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Multimodal Signaling by the ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) Promote Neurite Extension

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Abstract

Aggregating proteoglycans (PG) bearing chondroitin sulfate (CS) side chains associate with hyaluronan and various secreted proteins to form a complex of extracellular matrix (ECM) that inhibits neural plasticity in the central nervous system (CNS). Chondroitinase treatment depletes PGs of their CS side chains and enhances neurite extension. Increasing evidence from *in vivo* models indicates that proteolytic cleavage of the PG core protein by members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family of glutamyl-endopeptidases also promotes neural plasticity. The purpose of this study was to determine whether proteolytic action of the ADAMTSs influences neurite outgrowth in cultured neurons. Transfection of primary rat neurons with ADAMTS4 cDNA induced longer neurites, whether the neurons were grown on a monolayer of astrocytes that secrete inhibitory PGs or on laminin/poly-L-lysine substrate alone. Similar results were found when neurons were transfected with a construct encoding a proteolytically inactive, point mutant of ADAMTS4. Addition of recombinant ADAMTS4 or ADAMTS5 protein to immature neuronal cultures also enhanced neurite extension in a dose-dependent manner, an effect demonstrated to be dependent on the activation of MAP ERK1/2 kinase. These results suggest that ADAMTS4 enhances neurite outgrowth via a mechanism that does not require proteolysis but is dependent on activation of the MAP kinase cascade. Thus a model to illustrate multimodal ADAMTS activity would entail proteolysis of CS-bearing PGs to create a loosened matrix environment more favorable for neurite outgrowth, and enhanced neurite outgrowth directly stimulated by ADAMTS signaling at the cell surface.

Keywords

plasticity; protease; primary neuron; proteoglycan; extracellular matrix; matrix metalloproteinase; cell culture

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Introduction

Injury to the central nervous system (CNS) is often debilitating and compounded by little hope of recovery owing to the fact that once neural networks in the CNS are severed, they are difficult to re-establish. Predominantly, this is because the properties of myelin-associated proteins and other proteins that compose a glial scar impede the growth of axons toward their target. The glial scar is an accumulation of reactive astrocytes and extracellular matrix (ECM) molecules such as chondroitin sulfate (CS)-substituted PGs, tenascin and other proteins that inhibit the re-growth of axons and the migration of certain cells into the damaged region (Davies, et al., 1999, Davies, et al., 1997, Laywell, et al., 1992). Indeed, the CS side chains of PG molecules are classical inhibitors of neurite outgrowth both *in vitro* and *in vivo* (Carulli, et al., 2005, Silver and Miller, 2004, Snow, et al., 2001).

Lecticans is the term for the family of hyaluronic acid-binding PGs that regulate cell adhesion, migration and neurite outgrowth in the CNS and include brevican, aggrecan, neurocan and versican (Handley, et al., 2006). Long unbranched, sulfated, highly negatively-charged CS chains are covalently bound to the central domain of lecticans and discourage growth cone motility and neurite elongation, however, even when these glycosaminoglycan polymers are removed from the core proteins by chondroitinase treatment (Pizzorusso, et al., 2002), significant neurite inhibition is retained by versican (Schmalfeldt, et al., 2000), but not by brevican (Miura, et al., 2001), at least *in vitro*. The enduring biological action may be inherent to the core PG protein itself or it may result from interactions with other ECM molecules such as hyaluronan or tenascin-R. *In vivo*, intermolecular interactions among lecticans, hyaluronan and tenascin result in the formation of a mesh-like lattice in the matrix of the CNS that inhibits neural plasticity (Fig. 1A). To facilitate plasticity, there should be a means to relieve the inhibition afforded by the PG, however, the absence of an endogenous, extracellular chondroitinase to remove CS chains is a limiting factor. So exploiting a mechanism that occurs *in vivo* may be a feasible way to re-establish plasticity in the brain. Increased expression and activation of endogenous proteases that cleave the PG core would be one mechanism to enhance neural plasticity, by loosening the association and interaction among the matrix components that inhibit plasticity (Yamaguchi, 2000) (Fig 1B).

The ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) are multi-domain, metalloproteinases that have notable roles in angiogenesis, collagen processing, blood coagulation, cell migration, and arthritis and several family members are glutamyl-endoropeptidases that cleave lecticans (Porter, et al., 2005). These secreted proteases share similar functional domains, including a pro-protease, metalloproteinase, disintegrin-like, cysteine-rich and spacer domains. Activation of the pro-protease likely occurs by furin-mediated cleavage of the pro-domain at the N-terminus, and further C-terminal truncations are necessary to fully activate the enzyme (Wang, et al., 2004) (Gao, et al., 2004) (Kuno, et al., 1999). The interaction of the ADAMTS domains with their substrates is complex and may involve binding via the thrombospondin type 1 motif and/or sequences in the C-terminal spacer or cysteine-rich region of the molecule (Flannery, et al., 2002, Kashiwagi, et al., 2004, Tortorella, et al., 2000).

ADAMTSs, especially ADAMTS 1, 4, 5, 9 and 15 are expressed in brain and brain pathologies (Cross, et al., 2006, Cross, et al., 2006, Haddock, et al., 2006, Hurskainen, et al., 1999, Jungers, et al., 2005, Yuan, et al., 2002) (our unpublished observations), and each of these proteases is active in cleaving PGs. Several ADAMTSs have been shown to be elevated in human neurodegenerative disease and animal models of brain injury. ADAMTS1, but not ADAMTS5, appears to be up-regulated in Down syndrome, Pick's disease and Alzheimer's disease (Miguel, et al., 2005). ADAMTS4 and ADAMTS1 mRNA was markedly elevated in the hippocampus of rats in response to kainate-induced excitotoxic lesion (Yuan, et al., 2002), and ADAMTS1

expression was increased in the spinal cord of rodents having undergone axotomy (Sasaki, et al., 2001), indicating that these proteases may be increased in response to injury or during an inflammatory response.

