisting of six Histidine residues on either the N-terminus or C-terminus, Gas6 and Protein S DNA were amplified by polymerase chain reaction (PCR) starting from the codon coding for the asparagine residue 120 and 127, respectively. For N-terminal 6xHis tags primer sequences are 5'- CCG G AAT TCA CAT CAC CAT CAC CAT CAC CCT TGT GAT AAG AAG GG-3', 5'-G CTC TAG AGC CTA GGG GGT GGC ATG CTC CAC AGG-3' and 5'- C CGG AAT TCC CAT CAC CAT CAC CAT CAC AAT GAA GAT GGG TAT CTG GCC TGC C-3', 5'-C GGG ATC CCG CTA AAA ATT CTT CTG GAT CTT CCT CAC TGA CGG GCA CG-3' for ^{6x}HIS-^{N120}Gas6 and ^{6x}HIS-^{N127}ProS, respectively. For C-terminal 6xHis tags primer sequences are 5- CCG G AAT TCA CCT TGT GAT AAG AAG GGT ACT CAT ATC TGC-3', 5'-G CTC TAG AGC CTA GTG ATG GTG ATG GTG ATG GGG GGT GGC ATG CTC-3' and 5'- CCGG AAT TCC AAT GAA GAT GGG TAT CTG GCC TGC CAA GAT GGC C-3', 5'- C GGG ATC CCG CTA GTG ATG GTG ATG GTG ATG AAA ATT CTT CTG GAT CTT CC-3' for ^{N120}Gas6-^{6x}HIS and ^{N127}ProS-^{6x}HIS respectively. The primer sets included different restriction sites for digestion and subsequent ligation. All four PCR mixes contained 0.2 mM dNTPs, 1.0 mM $MgCl_2$, 30 ng DNA template, 0.4 µM of each forward and reverse primer and 1 U KOD DNA High Fidelity Polymerase (Novagen) in 50 µl 1X KOD DNA High Fidelity Polymerase buffer #1 (Novagen). After PCR, fragments were separated by agarose gel electrophoresis and bands of the appropriate size were excised after which DNA was purified using a Gel Extraction kit (Qiagen), following the manufacturer's protocol. Purified PCR products and plasmid pFLAG-CMV-1 (Sigma-Aldrich) were digested with either endonucleases EcoR1 and XbaI for Gas6 DNA, or with EcoR1 and BamH1 for Protein S DNA, following the manufacturer's guidelines (New England Biolabs). Subsequently, the plasmid was dephosphorylated using Antarctic Phosphatase, according to the guidelines provided online by the manufacturer (New England Biolabs). Inserts were ligated into the vector using the T4 DNA Rapid Ligation Kit, as recommended by the manufacturer (Roche). From these plasmids, the DNA encoding the truncated proteins carrying a His-tag and preprotrypsin sequence for secretion was amplified by PCR. Following PCR, digestion of the amplified DNA and ligation into pcDNA6-Myc/His A, that contains a blasticidin

resistance cassette was done as described above, with primer sequences 5'-CG CGG TAC 'CGT CAG AAT TAA TTC ACC ATG TCT GC-3' and 5'- TAG GCC' TCG AGC CAG GGC CAG GAG AGG CAC TGG-3' for all four constructs.

Gain or loss of function mutations were introduced in pcDNA6-myc/His constructs coding for TAM receptors in either the ITIM domain, the ITIM-like domain or the putative STAT1 activating domain by applying the QuikChange Site-Directed Mutagenesis Kit. For Tyro3, a gain of function mutation creating an ITIM domain was introduced. To introduce loss of function mutations in both the ITIM and ITIM-like domain of Axl and Mer, the QuikChange Multi Site-Directed Mutagenesis kit (Stratagene) was applied to the respective constructs. Primers were designed using the manufacturer's on-line software (http://www.stratagene.com/qcprimerdesign).

To amplify the constructs, chemo competent *E. coli* cells were electroporated with one of the engineered constructs. This was achieved by mixing 100 µl of cells with ~10 ng construct and incubating on ice for 15 min, followed by a heat shock of 42°C of 1 min. after which the cells were incubated in 1 ml LB-medium for at least 60 min. at 37°C. Selection of positive cells containing plasmid was performed by plating the cells on selective LB agar plates containing 100 µg/ml ampicillin and 16 hr incubation at 37°C. Positive colonies were cultured in 3 ml LB medium containing 100 µg/ml ampicillin at 37°C, shaking at 225 rpm for ~16 hr. Next, a Mini Prep Kit (Qiagen) was applied to each culture to isolate plasmid DNA. Purified plasmids were sequenced (ETON Bioscience Inc). After confirmation of the correct sequence, plasmids were used to transform chemo competent *E. coli* cells as described, followed by culturing in 3 ml LB medium containing 100 µg/ml ampicillin for ~8 hr. Next, cultures were transferred to 250 ml of the same medium, and incubated under the same conditions for ~16 hours. In order to isolate plasmid DNA for transfection of mammalian cells, a Maxi Prep Kit (Qiagen) was applied.

