Embryonic Neurons Adapt to the Inhibitory Proteoglycan Aggrecan by Increasing Integrin Expression

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The primary mediators of cell migration during development, wound healing and metastasis, are receptors of the integrin family. In the developing and regenerating nervous system, chondroitin sulfate proteoglycans (CSPGs) inhibit the integrin-dependent migration of neuronal growth cones. Here we report that embryonic sensory neurons cultured on the growth-promoting molecule laminin in combination with the inhibitory CSPG aggrecan rapidly adapt to inhibition. Adaptation is associated with a two- to threefold increase in the levels of RNA and surface protein for two laminin receptors, integrin $\alpha 6\beta 1$ and $\alpha 3\beta 1$, indicating that integrin expression is regulated by aggre-can. Increased integrin expression is associated both with increases in neuronal cell adhesion/outgrowth and with decreases in the ability of aggrecan to inhibit cell adhesion. Directly increasing integrin expression by adenoviral infection is sufficient to eliminate the inhibitory effects of aggrecan, indicating that upregulation of integrin receptors may promote neuronal regeneration in the presence of inhibitory matrix components.

Key words: integrin; CSPG; aggrecan; adaptation to inhibition; proteoglycan; regeneration; adenovirus-mediated gene transfer

Many matrix components are considered either growth promoting or inhibiting based on the ability of purified protein to support or block neurite outgrowth in culture. It is becoming increasingly evident, however, that the precise nature of a neuron's response to individual matrix proteins depends on both the context in which they are encountered and the past history of the individual neuron (Snow and Letourneau, 1992; Condic and Letourneau, 1997). Recent work has shown that a number of molecules can have either an attractive or a repulsive effect on neuronal outgrowth, depending on the levels of cyclic nucleotides present in the growth cone (Song et al., 1998). These results suggest that the history of a growth cone or the precise balance of molecules that it encounters can greatly influence the growth response.

Luo and Raper (1994) distinguish between two types of inhibitory elements for growth cones: molecules that directly impair intrinsic motility and molecules that interfere with substratum adhesion. Chondroitin sulfate proteoglycans (CSPGs) are a major class of inhibitory matrix proteins that have been proposed to act either as adhesion-inhibiting molecules or through receptors to directly affect cell motility (Luo and Raper, 1994; Faissner and Steindler, 1995; Hoke and Silver, 1996). In the adult CNS, the upregulation of CSPGs after injury is correlated with the failure of axons to extend despite the continued presence of growth-promoting molecules such as laminin (for review, see Hoke and Silver, 1996), suggesting that elevated expression of CSPGs contributes to the inability of neurons to regenerate after injury.

Aggrecan is one of the major CSPGs expressed in the CNS and PNS of embryos and adults (Caterson et al., 1983; Oakley and Tosney, 1991; Schwartz et al., 1996). Aggrecan is inhibitory to the extension of retinal and sensory neurons in culture (Snow and Letourneau, 1992; Challacombe et al., 1997). Embryonic neurons can adapt to the inhibitory effects of aggrecan over time when aggrecan is presented with growth-promoting molecules such as laminin (Snow et al., 1990a; Snow and Letourneau, 1992). Adaptation could be accomplished either through the upregulation of receptors for growth-promoting ligands such as laminin or through the downregulation of neuronal receptors for aggrecan. Although the effects of aggrecan are likely to be mediated in part through a receptor-based mechanism (Snow et al., 1994; Balsamo et al., 1995; Ernst et al., 1995), aggrecan receptors have not yet been identified. Neuronal outgrowth is mediated predominantly by receptors of the integrin family (Letourneau et al., 1994), which bind to a wide range of ECM and cell-surface ligands. In sensory neurons, neurite outgrowth on ECM proteins such as laminin is entirely dependent on integrin function (Tomaselli et al., 1993), suggesting that increased integrin expression in response to a combination of aggrecan and laminin may allow neurons to adapt to inhibition and extend on laminin. In this work, we have examined the expression of integrins in response to aggrecan to determine the role of integrin regulation in neuronal adaptation to inhibitory proteoglycans.