Anecdotal, but growing evidence indicates that metalloprotease activity is important in mechanisms of neural plasticity. Nerve growth factor treatment of PC-12 cells results in MMP-3 expression (Fillmore, et al., 1992, Machida, et al., 1989), and MMP-3 is essential in promoting PC12 cell growth cone invasiveness through an artificial basal lamina (Nordstrom, et al., 1995). More recently, excitotoxic lesion in the brain was shown to result in the expression of MMP-9 in the hippocampus (Szklarczyk, et al., 2002, Zhang, et al., 1998) and neuritic sprouting observed in the dentate gyrus after a lesion of entorhinal cortex was blocked by administration of a broad spectrum MMP inhibitor (Reeves, et al., 2003). These actions focus toward a role for the matrix-degrading metalloproteinases in neural plasticity. We recently demonstrated that active ADAMTSs which cleave lecticans are elevated in the dentate gyrus terminal zone during the period of neuritic sprouting after entorhinal cortex lesion (Mayer, et al., 2005). Taken together, these studies support the hypothesis that remodeling of ECM may be an important component in processes of neural and synaptic plasticity. The purpose of this study was to directly test the hypothesis that lectican-degrading activity may promote neurite outgrowth over an ECM that contains inhibitory PGs. We grew primary cultured neurons that were either secreting ADAMTS4 via a transfected expression vector or were exposed to ADAMTSs by direct addition of recombinant protein to the media. In some of these experiments, neurons were grown on an astrocyte monolayer that had previously been shown to deposit brevicin in the ECM (Hamel, et al., 2005, John, et al., 2006). Unpredictably, our results show that ADAMTS4, and other ADAMTSs promote neurite outgrowth in primary cultured rat neurons via a mechanism that appears to be independent of its proteolytic activity. In addition, intracellular signaling, appropriate for neurite outgrowth, is induced in ADAMTS-treated neurons.

Materials and Methods

PG degradation assay

Highly negatively charged molecules, including PGs, present in whole rat brain extracts were bound to and eluted from a DEAE matrix as described (Yamada, et al., 1994). Briefly, rat brain tissue (1g) was placed in 10 ml, ice cold, 4 mM HEPES pH 8.0, 0.15 mM NaCl, 0.1% Triton-X-100 containing 2 μ M 1,10 phenanthroline (Sigma, St. Louis, MO) and protease inhibitor cocktail (set III, Calbiochem/EMD Biosciences, San Diego). The tissue was disrupted in a teflon-glass homogenizer and the whole extract centrifuged at $30,000 \times g$ for 30 min at 4°C. The supernatant was removed, diluted 1:1 with 50 mM Tris-HCl, 0.15 M NaCl, 0.1% Triton-X-100, and passed over a DEAE column pre-equilibrated with the same buffer at a flow rate of less than 0.5 ml per minute. The flow through was collected, passed over the column again, and bound proteins were eluted with 5 column volumes of consecutive buffers containing 50 mM Tris-HCl pH 8.2, 0.15 M NaCl, 0.1% Triton-X-100, then 50 mM Tris-HCl pH 8.2, 0.25 M NaCl, 6 M urea, 0.1% Triton-X-100, and fractions containing PGs were eluted with 50 mM Tris-HCl pH 8.2, 1.0 M NaCl. PG-containing fractions were dialyzed against water for 24 h in SpectraPor 6000-8000MWCO (Millipore, Billerica, MA) membrane, the samples concentrated on a speed-vac and aliquoted. Total protein in the samples was 1.3 μ g/ μ l (Pierce, Rockford, IL). DEAE-purified PG samples were incubated with 25 nM recombinant ADAMTS1 (Chemicon/Millipore, Temecula, CA), ADAMTS4 or ADAMTS5 (expressed at Roche Biosciences, Palo Alto) (diluted in 10 mM Tris-HCl, 0.15 M NaCl, and 10 mM CaCl₂) for three hours at 37°C. Controls included samples with ADAMTS4 and ADAMTS5 that were inactivated by heating to 95°C for thirty minutes. After a three hour incubation period, beta-mercaptoethanol-containing SDS-PAGE sample buffer was added to the samples, the

samples were heated at 95°C for 4 minutes, and subjected to SDS-PAGE and Western blotting. Membranes were probed with mouse anti-brevican (BD Biosciences, San Jose) at 1:1000 and rabbit anti-EAVESE (Hamel, et al., 2005, Yuan, et al., 2002) at 1:100, and primary antibody detected with anti-mouse or anti-rabbit IgG conjugated to horse-radish peroxidase (Chemicon). Signal was detected using SuperSignal chemilluminescence substrate (Pierce, Rockford, IL).

Primary cell cultures

Primary cultures of astrocytes were prepared from whole brain (minus cerebellum) of postnatal day 1–3 Sprague-Dawley (SD) (Harlan, Indianapolis) rat pups in Dulbecco's Modified Eagles Medium containing 10% horse serum and 2.5% fetal calf serum as described (Gottschall and Yu, 1995, Hamel, et al., 2005). Astrocytes were grown in 100 mm tissue culture dishes pre-coated with 100 µg/ml poly-L-lysine for seven days until confluent. The cells were disrupted with trypsin and plated at 1×10^6 cells on 22 mm glass coverslips (Fisher Scientific, Pittsburgh) pre-coated with 500 µg/ml poly-L-lysine (Sigma). Seven days later, the astrocytes had formed a confluent monolayer and at this time, the cells were used as a neuronal growth substrate. Freshly prepared, trypsinized cells from cerebral cortex of embryonic day 18 SD pups (Hamel, et al., 2005) were transfected with ADAMTS4 pCMS expression vector or empty control vector and 1×10^6 cells were plated either directly on the confluent astrocyte monolayer or onto glass coverslips pre-coated with 500 µg/ml poly-L-lysine and 25 µg/ml mouse laminin. Twenty-four hours after plating the neurons, Dulbecco's Modified Eagles Medium plus 10% fetal calf serum (plating medium) was replaced with Neurobasal serum-free medium containing B-27 defined supplement, 0.5mM Glutamax II and 25µM glutamate containing 1% antibiotic/antimycotic (unless stated otherwise, cell culture materials obtained from Invitrogen, Carlsbad, CA). Neurons plated either on an astrocyte monolayer or in the absence of the monolayer were cultured for seven days.