Cell cultures and transfections

HEK-293 were maintained at 5% CO_2 in round Falcon dishes (100 mm radius) containing Dulbecco's Modified Eagle Medium (DMEM) (Gibco) with 10%(v/v) fetal bovine serum (FBS) and 1%(v/v) penicillin/streptomycin (Gibco)(culture medium). Before confluency, cell cultures were washed twice with PBS, and collected in a 50 ml Falcon tube after incubation with 3 ml 1x Trypsine-EDTA (Gibco) for ~4 min. at 37°C. After washing with PBS, ~10,000,000 cells were resuspended in 1 ml culture medium and incubated at room temperature for 5 min. with 10 µg

pcDNA6-myc-HIS plasmid containing either ^{6x}HIS-^{N120}Gas6, ^{6x}HIS-^{N127}ProS, ^{N120}Gas6-^{6x}HIS or ^{N127}ProS-^{6x}HIS, which was linearized by incubating at 65 °C for 10 min. After incubation, the cells were electroporated with a 0.23 V pulse and grown in round Falcon dishes with a radius of 3000 mm in culture medium. After ~8-16 hr, the selective antibiotic blasticidin was added to the culture medium to a final concentration of 10 µg/ml. Surviving cells were expanded for approximately 10 days, after which these were transferred to a 96-wells plate. After expansion, but before confluency, cells from each well were transferred to an individual well in a 24-wells plate and similarly the cells were transferred to 10 mm falcon dishes.
Culturing and stable transfection of MEF derived from triple KO mice (TKO) was achieved with individual constructs coding for a WT (WT) TAM receptor or a mutated receptor following the same procedure as for the HEK293 cells.
Transient transfection of NIH-3T3 cells, maintained under the same conditions as HEK293 cells, was achieved using the Effectene Transfection Reagent (Qiagen), following the manufacturers directions. The day after transfection, culture medium was refreshed and the activation assay was started on the second day after transfection.

Activation assay and Immunoprecipitation

NIH-3T3 cells were starved for FBS for ~16 hour in culture medium containing 0.25% (v/v) FBS. Next, the cells were cultured without FBS for 3 hours, after which they were activated by 10% (v/v) FBS for different times. Immediately following activation, the NIH-3T3 cells were lysed in 800 µl lysis buffer, containing 50 mM Tris, pH8 and 150 mM NaCl, 1% (v/v) Nonidet P-40, 1:100 Phosphatase Inhibitor Cocktail 2 (Sigma) and a Mini Protease Inhibitor Cocktail Tablet (Roche Complete, EDTA free) per 100 ml. The lysates was spun down at 14,000 g at 4°C for 10 minutes and the supernatant was precleared by end-over-end incubation with 20 µl Magna Bind Protein G Magnetic Beads (Pierce) at 4°C for 1 hour. The cleared lysate was then incubated end-over-end with 5 µl Goat-anti-Axl antibody at 4°C for ~16 hours, followed by incubation with 20 µl Magna Bind Protein G Magnetic Beads (Pierce) at 4°C for 2 hours. The beads were collected and immunoprecipitated proteins were eluted by resuspending in 20 µl SDS loading buffer and boiling at 100°C for 5 minutes.

Immunoblotting

Transfected whole cell MEF, HEK293 lysates or NIH-3T3 immunoprecipitates were resolved by SDS-PAGE, and proteins were transferred to PVDF membrane (Millipore). Proteins were detected by enhanced chemiluminescence. After detection of the phosphorylated tyrosines, the blot was stripped by boiling in TBS-T for ~30 seconds, washed several times in PBS-T, and used again with the antibodies for the receptor. Antibodies used for immunoblotting were as follows: Mouse-anti-Phospho-Tyrosine (P-Tyr-100; Cell Signaling); Goat-anti-mouse Axl (Santa Cruz Biotechnology, Inc.) Rabbit-anti-Tyro3 (*32*); and ECL™ Donkey-anti-rabbit IgG Horseradish Peroxidase and HRP-anti-mouse IgG Horseradish Peroxidase (Amersham Biosciences).

