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Citation for published version:

Digital Object Identifier (DOI):
10.1523/JNEUROSCI.0008-11.2011

Link: Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Journal of Neuroscience

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Brief Communications

Integrin Activation Promotes Axon Growth on Inhibitory Chondroitin Sulfate Proteoglycans by Enhancing Integrin Signaling

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Chondroitin sulfate proteoglycans (CSPGs) are upregulated after CNS lesions, where they inhibit axon regeneration. In order for axon growth and regeneration to occur, surface integrin receptors must interact with surrounding extracellular matrix molecules. We have explored the hypothesis that CSPGs inhibit regeneration by inactivating integrins and that forcing integrins into an active state might overcome this inhibition. Using cultured rat sensory neurons, we show that the CSPG aggrecan inhibits laminin-mediated axon growth by impairing integrin signaling via decreasing phosphorylated FAK (pFAK) and pSrc levels, without affecting surface integrin levels. Forcing integrin activation and signaling by manganese or an activating antibody TS2/16 reversed the inhibitory effect of aggrecan on mixed aggrecan/ laminin surfaces, and enhanced axon growth from cultured rat sensory neurons (manganese) and human embryonic stem cell-derived motoneurons (TS2/16). The inhibitory effect of Nogo-A can also be reversed by integrin activation. These results suggest that inhibition by CSPGs can act via inactivation of integrins, and that activation of integrins is a potential method for improving axon regeneration after injury.

Introduction

Chondroitin sulfate proteoglycans (CSPGs) are upregulated at sites of damage in the CNS, inhibiting axon regeneration (Tang et al., 2003; Silver and Miller, 2004). Of the several inhibitory CSPGs that are present, aggrecan has been shown to be strongly inhibitory to neurite outgrowth from several types of cultured neurons (Borisoff et al., 2003; Chan et al., 2008). It may act via several mechanisms, including the transmembrane protein tyrosine phosphatase σ (PTPσ) receptor (Shen et al., 2009) or the epidermal growth factor receptor (EGFR) (Koprivica et al., 2005), and through a variety of downstream signaling cascades, such as Rho/ROCK pathway or protein kinase C (PKC) (Monnier et al., 2003; Sivasankaran et al., 2004). Aggrecan is synthesized primarily by neurons and is endogenously present in the CNS (Asher et al., 1995; Li et al., 1996). It is also modulated after a CNS injury at the lesion site, where it is secreted by reactive astrocytes (Silver and Miller, 2004; Afshari et al., 2010).

Growth cone migration and axon extension require an adhesion molecule as a physical link between the cell and its substrate (Long and Lemmon, 2000; Previtali et al., 2001; Song and Poo, 2001). For interaction with molecules present in the extracellular matrix (ECM), the main receptors are integrins, which function as both adhesion and signaling molecules (Hynes, 2002; Lemons and Condic, 2008). Integrins are αβ heterodimeric transmembrane receptors, whose functions are regulated by two different but related processes, “inside-out” and “outside-in” signaling. The former (also termed “integrin activation”) indicates a change from a low ligand-binding affinity (inactive) conformation to a high-affinity (active) one. “Outside-in” signaling refers to the signaling cascades propagated intracellularly upon ligand–integrin binding. In the nervous system, integrins are involved in axon guidance, neurite extension, synaptic plasticity, and axon regeneration [for review, see Reichardt et al. (1989) and Lemons and Condic (2008)]. Previous work has indicated that the CNS inhibitory molecule Nogo-A exerts some of its effects through integrin inactivation (Hu and Strittmatter, 2008). Here we have investigated whether the effect of aggrecan in impairing axon regeneration could also be mediated through an integrin-related mechanism, and whether manipulation of integrins can allow axons to overcome this inhibition.