Transfection by electroporation

For the experiments described above, plasmid expression constructs of ADAMTS4 were made for transfection into primary rat cells trypsinized from cerebral cortex. A cDNA for ADAMTS4 (Gao, et al., 2002), and an inactive point mutant of ADAMTS4 362 (E→Q) (Gao, et al., 2004) that were inserted into pcDNA (Invitrogen), were restricted with Xba I and Kpn I (Promega, Madison), the ADAMTS4 (or mutant) fragment purified from an agarose gel and ligated into linearized pCMS plasmid, a dual promoter vector that expresses enhanced green fluorescent protein (EGFP) driven by SV40 (Clontech, Mountain View, CA). After cells from cerebral cortex of E18 SD pups were isolated, 5×10^6 cells were centrifuged and resuspended in 100µl rat nucleofactor solution (Amaxa Biosystems, Gaithersburg, MD). The cells were transferred to a cuvette and pCMS, pCMS-ADAMTS4 or pCMS-ADAMTS4 mutant were added and the cuvette and subjected to electric current using the Nucleofactor electroporator (Amaxa Biosystems) with either the O-03 or G-13 time and current pre-programmed by the manufacturer. Immediately after transfection, 500µl of pre-warmed, 37°C Dulbecco's Modified Eagles Medium with 10% fetal calf serum was added to the cell suspension, and the cells were transferred to a sterile microcentrifuge tube until plating. The transfected neurons were plated on glass coverslips pre-coated with the laminin/poly-L-lysine substrate or they were allowed to adhere directly to a previously cultured, live astrocyte monolayer. Neurons were allowed to extend neurites for seven days. In some experiments, 7-day old neurons were treated with 0.2, 1.0 or 5.0 µM monensin, a Na⁺ ionophore that inhibits release of proteins from the Golgi, for 5 h. After these times, cells were washed one time in PBS, fixed in 4% room temperature paraformaldehyde for twenty minutes, and washed an additional three times with PBS. Transfected cells were visualized under epifluorescence using a Zeiss Axioskop microscope interfaced with an Axiocam (Zeiss, Thornwood, NY) digital camera and images were obtained with Openlab software (version 3.1.4, Improvision, Lexington, MA). For all images, the brightness and contrast were adjusted equally and consistently for images within

a single experiment, and converted to grayscale using Adobe Photoshop (Version 8.0, San Jose). In other experiments, 3-day old transfected cultures were washed, total RNA isolated using Tri-reagent (Sigma), and RNA subjected to RT-PCR using primers designed to selectively recognize human, and not rat, ADAMTS4 (*forward* AGTTTGAATGGGCCTTTGAG and *backward* AGCCACCAGCCTGTGGAATATTGA, IDT, Coralville, IA).

Data collection and statistical analysis – transfection experiments

ImageJ software (Version 1.33u, NIH, Bethesda) was utilized to manually trace primary and secondary neurites to determine overall mean length of neurites per neuron and mean length of the longest neurite for each neuron. A primary neurite was defined as a neurite that sprouted directly from the soma and was at least two times the diameter of the cell body. A secondary neurite was defined as any neurite that branched from a primary neurite. Numbers of primary and secondary neurites were also recorded. The mean longest neurite was calculated by averaging the values of the longest neurite for each neuron measured, whereas the overall mean length of primary neurites was calculated by averaging values of every primary neurite measured. Secondary neurites were analyzed similarly. Means of experimental groups were statistically compared using ANOVA and pair-wise comparisons were made with Tukey-Kramer post-hoc test (SuperAnova, v 1.11, Abacus Concepts, Berkeley). A $p < 0.05$ was considered a significant difference between groups. Separate litters of rat pups were used to obtain cells for each of the three experiments.

ADAMTS4 protein treatment

Neurons grown at 2.5×10^5 cells per well were cultured on 22 mm glass coverslips for six days and treated at 2 and 5 days *in vitro* with human recombinant ADAMTS4 or ADAMTS5. Purified recombinant ADAMTS4 and 5 were provided by Roche Biosciences or Wyeth-Ayerst. Recombinant protein was diluted in Neurobasal medium plus 0.1% bovine serum albumin (BSA) and syringe filtered to sterilize. BSA was added to prevent adherence of the recombinant protein to the filter. Each of the ADAMTS protein preparations contained low, but detectable levels of endotoxin using the LAL assay (Cambrex, Walkersville, MD) that were comparable to the BSA controls. Control wells received a matched volume of filtered Neurobasal medium plus 1% BSA. Untreated controls were also measured in some experiments. At six days *in vitro*, neurons were fixed with 4% paraformaldehyde and processed for immunohistochemistry.

Immunohistochemistry

Neuronal cultures fixed as described above were incubated for one hour in blocking buffer containing 10% normal goat serum (NGS), 0.3% Triton-X-100 and 1M lysine in PBS at room temperature. Cells were incubated overnight at 4°C in dilution buffer (PBS, 10% NGS, 0.3% Triton-X-100) containing mouse anti-microtubule associated protein-2 (Chemicon) diluted to 1:200. The cells were washed three times in PBS and Alexa Fluor goat anti-mouse IgG fluorescent secondary antibody (Invitrogen) was incubated at a concentration of 1:500 for 1 h and the coverslips washed three times in PBS. Next, the coverslips were incubated with a fluorescent-tagged, actin binding protein, Alexa-Fluor 488 phalloidin (Molecular Probes/Invitrogen), at a dilution of 1:500 in 2% NGS and 0.3% Triton-X-100 in PBS for 45 minutes at room temperature, followed by an additional three rinses in PBS. The coverslips were then mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA) fluorescent mounting medium and the cells were visualized with epifluorescence. Neurons were photographed, brightness and contrast of all photomicrographs were enhanced equally, and images converted to grayscale using Adobe Photoshop.

Data collection and statistical analysis – protein treatment

Due to a higher density of neurites compared to the transfection experiments, it was impossible to discern individual neurites to make length measurements. Thus, neurites from a group that was blinded from the experimenter were traced on a new layer in Adobe Photoshop. The background layer containing the photomicrograph was deleted, leaving the tracings, and ImageJ software was utilized to calculate the area of the image occupied by the tracings. This area was divided by the number of neurons in the field image, generating μm^2 of neurite signal per neuron. A mean was calculated for the area occupied by neurites per neuron for several images and differences between the means were determined by ANOVA and the Tukey-Kramer multiple comparison test. A $p < 0.05$ was considered a statistically significant difference between groups.

Phosphotyrosine and ERK1/2 analysis

Neurons were cultured for five days *in vitro* and treated with 50 nM ADAMTS4. At 20, 40 and 60 minutes after addition of ADAMTS4, 20 mM Tris-HCl, 5 mM EDTA, 1% Triton-X-100, and protease inhibitor cocktail set III (Calbiochem) were added to cells for fifteen minutes on ice, the extract scraped from the dish, centrifuged and stored at -80°C . Neuronal lysates were subjected to Western blot analysis on 10% Tris-glycine SDS-PAGE gels, and the proteins were transferred onto Immobilon membranes (Millipore). Membranes were probed with rabbit anti-ERK1/2, rabbit anti-phospho-ERK1/2 (Chemicon) and/or mouse anti-phosphotyrosine (BD Biosciences) and the presence of primary antibody was detected with HRP conjugated to secondary antibody as described for anti-brevican and anti-EAVERSE above. The same blots were re-probed for phosphotyrosine and ERK analysis and densitometric measures of the immunoreactive protein bands were obtained from the ERK blots and the ratio of activated phospho-ERK1/2 was calculated by dividing phospho-ERK protein band density by total ERK1/2 band density. The time at which the maximum stimulation of ERK1/2 was observed was somewhat variable between experiments. Thus, representative blots and representative densitometric measures are shown in Fig. 6 and 7, with similar, but not identical, results seen in the replicate experiments.