Immunohistochemistry

At six weeks after birth, c57/Bl6 mice were deeply anesthetized by an injection of avertin (tribromoethanol, Sigma-Aldrich; intra peritoneal 0.6-0.7 ml of 2.5% solution in 0.9% NaCl) and transcardially perfused with heparine for 30 s, followed by 4% paraformaldehyde (PFA) in PBS, pH 7.4 for 5 minutes. Brains were removed and immersed in 4% PFA in PBS for 5 hours for additional fixation. Next, the tissue was equilibrated in 30% sucrose (w/v) in PBS for cryoprotection for ~ 16 hours, followed by immersion in TBS™ Tissue Freezing Medium (Triangle Biomedical Sciences) and frozen at -70°C. Cryostat sections of 10 µm were collected onto Precleaned Superfrost/Plus Microscope Slides (Fisher Scientific) and were allowed to air-dry over nigh. Next, the sections were boiled in Antigen Unmasking Solution (Vector Laboratories) for 10 minutes, washed in PBS 3 times for 5 minutes and treated with a 1:3 dilution in PBS of a trypsine solution (Zymed) for 5 minutes. After three 5 minutes washing in PBS the sections were blocked for 1 hour with block buffer, containing 5% (v/v) normal donkey serum (NDS), 1% bovine serum albumin (BSA) and 0.05% (w/v) Triton X-100 in PBS. After blocking, the sections were incubated with primary antibody in 1% (v/v) NDS, 1% BSA and 0.05% (w/v) Triton X-100 in PBS at 4 °C for ~16 hours. Primary antibodies were: goat-anti-mouse DTK (R&D systems) in a 1:200 dilution and rabbit-anti-calbindin D-28K (SWANT) in a 1:750 dilution. The next day, sections were washed 3 times 5 minutes in 1% (v/v) NDS, 1% BSA and 0.3% (w/v) Triton X-100 in PBS (wash buffer) and incubated with the secondary antibodies diluted in wash buffer at room temperature for 1 hour. Secondary antibodies were: biotinylated horse-anti-goat (Vector Laboratories Inc.)

and donkey-anti-rabbit-CY^{TM}2 (Jackson Immunoresearch laboratories Inc.), both in a 1:200 dilution. Following incubation, the sections were washed three times for 5 minutes in wash buffer and incubated with CY^{TM}3-conjugated streptavidin (Jackson Immunoresearch laboratories Inc.) in 1% (v/v) NDS, 1% BSA and 0.05% (w/v) Triton X-100 in PBS at room temperature for 30 minutes. The sections were washed 2 times in wash buffer, incubated with 0.002 ‰ (w/v) Hoechst nuclear stain (Sigma-Aldrich) in PBS, washed in PBS 3 times for 5 minutes, and coverslips were mounted using Aqua Poly/Mount (Polysciences). Sections were illuminated using an X-cite^{TM} 120 Fluorescence Illumination System (EXFO) connected to an AxioSkop 2 (Zeiss). Photographs were digitally obtained via an AxioCam MRm (Zeiss) connected to a PC running Windows XP Professional (Microsoft) with the software program AxioVision Release 4.5. Immunofluorescence staining was considered to be at background levels when the intensity of the signal matched that observed when the primary antibody was omitted.

Results

Downstream TAM receptor signaling

Description of the constructs

In order to express the two ligands, each with either an N-terminal or a C-terminal Histidine tag, constructs were engineered that contain the coding sequence for Protein S or Gas6 excluding the Gla-domain, as the Gla-domain might affect ligand bioactivity *in vitro*. This could be because of the need for γ-carboxylation of the Gla-domain in order for it to bind to the membrane and bring the SHBG-like domain in proximity of the receptors. After confirmation of successful cloning by sequence analysis, HEK293 cells were transfected with individual constructs for the ligands, positive clones were selected by means of blasticidin resistance. Over 200 clones were obtained after selection. Due to time restrictions, only a small number have been assessed by immunoblot for expression of the recombinant proteins. None of these clones, however, showed immunoreactivity for an anti-His tag, which should be present in these proteins.

The constructs encoding the individual receptors were electroporated into MEF TKOs, with the intention of being able to test the bioactivity of the ligands, and to set up a system in which a single receptor can be studied. The Mer-electroporated MEFs and over 40 clones for each Tyro3 or Axl receptor were screened for expression of the protein by Western blot. Unfortunately, in none of the clones the recombinant protein was detected.

A schematic representation of the domain distribution of the TAM receptors is shown in figure 2 A. In order to study the importance of conserved tyrosine residues in the intracellular domain of the receptors in SHP1/2 and STAT1 signaling, a number of putative loss of function mutations were engineered, as listed in the table in figure 2 B. Briefly, for Tyro3 a construct was engineered to code for the receptor with a putative gain of function mutation, i.e. introduction of a presumable functional ITIM domain and one to code for the receptor containing a loss of function mutation in the ITIM-like domain. For Axl and Mer putative loss of function mutations were engineered so that the proteins contain either a non functional ITIM domain, a non functional ITIM-like domain or both non functional ITIM and ITIM-like domains. Furthermore, loss of function mutations were introduced in the putative STAT1 activating tyrosine residues. Confirmation of the sequences was achieved through

sequence analyses, MEF cells were transfected by electroporation and individual transfected MEF clones were isolated as was done for the HEK293 cells.