Materials and Methods

Dorsal root ganglion neuron culture. Dorsal root ganglia (DRGs) were dissected from adult male Sprague Dawley rats (~3 months). The neurons were dissociated with collagenase and 0.1% trypsin, centrifuged through a 15% bovine serum albumin (BSA) density gradient, and cul-
tured in DRG culture medium [DMEM, insulin-transferrin-selenium (1×), penicillin-streptomycin-fungizone (1×), and NGF (10 ng/ml)]. Cultures were kept for 20 h at 37°C in 7% CO₂. Where relevant, 500 μM manganese was added only after neurons were attached to coverslips (~2 h after plating). For removing the glycosaminoglycan (GAG) chains on aggrecan, chondroitinase ABC (Sigma, C2905, 0.1 U/ml) was added to aggrecan for 1 h at 37°C before using it for coating/treatment on DRG cultures.

**Human embryonic stem cell-differentiated motoneuron culture.** Human embryonic stem cell (hESC) lines (H9 from the WiCell Research Institute and HuES9 from hES facility, Harvard University) between passages 50 and 90 were used for this study. hESC culture and neural induction were performed using a protocol adapted from Patani et al. (2009). Briefly, hESCs were propagated in defined medium supplemented with 8 ng/ml FGF2, 10 ng/ml Activin (Harrington et al., 2006), and 10 ng/ml insulin. To generate neural precursor cells (NPCs), hESCs were enzymatically and mechanically dissociated before being plated in chemically defined medium, comprising 50% Iscove’s modified Dulbecco’s medium (IMDM), 50% F12 and GlutaMAX, supplemented with 1.75 mM human recombinant insulin, 0.38 mM transferrin, 450 μM monothioglycerol, 10 μM lipids, and 5 mg/ml BSA fraction V on an orbital shaker. For spinal motoneuron specification, hESC-NPCs were cultured in 10–20 ng/ml FGF2 and 0.5 μM RA/1 μM purmorphamine. For terminal differentiation, hESC-NPCs were cultured on laminin-coated coverslips in DMEM supplemented with B27, PSF, 10 ng/ml BDNF, and 10 ng/ml GDNF.

For experiments involving the TS2/16 antibody (ATCC-HB243, Hybridoma Bank), hESC-NPCs were plated on coverslips coated with laminin or aggrecan–laminin and cultured in the differentiation medium (as described above) for 2 d at 37°C. Then, TS2/16 was added at 1:3 dilution, and the cultures were kept for another day.

**Postfixation immunostaining.** Cell cultures on coverslips were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100, blocked with goat serum, and incubated with primary antibodies at 4°C overnight. Then, coverslips were incubated with secondary antibodies for 1 h before mounting on slides. Primary antibodies were used against pY397 FAK (Biosource, 44-624G, 1:100), pY418 Src (Invitrogen, 44-660G, 1:100), pY861 FAK (Abcam, ab4804, 1:100), pY397 FAK (Biosource, 44-624G, 1:100), and pY418 Src (Invitrogen, 44-660G, 1:100), respectively.

**Live immunostaining.** 9EG7 antibody (BD Pharmingen, 550531, 1:50 in culture medium) was added to the cultures for 15 min at 37°C. After washing once with culture medium, the cultures were fixed with 4% PFA and incubated with FITC-conjugated goat anti-rat antibody for 1 h before mounting on slides.

**Western blotting.** Cell lysates from manganese- or aggrecan-treated DRG cultures were lysed and collected in RIPA buffer (Roche), supplemented with protease inhibitor and phosphatase inhibitor cocktails (Roche). The protein concentrations were then determined using BCA Protein Assay Kit (Pierce). Protein extracts were subjected to SDS-PAGE and blotted onto polyvinylidene difluoride (Hybond-P) membranes (GE Healthcare). Membranes were blocked with 5% skimmed milk in TBS-T, before incubation with rabbit anti-pY397 FAK (Biosource) or mouse anti-FAK (Millipore) antibodies at 4°C overnight. This was followed by incubation in HRP-conjugated anti-rabbit or anti-mouse antibodies at room temperature for 1 h. Protein bands were visualized using ECL-detecting reagents (GE Healthcare).