MAP kinase inhibition

Neurons were treated with MAP kinase inhibitors thirty minutes before the addition of 50nM ADAMTS4 to the cultures as described above. PD98059, (Tocris, Ellisville, MO), a selective inhibitor of activated ERK1/2, and U0126 (Tocris), an inhibitor of active and inactive ERK1/2, were added at 10, 25 and $50\mu\text{M}$ before the addition of 50nM ADAMTS4. U0124 (Tocris), the inactive analog of U0126, was used as a negative control at 10, 25 and $50\mu\text{M}$ as well. Cultures were treated and incubated as described above and images of neurons were captured, processed, examined and analyzed statistically for differences between groups in the same manner as in the ADAMTS protein treatment experiments.

Results

Degradation of purified PG by recombinant ADAMTSs

To ensure that the human recombinant ADAMTS proteases used in these experiments were effective in degrading brevican purified from rat brain, human recombinant ADAMTS1, ADAMTS4 and ADAMTS5 were incubated with DEAE-extracted PGs. Soluble homogenate of rat brain was applied to a DEAE column and highly negatively-charged species were eluted with 1 M NaCl. These proteins were incubated with active or heat-inactivated preparations of the human recombinant ADAMTSs. At the end of a 3 h incubation period with 25 nM of each recombinant ADAMTS, the samples were subjected to Western blot for brevican. Brevican detected in DEAE-eluant in the absence of recombinant protease was observed as a smear >

145 kD representing isoforms bearing various numbers of CS chains (Fig. 1C, control). Incubation of DEAE eluants with ADAMTS 1, 4 and 5 resulted in cleavage of brevican and the appearance of a 55kD N-terminal fragment (Fig. 1C). This same 55 kD fragment was detected when the membrane was probed with a rabbit antibody that recognizes the ADAMTS-specific, C-terminal, neoepitope rat sequence, EAVESE, exposed on the N-terminal fragment of ADAMTS-cleaved brevican (Fig. 1D) (Hamel, et al., 2005). After cleavage by each protease, the abundance of intact brevican was clearly diminished. Proteolytic activity was lost or significantly attenuated when the ADAMTS4 or ADAMTS5 preparations were heat inactivated prior to incubation with substrate (Fig. 1C and 1D). These results demonstrate that each of the recombinant preparations of the ADAMTSs was proteolytically active.

Neurite extension in primary rat neurons transfected with ADAMTS4 cDNA grown on an astrocyte monolayer

Since monolayers of rat astrocytes express brevican that remains associated with the cell layer (Hamel, et al., 2005), we were interested in determining whether overexpression of ADAMTS4 would stimulate cleavage of brevican and/or other PGs present in the astrocyte monolayer matrix and thus, promote neurite outgrowth in cells expressing ADAMTS4. To accomplish this, primary rat neurons were transfected with pCMS-EGFP vector that contained a human ADAMTS4 cDNA insert. Other neurons were transfected with pCMS-EGFP plasmid without the ADAMTS4 insert. The transfected neurons were plated on mature astrocyte monolayers, ie. two-week old cultures of postnatal day three astrocytes that had been subcultured one time. Seven days after plating, photomicrographs of fixed cultures were imaged (Fig. 2A, 2C) and tracings were made of neurites (Fig. 2B, 2D). Autofluorescence attributed to the astrocyte monolayer can be seen in the photomicrographs (Fig. 2A, 2C). Neurons transfected with ADAMTS4 cDNA more readily penetrated the astrocyte matrix, and extended 20–40% longer primary and secondary neurites as compared to neurons transfected with empty vector ($p \leq 0.05$) (Fig. 2E). There was no statistically significant difference in the number of primary neurites between control empty vector transfected neurons and those transfected with pCMS-EGFP ADAMTS4 cDNA, however, the number of secondary neurites was significantly increased in ADAMTS4-transfects ($p \leq 0.05$) (Fig. 2E, bottom right panel). Neurons from multiple coverslips taken from a single culture were utilized in this experiment, and similar results obtained in a replicate experiment.

Neurite extension in primary rat neurons transfected with ADAMTS4 cDNA grown directly on substrate without an astrocyte monolayer

If ADAMTS4 was proteolytically degrading a substrate on the astrocyte monolayer, releasing the neurites from exposure to the inhibitory matrix, then neurons transfected with ADAMTS4 cDNA and neurons transfected with empty vector should extend neurites equally as well when grown directly on plastic, in the absence of an astrocyte-derived ECM. To test this notion, neurons were transfected as above, plated directly on poly-L-lysine/laminin-coated coverslips, and allowed to extend neurites (Fig. 3). Interestingly, these sets of neurons exhibited neurite growth behavior similar to that observed when neurons were grown on the astrocyte monolayer. Neurons overexpressing ADAMTS4 (Fig. 3C, 3D) extended longer neurites as compared to control transfects (Fig. 3A, 3B) when grown on the poly-L-lysine/laminin substrate. ADAMTS4 overexpression induced significant increases in all parameters measured (Fig. 3E, $p \leq 0.05$), in this case, including the number of primary and secondary neurites. A single, independent neuronal culture generated the neurons shown in this experiment, and similar results obtained in a replicate experiment.