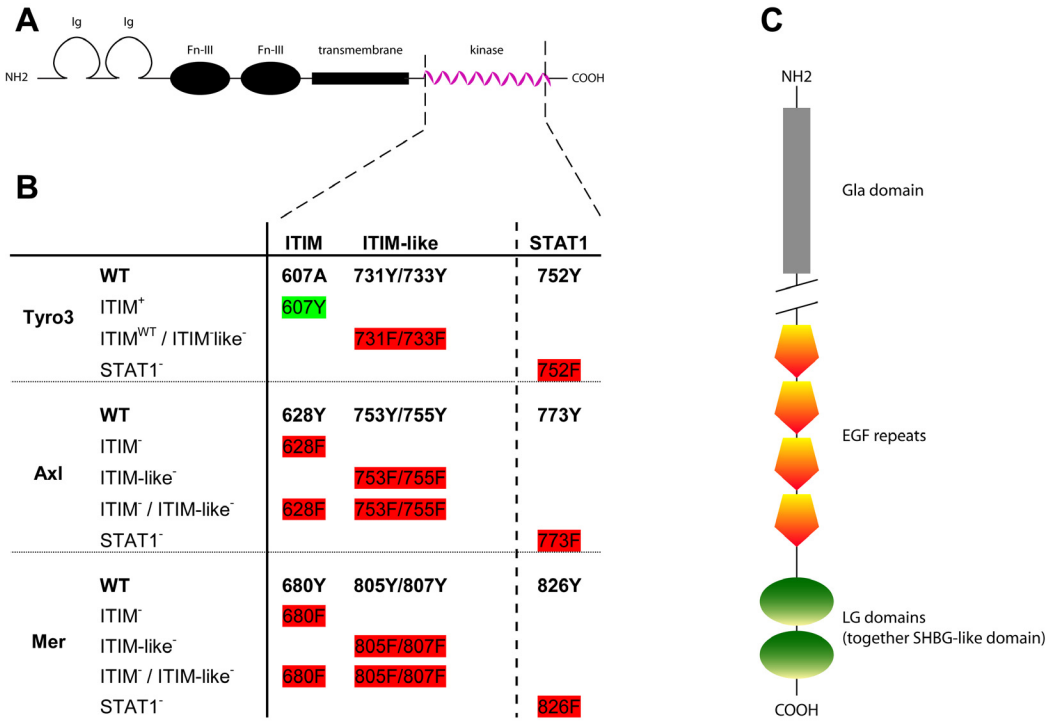


Figure 2 – A: *schematic representation of the general TAM receptor domain distribution.* ***B:*** *conserved motifs within the intracellular kinase domain and constructs designed for this study. For Tyro3, a putative gain of function mutation was made (green) so that the protein contains both an ITIM motif and an ITIM-like motif. For all three TAM receptors a putative loss of function mutation was introduced in the ITIM-like motif (red), and for Axl and Mer additional putative loss of function mutations were introduced in the ITIM motif separately and in the ITIM and ITIM-like motif together (red). For all three receptors a putative loss of function mutation was introduced, substituting the tyrosine residue that is conserved in the TAM receptors and the FGFR3 receptor for phenylalanine.* ***C:*** *schematic representation of the general domain distribution of Gas6 and Protein S. The constructs were truncated at the C-terminal end of the Gla domain, and contained a* ^{6x}*His-tag either at the truncated N-terminal or the C-terminal.*

Axl is phosphorylated upon treatment with FBS in NIH-3T3 cells

To set up a system in which it is possible screen small molecule inhibitors, NIH-3T3 cells were activated with FBS for different times. Next, the cells were lysed, Axl was immunoprecipitated and SDS-PAGE resolved lysates were transferred to PVDF-membrane. The membrane was probed for phospho-Tyrosine and subsequently probed for Axl expression (Figure 3). An increase n phosphorylated axl could be observed.