**Axon growth assay.** For adult DRG neurons, two parameters were quantified: (1) percentage of neurons with axons longer than the cell body diameter; (2) average of the longest axons extended by each neuron. For hESC-derived motoneurons, the lengths of βIII tubulin-positive (for differentiated neurons) axons extended by motoneurons were measured.

**Quantitative immunofluorescence.** At least 20 axons per coverslip were first selected at random and imaged. An area of axon (>30 μm long) was then traced, and the fluorescence intensity of immunostaining was analyzed using the Leica Application Suite (Leica Microsystems).

**Results**

**Axon growth is inhibited by aggrecan.** We cultured adult rat DRG neurons on the integrin substrate laminin, which is relevant because it is present in large amounts around Schwann cells and bands of Bungner, which support axon
regeneration after peripheral nerve injury. Axon growth after 20 h was assessed by measuring the percentage of neurons with axons, and the longest axon extended by each neuron. Axons grew profusely on laminin, the length of axons and the proportion of regenerating neurons varying with the concentration of laminin used for coating (Fig. 1A). Addition of aggrecan to the substrate inhibited axon growth at all laminin concentrations, although the effect gradually diminished with increasing laminin concentration (Fig. 1B). The addition of aggrecan to the substrate did not affect the level of laminin on the growth surfaces \((p = 0.84, t \text{ test}, n = 4)\) (Fig. 1C). That aggrecan inhibits growth even at saturating levels of laminin indicates that aggrecan is not acting simply by masking integrin binding sites on laminin. Removal of glycan chains from aggrecan by treatment with chondroitinase ABC (ChABC) 1 h before coating eliminated its axon growth-inhibitory effect \((p < 0.01, t \text{ test}, n = 3)\) (Fig. 1D). This showed that the main inhibitory action of aggrecan is mediated through the glycan chains. The reduction in the glycan level in the substratum after ChABC treatment was clearly observed by immunostaining of CS-56 (Fig. 1E).

### Aggrecan Impairs Integrin Signaling

We asked whether aggrecan-induced growth inhibition could be mediated via an effect on integrins. DRG neurons were cultured on laminin and allowed to extend axons for 20 h. The neurons were then treated with either aggrecan-containing medium (25 \(\mu_{g}/ml\)) or control medium for 2 min. Western blotting was performed on extracted proteins from the neurons to determine the level of intracellular integrin signaling, on the basis of the level of phosphorylated-tyrosine-397 FAK \((pY397 \text{ FAK})\) compared to total FAK. Aggrecan-treated neurons had a lower level of \(pY397 \text{ FAK}\), as compared to the control, with no difference in the level of total FAK (Fig. 2A). Since integrin-related events that mediate axon outgrowth primarily take place near the ends of the axons, we performed a quantitative immunofluorescence assay to assess the changes of \(pY397 \text{ FAK}\) in this region. As with the whole-cell Western blot, in the growth cone region, \(pY397 \text{ FAK}\) level decreased by 32.3\% \((p < 0.001, t \text{ test}, n = 6)\) upon aggrecan treatment, without any changes in the total FAK level \((p = 0.68, t \text{ test}, n = 3)\) (Fig. 2B). Treating aggrecan with ChABC before addition to the cultures significantly removed its inhibition on \(pY397 \text{ FAK}\) level \((p < 0.05, t \text{ test}, n = 3)\) (Fig. 2C), suggesting that aggrecan impairs integrin signaling via its glycan chains.

To see whether the inhibition of integrin signaling was due to a change in the surface level of integrin, we examined the amount of integrin on the axonal surface after aggrecan treatment using quantitative immunohistochemistry on nonpermeabilized axons. Because DRG axon binding to laminin is mediated by \(\beta1\) integrins, the level of surface \(\beta1\) integrin was assessed. No difference in the surface \(\beta1\) integrin level was observed between aggrecan- and control medium-treated axons \((p = 0.36, t \text{ test}, n = 3)\) (Fig. 2D), suggesting that aggrecan inhibits integrin signaling without altering surface integrin expression.