MATERIALS AND METHODS

Cell culture and substratum preparation. dorsal root ganglia were dissected from embryonic day 12 chicks and dissociated with brief trypsin treatment (Gomez and Letourneau, 1994). Dissociated cells were preplated on tissue culture plastic in Ham's F12 media containing 10% calf serum for 3 hr at 37°C to remove more adherent non-neuronal cells (Barres et

al., 1988). After 3 hr, >95% of cells remaining in suspension are neuronal. Neurons in suspension were harvested, rinsed in PBS, and cultured overnight on glass coverslips in serum-free media as described (Gomez and Letourneau, 1994). NGF was present in all cultures at 10 ng/ml, concentrations that are saturating for the TrkA receptor. For overnight cultures, glass coverslips were coated for 1 hr at room temperature with Engelbreth-Holm-Swarm tumor laminin (gift of Dr. S. Palm, University of Minnesota) at 20 μg/ml (LM) or 1 μg/ml (low LM), or Fibronectin (Life Technologies, Gaithersburg, MD) at 20 μg/ml in PBS. Aggrecan-containing substrata for culture were prepared by coating coverslips with chick embryonic limb-bud aggrecan (gift of Dr. David Corrino, Case Western Reserve University) at 50 μg/ml in PBS for 1 hr, followed by laminin at 20 μg/ml for 1 hr (PG/LM). These treatments resulted in the following bound concentrations of protein: (LM) 300 ng/cm^2 laminin; (low LM) 30 ng/cm^2 laminin; (PG/LM) 300 ng/cm^2 laminin, 180 ng/cm^2 aggrecan. These concentrations are likely to be above those predicted to form a molecular monolayer. Binding of laminin and aggrecan to coverslips and 96-well plates (below) was determined by inclusion of ^{3}H-labeled laminin and ^{35}S-labeled aggrecan at known specific activities in the coating medium, followed by extraction of the bound protein with 10% SDS and scintillation counting. Metabolically labeled chick embryonic aggrecan was obtained from Dr. David Corrino. Laminin was labeled with ^{3}H as previously described (Snow and Letourneau, 1992).

RNA and protein analysis. RNA was isolated from ~10^7 neurons by lysing the cells *in situ* in a phenol/SDS buffer (TriReagent, Molecular Research Center). RNA concentration was determined by UV absorption at 260 nm; 10 μg samples were analyzed on agarose gels and transferred to nylon membrane by pressure blot (Stratagene, La Jolla, CA) using standard procedures. Integrin α6, α3, and GAPDH transcripts were detected with ^{32}P-labeled oligonucleotide probes. Films and blots were quantitated using a Bio-Rad GS-700 imaging densitometer, a GS-363 phosphoimager, and NIH image 1.55 analysis software.

Cell surface receptor was labeled with biotin and immunoprecipitated using published methods and antibodies specific for α6- and α3-integrin (de Curtis et al., 1991). Immunoprecipitated proteins were size-fractionated under reducing conditions on acrylamide gels and electrophoretically transferred to nitrocellulose membrane using standard protocols. Biotin-labeled proteins were detected using streptavidin conjugated to HRP and a chemiluminescent reagent (Pierce, Rockford, IL) followed by exposure to film. Ratios of protein determined from quantitation of film were comparable to those obtained by direct phosphoimaging of chemiluminescently detected proteins (data not shown).

Cell adhesion assays. Cells were cultured overnight on either laminin, low laminin, or laminin in combination with low levels of aggrecan (see Cell culture and substratum preparation). Culture on both PG/LM (see Fig. 3) and low LM (Condic and Letourneau, 1997) increase integrin expression, apparently by different molecular mechanisms. After culture, neurons were removed from the substratum by a brief treatment with calcium-free media and gentle scraping in media containing 10% serum at 4°C to prevent the internalization of cell-surface integrins. Cells were spun down at 4°C, resuspended, and maintained at 4°C until plating. To measure cell adhesion, 96-well plastic plates (Dynatech Immunolon) were coated with laminin (40 μg/ml in PBS for 1 hr), low laminin (2 μg/ml laminin in PBS for 1 hr), or aggrecan and laminin (aggrecan at 50 μg/ml in PBS for 1 hr, followed by laminin at 40 μg/ml in PBS for 1 hr). On the basis of binding of isotopically labeled proteins, these concentrations of applied protein resulted in surface densities of bound protein similar to those used for culturing (data not shown). The plates were rinsed, blocked with PBS containing 5 mg/ml BSA and 0.2% sodium azide for 1 hr, and rinsed extensively. Cells were applied at 10,000 cells per well and allowed to adhere for 3 hr at 37°C. Wells were rinsed once with 100 μl PBS and then incubated for 10 min in PBS containing 2 μg/ml 2′,7′-*bis*-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, ace-toxymethyl ester (Molecular Probes, Eugene, OR) followed by detection of fluorescein emission using a microplate fluorescence reader (Cyt-ofluor II; Biosearch/Millipore).