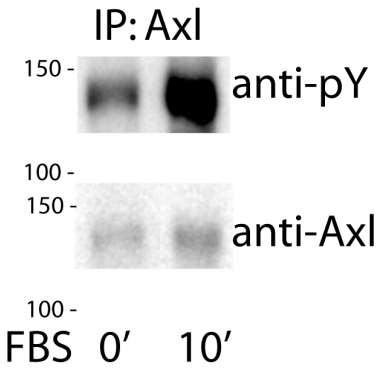


Figure 3 *–Immunoblot presenting Axl immunoprecipitation of NIH-3T3 cells. The blot was probed for phospho-tyrosine, and reused for probing with anti-Axl. Times indicate duration of stimulation with FBS after starvation.*

Immunohistochemistry

Immunohistochemical Tyro3 expression pattern in adult mouse brain

In order to determine the cellular expression pattern of Tyro3 in the mouse brain, immunohistochemical procedures were performed utilizing immunofluorescence to visualize the TAM receptor Tyro3, Calbindin D-28K, a marker for Purkinje neurons in cerebellum (*33*) and a nuclear marker (Hoechst Nuclear Stain) on cryo-sections of adult (P42) WT mouse brain.

In sagittal sections of the cerebellum, Tyro3 immunoreactivity was observed throughout the Purkinje cell bodies where it apparently co localizes with calbindin D-28K (Figure 4). The dendrites of Purkinje cells also showed Tyro3 immunoreactivity, gradually decreasing towards the distal end of the dendritic branches. Here too, apparent co localization was observed with Calbindin D28-K. Calbindin D28-K immunoreactivity was found in the cytoplasm of Purkinje cell axons, whereas Tyro3 was found exclusively along the edges of presumable Purkinje cell axons. Importantly, very distinct Tyro3 immunoreactivity was present in fibers radiating from the Purkinje cell layer. These are likely the Bergmann glia. Note that in order to visualize these fibers, the immunohistochemical procedure was slightly altered, i.e. omission of boiling in Antigen Unmasking Solution. This alteration, however, resulted in loss of some Tyro3 immunoreactivity that was present before, e.g. in the Purkinje cells. This immunoreactivity is non specific, as the signal was present at comparable levels in the control without the primary antibody. A similar artifact was found in the

Dentate Gyrus. Using either CY^{TM}3-, CY^{TM}2 or FITC-conjugated streptavidin yielded the same non specific binding of these fibers, suggesting that streptavidin associates with Bergmann glia under these conditions.