Investigating the changes in \(pY397 \text{ FAK}\) over a longer time period, we observed that \(pY397 \text{ FAK}\) level was maximally decreased at 2 min, was less decreased at 5 min, and returned to control level within 15 min \((n = 3)\) (Fig. 2E). Since the inhibitory effect of aggrecan on axon outgrowth is sustained and lasts much longer than 15 min, we asked whether integrin downstream signaling may exhibit a longer-lasting change. Downstream of
tyrosine-397 residue phosphorylation, “outside-in” integrin signaling is propagated by the phosphorylation of other tyrosine residues on FAK, followed by the activation of other signaling molecules, such as Src (Parsons, 2003; Mitra and Schlaeffer, 2006). We therefore examined the phosphorylation of tyrosine-861 FAK (pY861 FAK) and tyrosine-418 Src (pY418 Src) after aggrecan treatment on DRG neurons. We found that the pY861 FAK level begins to decline 5 min after addition of aggrecan and returns to control level within 15 min ($n = 3$), while pY418 Src, which is further downstream, decreases after 5 min, and stays at this depressed level for at least 15 min ($n = 3$) (Fig. 2E). These results are consistent with our hypothesis that aggrecan impairs integrin signaling. Moreover, they corroborate the sequential phosphorylation events known to occur during integrin signaling.

### Integrin activation increases axon growth by enhancing integrin signaling

To examine the effect of integrin activation on axon growth, we cultured DRG neurons on different laminin concentrations (0.1–10 μg/ml), in the presence or absence of manganese (500 μM), and assessed the axon growth after 20 h. Manganese is a potent integrin activator that holds integrin receptors in a conformation with a high ligand-binding affinity (Mould et al., 1995). At very low (0.1 μg/ml) or high (10 μg/ml) laminin concentrations, addition of manganese did not alter the extent of axon growth. However, at low (1 μg/ml) laminin concentration, manganese treatment induced an increase in axon growth (percentage of neurons with axons, 22.0% to 30.2%, $p < 0.01$; axon length, 144.8 μm to 184.5 μm, $p < 0.001$, $t$ test, $n = 6$) (Fig. 3A, B). To verify that there is an integrin-activating effect of manganese, DRG cultures were immunostained live using a monoclonal antibody, 9EG7, which recognizes activated integrins (Bazzoni et al., 1995; Montanez et al., 2008). Neurons cultured in manganese-containing medium exhibited a higher level of 9EG7 immunostaining (+54.2%; $p < 0.01$, $t$ test, $n = 3$) on the axons compared to control (Fig. 3C), indicating more “active” integrins on the surface.

The growth-enhancing effect of manganese is probably mediated via increased “outside-in” integrin signaling. Therefore, the status of pY397 FAK in DRG neuron cultures after manganese treatment was assessed. From Western blot of whole-cell lysate, as well as quantitative immunofluorescence of axon/growth cone regions, pY397 FAK level was markedly enhanced (quantitative immunofluorescence, +56.1%; $p < 0.05$, $t$ test, $n = 3$) in the presence of manganese (Fig. 3D, E), while total FAK level remained unchanged ($p = 0.80$, $t$ test, $n = 3$). Furthermore, a time course study revealed that the pY397 FAK level began to increase after 2 min and reached a statistically significant level after 5 min ($p < 0.05$, $t$ test, $n = 3$), before settling at a plateau of ~150% of control level after 15 min (Fig. 3F).