Effects of ADAMTS4 on neurite outgrowth appear independent of proteolysis

To more directly determine whether the proteolytic action of ADAMTS4 was leading to increases in neurite outgrowth, experiments were carried out with a group that was transfected with a pCMS construct that contained an ADAMTS4 cDNA bearing a point mutation in the protease (Fig. 4, inactive ADAMTS4 mutant). This cDNA encodes a protein with a single amino acid substitution 362 (E→Q) in the catalytic domain resulting in an inactive protease, yet it contains all other functional domains of ADAMTS4. Quite surprisingly, neurons transfected with either active ADAMTS4 cDNA or neurons transfected with proteolytically inactive ADAMTS4 cDNA extended longer neurites in the absence of an astrocyte monolayer. Representative photomicrographs show increases in neuritic growth (Fig. 4A, 4B: control; 4C, 4D: ADAMTS4; 4E, 4F: inactive ADAMTS4 mutant). Transfection with either the active or inactive ADAMTS4 construct resulted in enhanced length of the longest primary and secondary neurites, and the mean length of all primary and secondary neurites. (Fig. 4G). There was even a modest, but significant increase in the mean length of primary neurites in neurons transfected with the inactive ADAMTS4 mutant, compared to the wild-type ADAMTS4 cDNA (Fig. 4G, top middle panel). A single independent neuronal culture was used in this experiment, and similar results obtained in a replicate experiment.

Finally, to confirm that ADAMTS4 protein was being expressed by the individual constructs at 7 DIV, neurons were transfected with empty pCMS, pCMS ADAMTS4 and pCMS ADAMTS4 mutant, treated with monensin for 5 h to block secretion, fixed and immunostained. Neurons robustly expressing the ADAMTS4 and ADAMTS4 mutant construct co-localized with GFP containing neurons (supplemental Figure 1).

Effect of recombinant ADAMTSs on neurite outgrowth

To test the effects of ADAMTS4 protein on neurite extension and potentially confirm the results seen with transfection, increasing concentrations of human recombinant ADAMTS4 were added to the medium of neurons at 2 and 5 DIV and neurite length measured at 8 DIV. Neurons were also treated with ADAMTS5 to determine whether this was an effect selective to ADAMTS4. Each recombinant protein was diluted with BSA in culture medium and filter sterilized prior to addition to the culture. Control wells consisted of untreated neurons and neurons treated with filtered medium and BSA (Fig. 5B, 5C) to ensure that the BSA was not affecting neurite outgrowth. At 8 DIV, neurons were immunostained with microtubule-associated protein-2 and stained with phalloidin, images collected, and the lengths of neurites traced. Compared to the transfection experiments, where low numbers of neurons were visible due to low transfection efficiency, all neurons were stained and visible with dense networks of neurites. For quantification, all neurites were traced in an individual field, and the “area occupied by neurite tracing” calculated. When divided by the number of neurons in the field, it resulted in the area occupied by neurite tracing per neuron (μm^2 tracing/neuron).

The addition of BSA in culture medium had no significant effect on neurite outgrowth compared to untreated cultures of neurons. When neuronal cultures were treated with ADAMTS4, there was a dose-dependent increase in neuritic growth, with a statistically significant increase at 10 (Fig. 5D, 5E) and 50nM ADAMTS4 (Fig. 5F, 5G and 5A, $p \leq 0.05$). ADAMTS5, a member of this protease family not highly expressed in the nervous system, also significantly increased growth of neurites compared to control cultures (Fig. 5H, 5I and 5A, $p \leq 0.05$). Number of perikarya per field did not differ among the treatment groups, indicating that the ADAMTSs did not significantly influence neuroan

Modulation of tyrosine phosphorylated proteins in response to ADAMTS4

Since ADAMTS4 appears to exert its effects on neurite outgrowth independent of its proteolytic activity, it is possible that ADAMTS may be signaling the cell by binding and acting

at the cell surface. Significant evidence is available showing that ADAMTS protein is localized to the ECM and cell surface (Gao, et al., 2004, Kuno and Matsushima, 1998, Kuno, et al., 1999). Since neurite outgrowth has been shown to be mediated in part by tyrosine kinase signaling, neurons treated with ADAMTS4 were examined for elevated abundance of intracellular proteins containing phosphorylated tyrosine residues using an anti-phosphotyrosine antibody in neuronal lysates. Neurons were treated with 50nM ADAMTS4 and extracted after 20, 40 and 60 minutes of incubation, lysates subjected to Western blot and probed with anti-phosphotyrosine antibody. Increases in the abundance of several phosphotyrosine-containing proteins of differing molecular weights were observed after stimulation with ADAMTS4 (Fig. 6F). Robust up-regulation of a 50kD (Fig. 6C), 115kD (Fig. 6D) and 125kD (Fig. 6E) signal was detected at 60 minutes after addition of ADAMTS4, although the peak of the time-course was somewhat variable among experiments. Modest increases were seen in a phosphotyrosine-containing protein at ~40kD (Fig. 6A, B).

Modulation of MAP kinase (ERK1/2) in response to ADAMTS4

The increase in the abundance of phosphotyrosine-containing proteins around 40 kD led us to examine the possibility that the effects on neurite outgrowth were mediated by ERK1/2, an intracellular factor known to have effects on neurite outgrowth. Thus, neuronal extracts were examined by Western blot and probed with anti-“pan” ERK1/2 antibody and anti-phospho-selective ERK1/2 antibody to determine if any changes were occurring in the ratio of activated (phospho) to total ERK1/2 protein in response to ADAMTS4. Marked increases in activated 42/44kD ERK1/2 were detected after ADAMTS4 treatment (Fig. 7A–D), but there was some variability in the time of maximum stimulation among different experiments. However, elevations in the phospho-ERK1/2/total ERK1/2 ratio were observed in four separate independent experiments (not shown).

Neurite outgrowth in the presence of MEK inhibitors

To determine whether the MAP kinase pathway(s) was mediating the neurite growth promoting effects of ADAMTS4, neurite outgrowth was quantified in cultures treated with selective MAP kinase inhibitors or their inactive analogs (Fig. 8). The MAP kinase inhibitor PD98059 (Fig. 8A) was added at 10 and 25 μ M, and neurons were treated with U0126, a potent inhibitor of MEK-1 and -2, at 10, 25 and 50 μ M (Fig. 8B). Neurons were treated with the inactive analog of U0126, U0124 as a control. The inhibitors were used alone or thirty minutes before the addition of 50nM ADAMTS4, and the neurons were fixed, immunostained and photographed. Neurite outgrowth was quantified by the same procedure as the protein treatment experiments. All neurites were traced in a particular field, area occupied by the tracing was determined and expressed as area occupied by neurite tracings divided by the number of neurons in the field to yield μ m² neurite tracing/neuron. ADAMTS4 at 50 nM was effective in enhancing neurite outgrowth (Fig. 8A–C). PD98059 treatment alone did not significantly alter neurite outgrowth in the absence of ADAMTS4, and was comparable to control levels at 10 and 25 μ M (Fig. 8A). However, when added to cultures stimulated with ADAMTS4, PD98059 completely inhibited the neurite outgrowth promoting effects of ADAMTS4 (Fig. 8A). When the MEK-1,-2 inhibitor, U0126 was incubated with neurons in the presence of ADAMTS4, it also inhibited neurite outgrowth induced by the protease (Fig. 8B), yet at the highest dose tested, the drug appeared to be neurotoxic (not shown). The inactive analog of U0126, U0124, did not influence ADAMTS4 induced neurite outgrowth at 25 and 50 μ M (Fig. 8C). At the concentrations for these experiments, the diluent used for each drug, DMSO, alone did not affect neurite outgrowth (data not shown). These results suggest an involvement of the MAP kinase signaling pathway in ADAMTS4-stimulated neurite outgrowth.