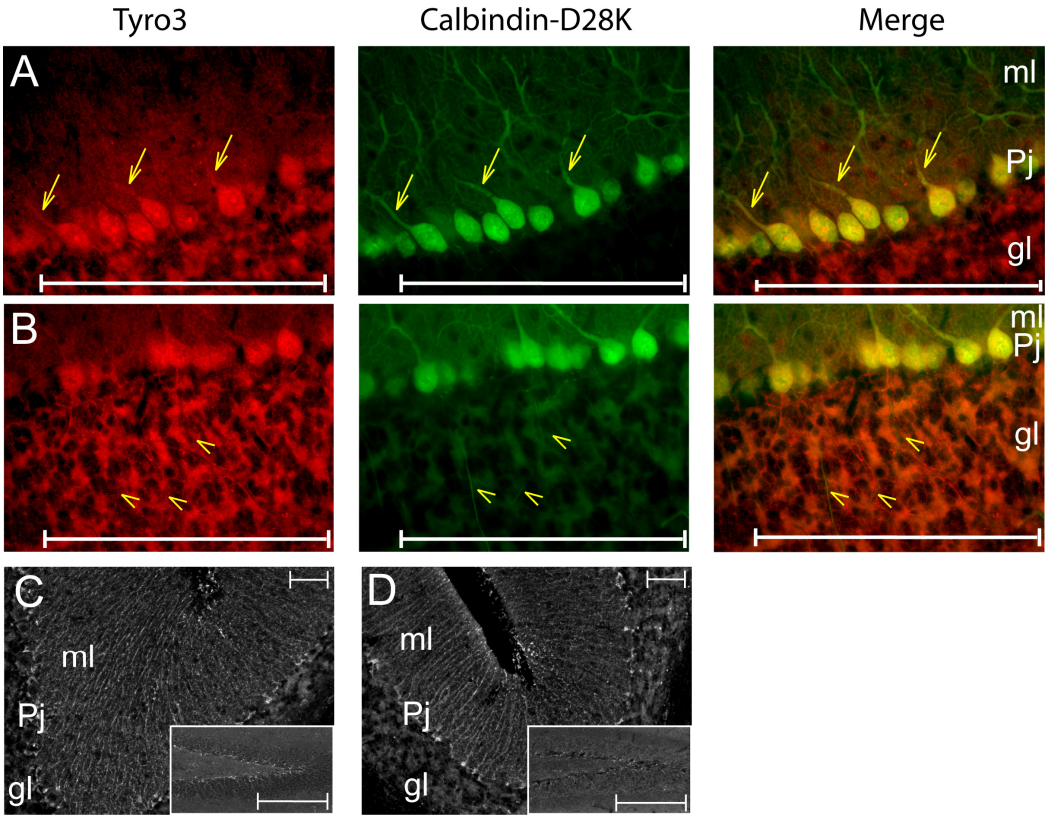


Figure 4 - *Expression of Tyro3 protein in the adult cerebellum; sagittal sections of adult mice were stained with anti-Tyro3 (panel **A**, **B, C** and **D**) and anti-Calbindin-D28K (panel **A** and **B**) antibodies, and visualized with CY3 and CY2 respectively. **A** and **B**: Tyro3 (left) expression in Purkinje cell bodies, proximal dendrites (arrows) and proximal axons (arrowheads); Calbindn-D28K (middle) expression in Purkinje cell bodies, throughout the dendrites and in the axons; merged image (right), showing apparent overlap in Purkinje cell bodies and dendrites (arrows). Note the distinct Tyro3 immunoreactivity lining the presumable Purkinje cell axons (arrowheads).**C**: negative control for **D**, i.e. treated identically as **D** except for omission of the primary antibody. Insets in **C** (negative control) and **D** show the Dentate Gyrus. Abbreviations: gl: granular layer; ml: molecular layer; Pj: Purkinje cell layer. Scale bars: 200 µm.*

In addition to cerebellum, coronal sections of WT mouse brain were screened and analyzed for Tyro3 immunoreactivity. In general, a striking similarity was found with Tyro3 mRNA expression levels that were reported earlier (*10*). In hippocampus, the very clear distinction between the CA1 and the CA2 region that was observed for Tyro3 mRNA, is reflected by protein; a diffuse immunoreactivity for Tyro3 was observed in the stratum radiatum and oriens of the CA1 region, while in the CA1 pyramidal neurons, Tyro3 seemed to be located much more in the dorsal cell layers.

In the pyramidal cell layer, a distinction was found between cells expressing Calbindin-D28L, or Tyro3. In contrast, very low levels of immunoreactivity for Tyro3 were observed in the CA2 region. Intense Tyro3 immunoreactivity was observed in the lateral septal nuclei (lsi) (Figure 5). Within the dorsal layers of the CA1 region and the lateral septal nuclei, distinct Tyro3 expression was found resembling the expression in cerebellum, apparently along Purkinje cell axons.