To determine the effect on neuronal adhesion of applying proteins to the substrata in different order (see Fig. 2), aggrecan was bound to 96-well plates before, after, or in combination with laminin. Aggrecan and laminin binding to the substrata were determined for all applied concentrations by inclusion of isotopically labeled protein (see Cell culture and substratum preparation). All three treatments resulted in identical concentrations of bound laminin (300 ng/cm^2) for all concentrations of bound aggrecan. Plates were prepared as follows. BEFORE:

aggrecan was applied first at concentrations of 1, 2.5, 5, and 50 μg/ml, resulting in bound concentrations of aggrecan equal to 20, 60, 100, and 180 ng/cm^2 followed by rinsing and application of laminin at 40 μg/ml. AFTER: laminin was applied first at a concentration of 40 μg/ml, followed by rinsing and application of aggrecan at a concentration of 10, 25, 100, and 500 μg/ml, resulting in bound concentrations of aggrecan equal to those achieved by application of aggrecan before laminin (i.e., 20, 60, 100, and 180 ng/cm^2). MIXED: laminin at 40 μg/ml was mixed with aggrecan at 5, 15, and 25 μg/ml for 30 min before application to the plates, resulting in bound concentrations of aggrecan equal to 20, 60, and 100 ng/cm^2. Note that only three concentrations were tested because aggrecan concentrations higher than 25 μg/ml inhibited binding of laminin (data not shown).

Cell outgrowth assays. To determine neurite initiation (see Fig. 1), cells were cultured on either LM or PG/LM substrata (see Cell culture and substratum preparation) for 3 or 20 hr, and 10 random fields were recorded as video images. The total number of cell bodies and neurites greater than one cell diameter in length were counted for each field and expressed as the average number of neurites per cell. To determine the rate of neurite extension, cells were cultured as above on either LM or PG/LM substrata for 3 or 20 hr and moved to a heated microscope stage. Dishes were allowed to equilibrate for 30 min, then images were captured for 1 hr, one image every 5 min. Images were captured every 5 min to confirm that the path of the neuron was linear over the culture period, so the average rate of growth cone advance could be determined from the linear distance traversed. The rate of axon extension was determined from the distance traversed by the growth cone in 1 hr as measured between the positions of the leading edge of the growth cone at the beginning and end of the period of image acquisition using Image-1 software. To measure outgrowth of neurons with different levels of integrin expression (see Fig. 4), neurons were cultured on LM, PG/LM, or low LM substrata overnight, as described above (Cell adhesion assays), and were subsequently replated on laminin, low laminin, or PG/LM containing substrata and allowed to extend axons for 3 hr. Cultures were fixed with 4% paraformaldehyde, and the percentage of cells with neurites one cell diameter or greater in length was determined.

Adenoviral infection. Replication-deficient adenoviral constructs were obtained from the laboratory of Dr. Clayton Buck (Wistar Institute). The integrin-expressing adenoviral constructs were prepared using standard methods (Graham and Prevec, 1995). Briefly, full-length mouse α1-integrin or β-galactosidase (as a control) cDNA were cloned into the pAd.CMV-link plasmid under the control of the cytomegalovirus immediate early enhancer/promoter element. This promoter has been shown to yield efficient expression of transgenes in chick embryonic neurons (Yamagata et al., 1994) and in the nervous systems of other species. NIH 293 cells were cotransfected with linearized pAd.CMV-link plasmid (with insert) and the replication-deficient sub 360 or dl70001 adenoviral backbone. Recombinant virus was collected from plaques, and the inserts were confirmed by PCR. The virus was subjected to three rounds of plaque purification to ensure that a single recombinant was selected and further purified by centrifugation on a cesium gradient. The titer of the purified recombinant virus was determined with plaque assay.