Discussion

In vivo studies have provided the most direct evidence to date for the involvement of matrix-degrading metalloproteinases in neural plasticity in the CNS. These demonstrated increased expression of matrix-cleaving proteinases during conditions of regeneration and/or neuronal sprouting (Mayer, et al., 2005, Szklarczyk, et al., 2002, Yuan, et al., 2002) or synaptogenesis (Kim, et al., 2005) and that blockade of metalloproteinase activity during a critical period may impede neural plasticity mechanisms (Reeves, et al., 2003). In at least two of these studies, a PG crucial to the plastic response was identified and shown to be an *in vivo* substrate for an active metalloproteinase (Mayer, et al., 2005, Yuan, et al., 2002). Additional data that supports the concept of increased neural plasticity as a result of extracellular matrix proteolysis comes from *in vitro* models utilizing peripheral neurons. These have shown that metalloproteinases enhanced penetration of neurites into the basal lamina (Nordstrom, et al., 1995), potentiated nerve growth factor-induced neurite extension in PC12 cells (Shubayev and Myers, 2004) and cleavage of a PG converted the growth environment from an inhibitory to a permissive one for the growth of dorsal root ganglion neurites (Zuo, et al., 1998). In the present study, ADAMTSs were examined for their ability to induce neurite outgrowth *in vitro* in the absence of any other classical growth factors. The ADAMTSs used in this study, ADAMTS1, ADAMTS4 and ADAMTS5 are proteases particularly adept at cleaving aggregating, CS-containing PGs that are abundantly expressed in the CNS.

Transfection of ADAMTS4 cDNA into primary rat neurons resulted in enhanced neurite growth compared to neurons transfected with vector alone, when neurons were grown on an astrocyte monolayer. The initial interpretation was that elevated ADAMTS4 expression resulted in increased cleavage and/or degradation of CS-containing PGs present in the astrocyte monolayer (Hamel, et al., 2005). Thus, the matrix was more permissive for robust neurite outgrowth after being released from the inhibitory effects of the PGs (Zuo, et al., 1998). However, this interpretation was not supported when it was found that neurons cultured directly on a poly-L-lysine/laminin substrate without an astrocyte monolayer also enhance neurite outgrowth with overexpression of ADAMTS4. This was puzzling since the abundance of matrix proteins produced in neuronal cultures is markedly lower than that found on astrocyte monolayers. So in an effort to determine whether proteolytic activity was an essential component in the action of the ADAMTSs in promoting neurite growth, neurons were transfected with a construct encoding an inactive, catalytic domain, point mutant of ADAMTS4, with all other domains identical to the wild-type protein. Neurons transfected with mutant ADAMTS4 construct (Gao, et al., 2002) were at least as effective as the DNA encoding the wild-type, active protease in stimulating neurite outgrowth, indicating that proteolytic activity was not required for ADAMTS4 to stimulate neurite extension, at least on an artificial laminin substrate. It also suggested that ADAMTS4 may be acting by engagement with a cell surface protein, as mounting evidence indicates that certain biological actions of metalloproteinases are stimulated by binding to cell surface signaling molecules, such as integrins (Conant, 2005), independent of proteolytic function. Unlike MMPs, ADAMTSs avidly bind to heparan and are found anchored to the cell surface or the ECM via its thrombospondin motif and/or spacer region (Kuno and Matsushima, 1998, Kuno, et al., 1999). ADAMTSs bind to heparan sulfate or CS chains found typically attached to core protein PGs, and this protein-glycosaminoglycan interaction is important for substrate recognition (Flannery, et al., 2002, Kashiwagi, et al., 2004). Indirect evidence suggests that ADAMTS4 may bind to the cell surface heparan sulfate PG, syndecan-1, in chondrocyte-like cells in culture (Gao, et al., 2004) and orient the protease for C-terminal truncation by another cell surface metalloproteinase. Whether binding of syndecan-1 by ADAMTS4 directly activates intracellular signaling mechanisms is not known.

Compelling evidence presented here indicates that one intracellular signaling mechanism induced by ADAMTS4 and involved in stimulated neurite outgrowth is the MAP kinase cascade. ADAMTS-induced neurite extension is dependent on activation of this pathway since pharmacological inhibition of ERK1/2 kinase activity by treatment with PD98059 and U0126 blocked the ADAMTS-stimulated growth in a dose-dependent manner, and inactive negative control compounds were without effect. MAP kinase signals are required for cellular process outgrowth and plasticity in a variety of neuronal-like cell types. Neurite outgrowth on the adhesion protein L1 in B35 neuroblastoma cells required MAP kinase activation (Schmid, et al., 2000) and expression of Eph8 stimulated MAP kinase and neurite outgrowth in NG108-15 cells (Gu, et al., 2005). Neuregulin-1 binding to erbB receptors activated MAP kinase and stimulated hippocampal neuronal differentiation, neurite extension and arborization (Gerecke, et al., 2004). MAP kinase was involved in suramin-induced neurite elongation (Nakata, 2007). These data clearly support the concept that MAP kinase activation is essential in ADAMTS-induced neurite extension of rat primary neurons in culture.

Additional data indicate that heparan sulfate-containing proteins transduce signals resulting in the activation of MAP kinase. Basic fibroblast growth factor (FGF)-2 was shown to bind heparan sulfate on the PG, agrin, which activated the ERK1/2 signal and modulated neurite outgrowth in PC-12 cells and retinal neurons (Kim, et al., 2003). It may be that ADAMTS mobilizes and activates bFGF by interacting with a heparan sulfate-containing PG, such as agrin, at the surface of cortical neurons, although agrin expression is much lower on CNS neurons compared to those from the periphery. As mentioned above, another possible ADAMTS binding partner is syndecan. Connective tissue growth factor, (CCN-2), a protein that like ADAMTS4, contains a thrombospondin type-1 motif, was shown to bind syndecan-2 and activate ERK1/2 (Gao, et al., 2004). It is plausible that the ADAMTSs bind syndecan and activate the MAP kinase cascade, thereby increasing neurite extension. We have demonstrated here that ADAMTSs induce MAP kinase signaling in primary rat cortical neurons, an action essential for ADAMTS-induced neurite outgrowth since inhibitors of the cascade block ligand-induced neuritic extension. Compared to neuron-like cell lines which are the cells typically used to demonstrate signaling by the MMPs, the primary cultured neurons from embryonic rat cortex used in these studies are a model that more closely parallel neurons *in vivo* in their receptor and signaling ensemble.