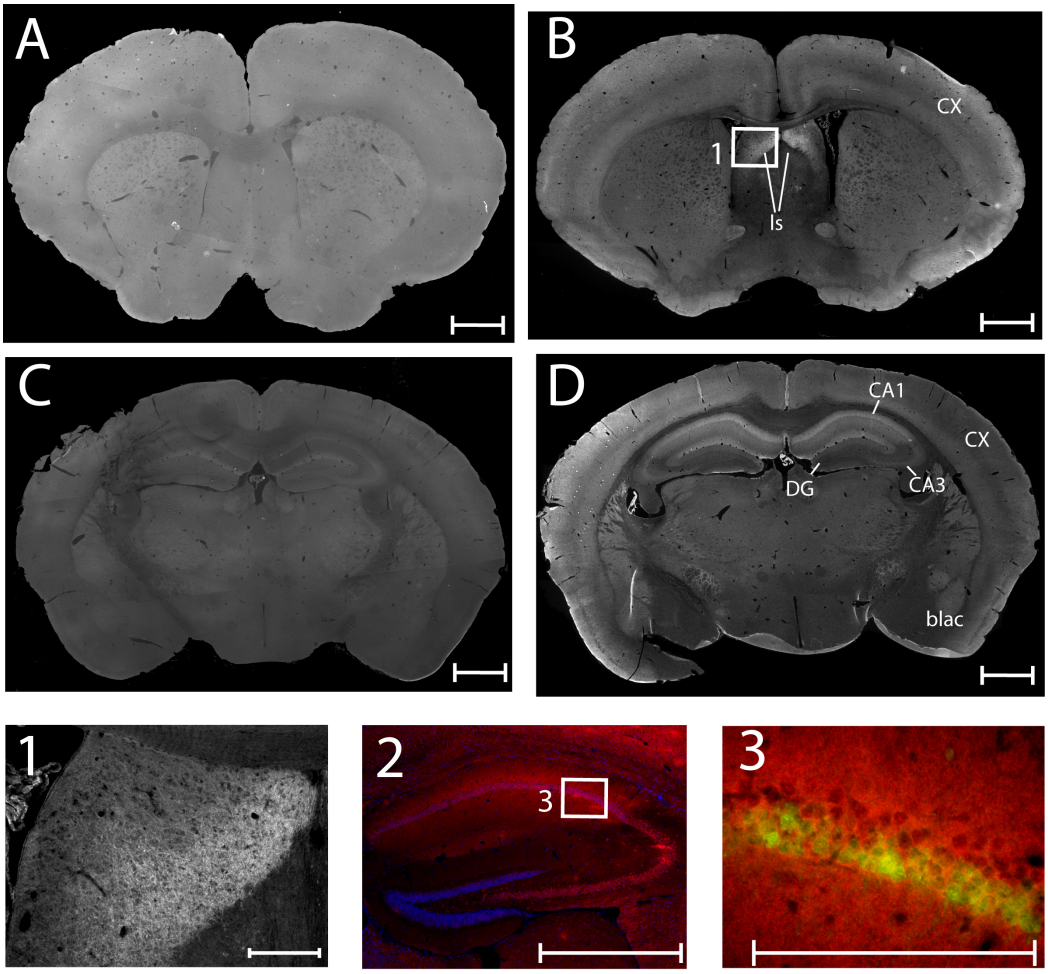


Figure 5 – *Expression of Tyro3 protein in the adult brain; coronal sections of adult WT mice.* ***A*** *and* ***C****: negative controls, i.e. treated identically as* ***B*** *and* ***D*** *except for omission of the primary antibody.* ***A*** *and* ***B*** *are more rostrally located sections (Bregma 0.86 mm and 0.62 mm respectively) than* ***C*** *and* ***D*** *(Bregma -2.18 mm and -2.06 mm respectively).* ***1****: Tyro3 expression in a higher magnification of the squared area in* ***B****.* ***2****: Tyro3 (red) expression in a higher magnification of the hippocampal area, including the Hoechst signal (blue) for orientation.* ***3****: Tyro3 (red) and Calbindin-D28K (green) expression in a higher magnification of the squared area in* ***2****. Note that Tyro3 is mainly expressed dorsal to the Calbindin-D28K expressing neurons. Abbreviations: blac: basolateral amygdaloid complex; CA1 and CA3: Cornu Ammonis 1 and 3, hippocampal areas; cx: cortex; DG: Dentate Gyrus; ls: lateral septal nuclei. Scale bars: 1000 µm (****A****-****D****) and 200 µm (****1****-****3****).*

Discussion

In order to thoroughly study the effects of activating a single TAM receptor, a system was designed in which only one of the TAM receptors would be expressed: a MEF cell line derived from TAM TKO transfected with one of the full length TAM receptors. To this end an essential panel of constructs were engineered encoding Tyro3, Axl or Mer, or receptors encoding gain-or loss-of-function mutations at specific conserved tyrosines. In order to obtain cells expressing either only one of the WT TAM receptors, or one of the mutated receptors, MEF derived from TKO mice were electroporated with these constructs. No expression of the proteins was found so far. Based on these results, electroporation might not be an efficient method to transfect MEFs, and another approach is needed. Lentiviral cDNA expression has been successfully applied extensively (*34*), therefore a possibility to increase the efficiency is to prepare lentiviral cDNA expression constructs that carry the described specific, single amino acid loss-of-function mutations and infect cells derived from triple mutants with these constructs, and verify receptor expression.

Furthermore, to obtain biologically active ligands for the TAM receptors that are independent of Vitamin K dependent γ-carboxylation, different critical constructs were engineered for Gas6 and Protein S. HEK293 cells that were electroporated with the individual constructs and cultured in selective medium yielded over 100 clones. Due to time restraints, not all clones have been screened for expression yet. The clones that were screened showed no immunoreactivity to an anti-His antibody on Western blot indicating that the His-tagged recombinant proteins are not expressed in these cells. HEK293 cells are widely used for production of recombinant protein, and the individual clones were selected for stable transfection by means of a resistance cassette. Therefore, it is reasonable to assume that clones expressing the constructs are amongst the ones not yet screened for expression.

A system that provides the opportunity to activate the individual receptors would be ideal for screening small molecule inhibitors. Once such molecules have been identified, these could be applied to specifically prevent activation of the receptors prior to activation tests. Therefore, initial experiments were performed to test function and specificity of uncharacterized RPTK inhibitors. NIH-3T3 cells, that express Axl, did show activation of Axl upon treatment with FBS; however, only a small increase was observed in Axl phosphorylation. Therefore, it might be better to assess activation levels on NIH-3T3 cells that are transiently transfected with full length Axl. In case Axl protein levels indicate a greatly enhanced activation level,

experiments can be set up to test small molecules. These experiments would comprise the activation of TAM receptors in the presence of these inhibitors to see decreased activation levels. To assess specificity, time course experiments and subsequent immunoprecipitation assays of various downstream molecules of TAM receptors and other RPTK will provide valuable data.