Embryonic chick neurons were cultured on laminin (300 ng/cm^2) substrata and infected overnight at a viral concentration of 8 × 108 pfu/ml. After 16 hr, the virus was removed, and neurons were cultured for an additional 48 hr to insure strong expression of the transgene. Cell surface expression of the integrin transgene was confirmed by staining cells live with antibodies that specifically recognize mouse α1-integrin (Ha31/8; PharMingen, San Diego, CA). All cells in the mouse α1-integrin-infected culture expressed the transgenic protein at the cell surface. Endogenous α1 is expressed at low levels in both control and α1-integrin-infected cultures (see Fig. 5B). β-galactosidase expression was confirmed by antibody staining (5 Prime to 3 Prime Biologicals) of fixed and Triton X-100-extracted cells. Infected neurons were removed from the dishes as described above (Cell adhesion assays), replated on substrata containing laminin or PG/LM, and assayed for outgrowth in 3 hr as above (Cell outgrowth assays). Levels of total α1-integrin expressed at the cell surface were determined by immunoprecipitation of cell-surface biotinylated α1-protein using an antibody that recognizes both the exogenous (mouse) and the endogenous (chick) α1 subunits (Chemicon, Temecula, CA), followed by Western analysis as described (RNA and protein analysis).
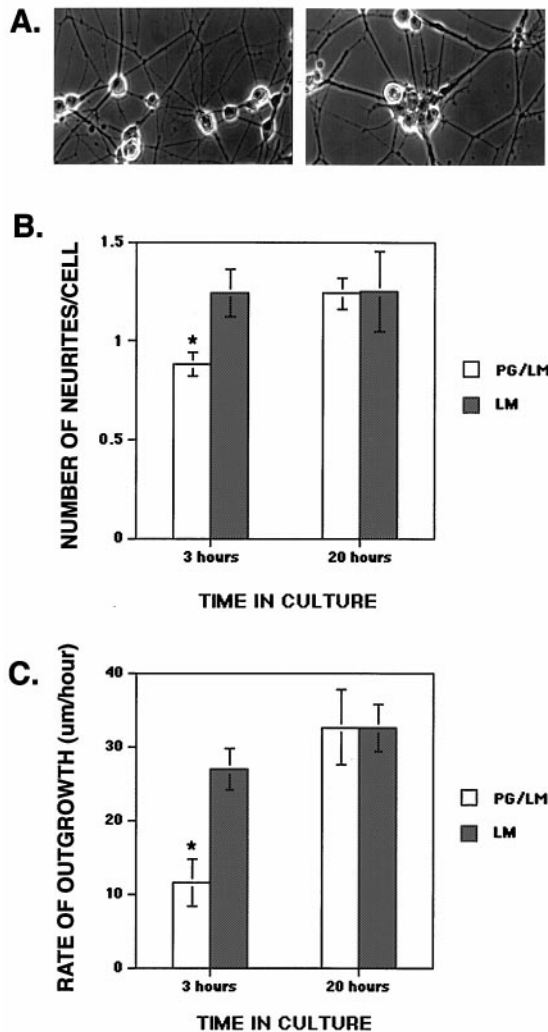
Figure 1. Neurons cultured on substrata containing a low concentration of the inhibitory proteoglycan aggrecan adapt to inhibition and extend neurites. *A*, Neurons were cultured on substrata containing either laminin alone (*left*) or laminin in combination with aggrecan (*right*). After 16–20 hr in culture, neurons have extended axons on both substrata. Outgrowth was less profuse and axons were more fasciculated on substrata containing aggrecan relative to outgrowth on laminin alone. *B*, The initiation of neurites is delayed on substrata containing aggrecan. At 3 hr, significantly fewer cells ($p < 0.002$, *t* test) have initiated neurites on substrata containing aggrecan relative to cells on laminin alone. By 6 hr in culture, the number of neurites initiated on aggrecan-containing substrata has greatly increased, suggesting that the neurons have adapted to inhibition. Means + SEMs of three experiments, each measuring at least 174 neurons/condition, are given. *C*, The rate of axon extension on laminin at 3 hr in culture is reduced by the presence of aggrecan ($p < 0.001$, *t* test), but by 20 hr in culture the rate of outgrowth on the two substrata is identical. Means + SEMs of three experiments, each measuring at least 14 neurons/condition, are given.

RESULTS

Neuronal adaptation to inhibition by aggrecan

Embryonic neurons purified from dissected dorsal root ganglia (Barres et al., 1988) were cultured on substrata containing both the growth-promoting ECM molecule laminin and low levels of the inhibitory proteoglycan aggrecan. Neurons were able to adhere to the substratum and extend axons after 20 hr in culture (Fig. 1*A*). However, the outgrowth of neurons cultured in the presence of aggrecan (*right panel*) was less profuse than that of
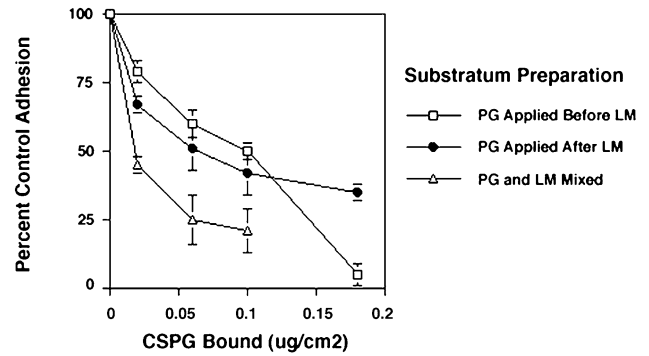