One primary candidate, functional motif in the structure of ADAMTS4 that may be responsible for enhancing neurite elongation is the type-1 thrombospondin motif. Thrombospondin itself enhances neurite outgrowth (O'Shea, et al., 1991) and the thrombospondin type-1 repeats of SCO-spondin bind to $\alpha_1\beta_1$ integrin to stimulate cell process extension in B104 cells, a neuron-like cell line (Bamdad, et al., 2004). Furthermore, other ECM proteins that contain thrombospondin-like motifs, such as heparin-binding growth-associated molecule (HB-GAM, also known as pleiotrophin) and midkine, increase neurite outgrowth in hippocampal neurons (Raulo, et al., 2005). Thus, there is strong evidence that thrombospondin-like regions of various proteins expressed in and secreted by neurons (or astrocytes via a paracrine action) may affect neural plasticity.

In sum, these results suggest that the ADAMTSs, similar to the MMPs (Conant, 2005), exert biological activities independent of their proteolytic activity, one of them that induces extension of neurites in primary cultured embryonic neurons. The particular motif on the ADAMTSs responsible for stimulating this effect is not known, but evidence from others points to the thrombospondin type 1 repeat or the spacer region. Since this carboxy-terminal may bind to heparan sulfate chains, the cell surface molecules that bind the ADAMTSs likely differ markedly from the MMPs, molecules that do not contain a thrombospondin repeat. Preliminary, unpublished data from our laboratory indicates that the ADAMTSs stimulate neurite outgrowth on a CS-containing substrate that is laid on plastic *in vitro*. It will be interesting to determine

whether this activation of neurite extension is dependent on or independent of ADAMTS proteolytic activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Proteoglycan (lectican)-tenascin-hyaluronan matrix complex in the CNS and lectican cleavage by ADAMTSs. (A), Intact complexes of extracellular matrix form an inhibitory boundary toward neurite outgrowth by hyaluronic acid binding to the N-terminus, tandem repeats of the lecticans and tenascin binding to the C-terminus of the lecticans. (B), Cleavage of lectican by an ADAMTS loosens the stable, matrix network, potentially increasing fluidity and allowing for increased plasticity. (C, D), Cleavage of the lectican, brevican, by human recombinant ADAMTSs. Proteoglycans were purified from rat brain extracts contained in fractions eluted from a DEAE column with 1 M NaCl. Lectican-containing fractions were incubated with active ADAMTSs, or heat-inactivated protease (Inactive), subjected to SDS-PAGE, and probed with anti-brevican antibody (C) to reveal holoproteins and the N-terminal cleavage product or with anti-EAVESE antibody to selectively reveal the N-terminal fragment that contains the EAVEAE neopeptide exposed by ADAMTS-cleavage of brevican (D). "Control" lane shows brevican immunoreactivity as a smear at a molecular weight of >145 kD. Samples containing inactive ADAMTS show little cleavage, whereas brevican substrate incubated with human recombinant ADAMTSs 1, 4 and 5 (25 nM) for 3 hours reveal an abundance of the 55 kD, N-terminal cleavage fragment (C) that is observed when probed with antibody selective for the ADAMTS-cleaved fragment (D).

Figure 2.

ADAMTS4 cDNA induces neurite outgrowth in neurons grown on astrocyte monolayers. Primary cortical neurons transfected with empty pCMS vector or pCMS inserted with ADAMTS4 cDNA were grown on a previously prepared astrocyte monolayer for 7 days. Representative photomicrographs depict neurons transfected with empty pCMS (A) or with the ADAMTS cDNA insert (C). Photographs were taken of GFP-expressing neurons and neurites were traced as a measure of neurite outgrowth (B, pCMS; D, pCMS-ADAMTS4). Means $\pm$ SEM of traced neurons reveal ADAMTS4 increases several parameters of neurite outgrowth including the mean longest primary and secondary neurites, overall mean primary and secondary neurites and number of secondary neurites (* signifies $p \leq 0.05$ compared to control pCMS vector alone). No significant difference was observed in the mean number of primary neurites in response to ADAMTS4 cDNA transfection ($p > 0.05$). A single independent neuronal culture was utilized to measure 117 primary and 84 secondary neurites from 50 empty pCMS-transfected neurons and 127 primary and 124 secondary neurites from 37 pCMS-ADAMTS4-transfected neurons. Similar data was obtained when the experiment was repeated. Scale bars, 50µm.

Figure 3.

ADAMTS4 cDNA induces neurite outgrowth in neurons grown on a poly-L-lysine/laminin substrate. Primary cortical neurons transfected with empty pCMS vector or pCMS inserted with ADAMTS4 cDNA were grown on poly-L-lysine/laminin coated coverslips in the absence of an astrocyte monolayer for 7 days. Representative photomicrographs depict neurons transfected with empty pCMS (A) or with the ADAMTS cDNA insert (C). Photographs were taken of the GFP-expressing neurons and neurites were traced as a measure of neurite outgrowth (B, pCMS; D, pCMS-ADAMTS4). Means $\pm$ SEM of traced neurons reveal ADAMTS4 induced statistically significant (* signifies $p \leq 0.05$ compared to pCMS vector alone) increases in several parameters of neurite outgrowth including the mean longest primary and secondary neurites, overall mean primary and secondary neurites and the number of primary and secondary neurites. 112 primary and 48 secondary neurites from 45 empty pCMS-transfected neurons and 136 primary and 107 secondary neurites from 44 pCMS-ADAMTS4 transfected neurons derived from a single independent neuronal culture were analyzed. Similar data was obtained when the experiment was repeated. Scale bars, 50 μ m.

Figure 4.