TAM receptors were found to be expressed in brain (*10, 11*), yet their function in the nervous system is still poorly understood. Therefore, an attempt was made to establish the expression of the protein for Tyro3, the member of the TAM family which is the most widely expressed in the adult brain. In the present study immunohistochemical procedures were applied on mouse brain and the expression of Tyro3 in brain was successfully revealed. In support of the present findings, the expression pattern of Tyro3 closely resembles mRNA expression found in previous studies. Tyro3 protein was found in Purkinje cells, mainly the cell body and dendrites. In order to firmly establish the expression of Tyro3 protein in Purkinje cells and their dendrites, confocal z-series imaging would be of great value. Off course, comparison to the Tyro3 KO and TAM TKO mouse, where no signal is expected, would be the conclusive analysis.

Previously, Tyro3 was reported to be expressed by Bergmann glia in cerebellum (*10*). In the current study it was found that this is not the case. Bergmann glia could only be visualized when the immunohistochemical procedures were altered, and further analysis lead to the conclusion that immunoreactivity of the Bergmann glia was non specific. Similar to the pictures presented in this study, the pictures reported by Prieto *et al.* (*10*) show either Tyro3 immunoreactivity in Purkinje cells, or Bergmann glia. This indicates that in the former study too, the conditions might have been altered, although that is not mentioned. The discrepancy between the present study and the earlier study could also arise from a difference between species: mice and rat, or the use of a different set of antibodies. As Tyro3 and TAM TKO mice are available, definitively establishing whether Bergmann glia express Tyro3 in mice, could be achieved by comparing KOs to WT sections. Another possibility is to engineer knock in mice that carry a tagged Tyro3 receptor, e.g. GFP-Tyro3.

The cerebellum is important for various motor related tasks, and is also recognized for being implicated in cognitive tasks (*35*). The cerebellum receives two sources of excitatory input: one comprises the mossy fibers, coming from numerous

sources in peripheral nerves, the spinal cord, and the brain stem, which form synaptic contacts with the granular cells The other excitatory input comprises the climbing fibers, coming from the inferior olive and form synaptic contacts with dendritic spines of the Purkinje cell (*36*). In combination with parallel fibers from the granular layer, the climbing fibers can induce long term potentiation (LTP) or long term depression (LTD) in Purkinje cell dendritic spines (*37, 38*). A structure that is well known for its important role in learning and memory is the hippocampus. The major input to the hippocampus arises from the entorhinal cortex, with its projections terminating on dendrites in the Dentate Gyrus, CA3 and CA1 regions. In turn, the CA3 pyramidal neurons project to CA1 (*39*). In the hippocampus LTP and LTD have been characterized extensively, and a form of LTP, called late-LTP depends on protein synthesis and dendritic spine formation. (*38, 40, 41*). Based on the finding that Tyro3 is expressed in both the hippocampus and cerebellum and the finding in a study by K. K. Murai *et al. (42)* that RPTKs can be important for late-LTP through controlling the morphology of spines, it is tempting to speculate about the possibility that Tyro3 might also play a role in neuronal plasticity. Tyro3 KO mice show some disrupted behavior, and preliminary analysis of Tyro3 KO brain sections did not reveal gross abnormalities in cerebrum. This might indicate that Tyro3 is not necessary for neuronal development but might play a role in the functionality of hippocampal and Purkinje neurons. It would be interesting to analyze the effect of activating Tyro3 with Gas6 in a similar manner as has been done for the EphA4 receptor (*42*), as Gas6 mRNA was found in pyramidal neurons throughout the hippocampus and Purkinje cells in the cerebellum, indicating that Gas6 might be a functional ligand in hippocampus. It is important to keep in mind however, that Gas6 KO mice do no show an identical phenotype as TAM KO mice in eye and testis (*8, 43*), supporting a role for Protein S in these systems. Additional support for an important role for Protein S in brain comes from studies showing that Protein S protects ischemic hippocampal neurons from apoptosis (*44*). Now that the global expression pattern of Tyro3 in brain has been achieved, and more detailed characterizations are in progress, issues regarding whether Protein S functions through a TAM receptor, specifically Tyro3, can be more easily assessed. More three TAM receptors share an immunon S and Tyro3 might arise from a the available Tyro3 KO mice and a conditional KO (cKO) mouse, lacking Protein S expression in brain. In fact, this Protein S cKO mouse is in the process of being engineered in this lab (*45*) (i.e the lab of Dr. Greg Lemke Molecular Neurobiology

lab (*45*) (i.e the lab of Dr. Greg Lemke Molecular Neurobiology Laboratory, Salk Institute for Biological Studies, La Jolla, CA, USA).

Once the roles of Tyro3 have been thoroughly established, downstream pathways might be elucidated by using dissociated cell cultures, and this will possibly highlight some pathologies that might be linked to certain deficits in Tyro3 signaling.

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