### Integrin activation overcomes aggrecan-mediated growth inhibition by enhancing integrin signaling

Since aggrecan inactivates integrins and decreases integrin signaling, we asked whether forced activation of integrins might overcome its inhibitory effect. DRG neurons were cultured for 20 h as before, then treated with aggrecan or mixed aggrecan–manganese solutions to assess the immediate effect on integrin signal-
ing. Quantitative immunofluorescence analysis showed that the decrease in pY397 FAK level brought about by aggrecan was alleviated by manganese treatment ($p < 0.01$, $t$ test, $n = 5$) (Fig. 4A).

The ability of integrin activation to rescue axon growth on inhibitory aggrecan was also assessed. To further dissect the potential differential effects at varying substrate concentrations, two laminin concentrations were selected to reflect “low” (1 μg/ml) or “high” (10 μg/ml) ligand availability. At both concentrations, aggrecan inhibited axon growth from DRG neurons, with the effect being stronger at the low laminin concentration. Addition of manganese restored the growth to control levels on both surfaces (percentage of neurons with axons: low laminin, 9.0% to 17.3%, $p < 0.01$; high laminin, 27.7% to 35.5%, $p < 0.001$; axon length: low laminin, 85.6 μm to 146.9 μm, $p < 0.01$; high laminin, 167.4 μm to 212.0 μm, $p < 0.01$, $t$ test, $n = 6$) (Fig. 4B,C), suggesting that integrin activation enables neurons to overcome aggrecan-mediated inhibition to enhance axon growth.

The effects of manganese are not entirely specific to integrin activation. To confirm the growth-enhancing effect of manganese is attributable to integrin activation, we repeated our assays using a specific integrin activator, the monoclonal antibody TS2/16 (Tsuchida et al., 1997; Hu and Strittmatter, 2008). As the antibody specifically recognizes human integrins, we performed our experiments using motoneurons differentiated from hESCs, following an established protocol (Patani et al., 2009). The results we obtained were the same as those from DRG neurons. Activation of integrin, induced by TS2/16 antibody, alleviated the inhibitory effect of aggrecan on axon outgrowth, and enabled the motoneurons to extend longer axons on aggrecan–laminin substrates (low laminin, 72.5 μm to 117.1 μm, $p < 0.05$; high laminin, 138.1 μm to 177.5 μm, $p < 0.05$, $t$ test, $n = 3$) (Fig. 4D,E). This indicates that earlier results obtained with manganese treatment were attributable to its integrin-activating effect.

To confirm the generality of integrin modulation as a mechanism for inhibition of axon growth in the damaged CNS, we performed a similar experiment as previously reported (Hu and Strittmatter, 2008), demonstrating that Nogo-A inhibits axon growth on laminin, but that this inhibition can be overcome by activation of integrins with manganese ($p < 0.05$, $t$ test, $n = 3$) (Fig. 4F).

**Discussion**

**The inhibitory effect of CSPGs on integrin signaling**

There is evidence for various mechanisms mediating the inhibitory action of aggrecan and other CSPGs on axon growth. Several signaling pathways have been implicated, including Rho/ROCK activation (Monnier et al., 2003), PKC activation (Sivasankaran et al., 2004), and EGFR signaling (Koprivica et al., 2005). A recent paper proposed that the transmembrane protein tyrosine phosphatase $\beta$, PTP$\beta$, is a receptor for CSPGs such as aggrecan and neurocan, and mediates the growth-inhibitory effect of these molecules (Shen et al., 2009). Our results suggest that these pathways may converge on the impairment of integrin function by affecting their activation state, resulting in decreased integrin signaling. An earlier study had showed that aggrecan inhibits NGF-supported axon assembly on laminin without affecting to hESC-derived motoneurons (D, E, scale bar, 100 μm) reverses the inhibition of aggrecan on axon growth. $F$, Manganese reverses Nogo-A-mediated inhibition on axon growth. All data are mean ± SEM, and analyzed with Student’s $t$ test. $***p < 0.001$; $**p < 0.01$; *$p < 0.05$; n.s., not significant.
NGF-induced ERK phosphorylation, which led the authors to infer that ECM-integrin signaling was a likely target for CSPGs (Zhou et al., 2006). Here, we demonstrate that aggrecan, via its glycan component, causes a suppression of integrin signaling, starting with a decrease in pY397 FAK level, maximal at 2 min, and decreasing thereafter. This is followed by decreases in further downstream phosphorylation events via pY861 FAK and pY418 Scr. Crucially, levels of surface β1 integrin were unaffected by aggrecan treatment. These changes are in agreement with previous data with regards to the sequence of phosphorylation events from integrin signaling (Parsons, 2003; Mitra and Schlaepfer, 2006). It is possible that interference with integrin signaling is a general mechanism for inhibition of axon growth. Interestingly, amino-Nogo also impairs integrin function and fibronectin-induced pY397 FAK upregulation in cells (Hu and Strittmatter, 2008). Semaphorins, which are also expressed in the damaged CNS, also impact integrin function (Pasterkamp and Giger, 2009).