An inactive mutant ADAMTS4 cDNA enhances neurite outgrowth. Primary cortical rat neurons transfected with empty pCMS vector, pCMS inserted with ADAMTS4 cDNA, or pCMS vector inserted with an inactive mutant form of ADAMTS4 cDNA were grown on poly-L-lysine/laminin coated coverslips in the absence of an astrocyte monolayer for 7 days. Due to a point mutation in the catalytic domain (362 E→Q), this ADAMTS4 protein possesses the same structural domains as native ADAMTS4, but lacks any proteolytic capacity. Representative photomicrographs depict pCMS (A), pCMS-ADAMTS4 (C) and pCMS inactive ADAMTS4 mutant (E) expressing neurons. Photographs were taken of the fluorescing neurons and neurites were traced as a measure of neurite outgrowth (B, pCMS; D, pCMS-ADAMTS4, F, pCMS inactive ADAMTS4 mutant). Means ± SEM of traced neurons reveal ADAMTS4 and inactive ADAMTS4 mutant induced statistically significant (* signifies $p \leq 0.05$ compared to pCMS vector alone) increases in several parameters of neurite outgrowth including the mean longest primary and secondary neurites, overall mean primary and secondary neurites and the number of secondary neurites. There was an additional increase in the mean length of primary neurites of neurons transfected with pCMS inactive ADAMTS4 mutant cDNA compared to neurons transfected with pCMS-ADAMTS4 cDNA, (** signifies $p \leq 0.05$ compared to pCMS-ADAMTS4). No significant differences ($p > 0.05$) were observed in the mean number of primary neurites in response to ADAMTS4 cDNA transfection. A single independent neuronal culture was utilized and 288 primary and 181 secondary neurites from 111 empty pCMS-transfected neurons were examined. 207 primary and 171 secondary neurites from 70 pCMS-ADAMTS4-transfected neurons were measured, and 325 primary and 316 secondary neurites from 129 pCMS-inactive ADAMTS4-transfected neurons were quantified. Similar data was obtained when the experiment was repeated. Scale bars, 50μm.

Figure 5.

Human recombinant ADAMTS4 induces neurite outgrowth. Representative photomicrographs and tracings are shown of primary cortical rat neurons grown on poly-L-lysine/laminin and treated with ADAMTS4 at 1, 10 (D, E) and 50nM (F, G) and ADAMTS5 at 10nM (H, I) or culture medium containing BSA (B, C) at days 2 and 5 of culture and neurite outgrowth measured at day 6. At the end of culture, neurons were visualized by using anti-microtubule associated protein-2 immunocytochemistry and the Alexafluor 488-conjugated actin binding protein, phalloidin. The photomicrographs (B, D, F, H) were converted to grayscale and neurites traced (C, E, G, I). Data was calculated by dividing the area of the photograph occupied by neurite signal (μm^2) divided by number of neurons in the field to generate mean $\pm$ SEM

($\mu\text{m}^2/\text{neuron}$) that are shown in panel A. Treatment with ADAMTS4 resulted in a dose-dependent increase in neurite outgrowth (see panel A) and 10nM ADAMTS5 also significantly increased neurite outgrowth (* $p \leq 0.05$ compared to untreated or medium + BSA treated wells). Three separate cultures were utilized in these experiments, with at least 10 fields in total for each treatment group, representing over 1,000 neurons for each group. Scale bars, 100 μm .

Figure 6.

Tyrosine residue phosphorylation in total protein extract from neurons after treatment with human recombinant ADAMTS4. Representative immunoblot showing protein bands immunoreactive with anti-phosphotyrosine antibody in extracts from neurons, at 5 days in culture and treated with culture medium or 50 nM ADAMTS4 for 20, 40 and 60 min. Proteins immunoreactive for phospho-tyrosine were observed at several molecular weights, 42 (A), 44 (B), 50 (C), 115 (D), >120 (E). Protein bands from this single experiment were quantified by densitometry and values are shown in panel (F) indicate increases in band density for proteins at several different molecular weights, especially prominent is a band at about 40 kD. This blot is from a representative experiment and results were similar in three replicate experiments.

Figure 7.

ERK1/2 is activated in neurons in response to treatment with human recombinant ADAMTS4. Representative blots (A and B) and densitometric values of immunoreactive bands (C and D) of ERK1/2 immunoreactivity in extracts from neurons at 5 days in culture and treated with ADAMTS4. Culture medium vehicle or human recombinant ADAMTS4 at 50 nM was added to neuronal culture medium and cell lysates were collected 20, 40 and 60 minutes after treatment, the lysates subjected to SDS-PAGE and Western blotting using anti-42/44kD phospho-ERK1/2 (A) and anti-pan-ERK1/2 (B) antibodies. Densitometry of the immunoreactivity bands was quantified and the ratio of phospho-ERK1/2 to pan-ERK1/2 was calculated for each sample and for each isoform of ERK 1/2. (C) ratio of 42 kD activated

phospho-ERK to pan- 42 kD ERK, (D), ratio of 44 kD activated phospho-ERK to pan- 44 kD ERK. Marked and apparently sustained increases in activated ERK1/2 were observed in response to treatment with ADAMTS4 This experiment was repeated four times, with increases seen in each experiment, however, the time of the peak magnitude of the increase was variable among experiments, Thus, results from a single, representative experiment is shown.

Figure 8.

MAP kinase inhibitors attenuate neurite outgrowth induced by human recombinant ADAMTS4 treatment. PD98059, an inhibitor of MAP kinase (A) U0126, a potent inhibitor of MEK-1 and -2 (B) and U0124, an inactive analog of U0126 (C) were added to the culture medium of neurons at 2 and 5 days in culture to determine whether inhibition of the MAP kinase could influence the growth of neurites induced by ADAMTS4. Cells were treated with vehicle (DMSO) or inhibitor alone or with inhibitor thirty minutes before the addition of 50nM ADAMTS4, followed by fixation, immunostaining and quantification of neurite outgrowth by tracing neurites on day 6 of the culture. Area occupied by neurites (μm^2) from neurite tracings was divided by number of neurons in the field to generate a mean $\pm$ SEM (μm^2 /neuron). Each MAP

kinase inhibitor significantly and dose-dependently impaired the neurite outgrowth promoting effects of ADAMTS4 with little effect on basal neurite outgrowth with the exception of high doses of U0126 which appeared to be toxic toward the neurons. * signifies $p \leq 0.05$ compared to “control” treatment; ** signifies $p \leq 0.05$ compared to treatment with 50 nM ADAMTS4 alone. This experiment was performed using an independent culture and at least 10 fields were examined for each treatment group representing about 75 neurons per treatment group. Similar data was obtained when the experiment was repeated.