Integrin activation promotes axon growth by enhancing “outside-in” integrin signaling

Earlier studies have demonstrated that integrin activation increases axon growth from PC-12 cells and mouse DRG neurons (Ivins et al., 2000; Lein et al., 2000). We found that this effect is dependent on substrate (laminin) concentration; i.e., integrin activation only enhances axon growth at low laminin concentrations but not on very low- or high-laminin substrata. A likely explanation is that when presented with very low concentrations of ligand, activation of integrins cannot override the paucity of ligands, while at very high ligand concentrations, most integrins are already activated and cannot be further activated. At low ligand concentrations, a high proportion of integrins are “inactive,” allowing manganese to switch them into the “active” state (Lemons and Condic, 2006).

The increase in ligand-binding affinity conferred by integrin activation increases “outside-in” integrin signaling, as demonstrated by enhanced phosphorylation of Y397 of FAK. Integrin signaling enhancement in response to integrin activation was rapid with pY397 FAK starting to rise within 2 min of manganese addition, reaching a plateau in 5 min at ~150% of the control level. That manganese induces a rapid integrin activation effect is in agreement with previous work using flow cytometry and quantitative immunofluorescence to detect activated integrins (Bazotti et al., 1998; Zhao et al., 2005; Lemons and Condic, 2006), as well as functional assays (Nieswandt et al., 2007; Moser et al., 2008).

Integrin activation allows axons to overcome inhibitory influences

The inhibition of axon growth caused by CSPGs was successfully overcome by activating integrins in neuronal cultures, using both manganese and the integrin-activating antibody TS2/16 as the activating agents. This could result either from integrin activation overcoming a ligand-masking effect of CSPGs or through overcoming CSPG-mediated integrin inactivation. Our data speak against the first option: we grew axons in the presence of a large excess of laminin (10 μg/ml), yet aggrecan was still inhibitory at these concentrations and treatment with integrin activators (manganese or TS2/16) reversed this inhibition. We conclude that integrin activation enhances axon growth on a CSPG-containing substratum by reversing the inactivation caused by the CSPG. Inhibition of axon growth through integrin inactivation may be a general mechanism, because Nogo-A exerts part of its inhibitory effect in this way (Hu and Strittmatter, 2008).

Our results together with previous experiments demonstrate that molecules that inhibit axon regeneration in the CNS act in part by interfering with integrin function. Crucially, this inhibition can be overcome by enhancing the ligand-binding affinity of integrin receptors on axons through integrin activation. Activation of axonal integrins should therefore promote axon regeneration in the damaged CNS, where these inhibitors are upregulated (Silver and Miller, 2004; Xie and Zheng, 2008). We have shown that expression of an integrin that interacts with tenasin-C, a glycoprotein upregulated in the damaged CNS, can enhance axon regeneration after CNS damage (Andrews et al., 2009). Transgenic expression of this integrin, coupled with integrin activation, should be a powerful method of promoting axon regeneration in the damaged CNS.

References