Figure 2. The adhesion of neurons to substrata containing constant amounts of bound laminin (300 ng laminin/cm^2) and increasing amounts of bound aggrecan is affected by the order of application of the two proteins to the substrata. Aggrecan mixed with laminin in solution (*shaded triangles*) is more inhibitory than aggrecan applied before or after adsorption of laminin to the substrata (*Mixed* points are statistically different from either *Before* or *After* at $p < 0.05$, *t* test), suggesting that conformational changes in laminin may underlie some of the inhibitory effect of aggrecan. At the highest concentrations tested, aggrecan is more inhibitory when applied before laminin, supporting the idea that interactions of soluble laminin with aggrecan result in greater inhibition. Adhesion is given as a percentage of adhesion to control (laminin only) substrata. Means + SEMs from three experiments are given. When error bars do not appear, they are smaller than the size of the symbol.

cells cultured on laminin alone (*left panel*), suggesting that aggrecan affects either the initiation of neurites or the rate of neurite extension once initiated. Observation of cultures at 3 and 20 hr indicated that the initiation of axons on aggrecan-containing substrata was delayed relative to controls ($p < 0.002$, *t* test) (Fig. 1*B*). Once initiated, axon outgrowth was significantly slower on substrata containing aggrecan for the first several hours of culture ($p < 0.001$, *t* test) (Fig. 1*C*). By 20 hr in culture, however, neurons grown on substrata containing aggrecan had significantly increased their rate of growth such that the rates of axon extension in the presence or absence of low levels of aggrecan were identical (Fig. 1*C*). These data indicate that the different substrata are not selecting different neuronal subpopulations with differing abilities to adhere to and extend processes on aggrecan. If neurons that are constitutively able to adhere and extend processes in the presence of aggrecan were being preferentially selected by aggrecan-containing substrata, then there would be no difference between the behavior of cells measured at 3 versus 20 hr in culture. Rather, the improved performance of neurons on aggrecan-containing substrata over time indicates that neurons are able to adapt to the inhibitory effects of aggrecan and extend axons under conditions that initially suppress neurite outgrowth. This adaptation could be mediated either through the upregulation of receptors for growth-promoting molecules that are also present in the substrata or through the downregulation of neuronal receptors for aggrecan in response to low levels of the inhibitor.

The inhibitory effects of aggrecan on neurite initiation and the rate of neurite outgrowth were confirmed by the observation that aggrecan inhibits neuronal cell adhesion to laminin in a dose-dependent manner (Fig. 2). In the presence of constant amounts of bound laminin, increasing concentrations of aggrecan significantly reduced the attachment of neuronal cells. It is not currently known how aggrecan inhibits neuronal attachment and outgrowth when co-presented with ECM molecules that promote both neuronal adhesion and outgrowth, such as laminin. If aggre-

can blocks access of neurons to laminin, the inhibitory effect of aggrecan on outgrowth should not be strongly influenced by the order of application of the two proteins to the substratum, as long as comparable amounts of both molecules are bound in all conditions. However, we observed that the inhibitory potency of aggrecan was significantly affected by the order of application of aggrecan and laminin to the substrata (Fig. 2). Aggrecan adsorbed to the substrata in the presence of soluble laminin was significantly more inhibitory than comparable amounts of proteoglycan bound either before or after the application of laminin. For the highest concentrations of proteoglycan tested, aggrecan bound after laminin (the condition in which simple masking of laminin should be, if anything, most pronounced) was less inhibitory than aggrecan bound before laminin.

Changes in integrin expression are associated with adaptation

The molecular basis for neuronal adaptation to aggrecan was examined by determining the levels of neuronal receptors for laminin in cells that had been cultured either on laminin alone (300 ng laminin/cm^2) or on laminin in combination with low levels of aggrecan (300 ng laminin/cm^2, 180 ng aggrecan/cm^2). Integrin $\alpha6\beta1$ and $\alpha3\beta1$ are the major laminin receptors in chick ciliary neurons (Weaver et al., 1995) and contribute significantly to the attachment (Condic and Letourneau, 1997) and outgrowth (Tomaselli et al., 1993) of dorsal root ganglion neurons cultured on laminin. Antibodies that block the function of $\beta1$-containing integrins completely block the attachment and outgrowth of chick sensory neurons on laminin (cf. Tomaselli et al., 1993) and on laminin in combination with aggrecan (data not shown), indicating that neuronal outgrowth on laminin, in either the presence or absence of aggrecan, is integrin dependent.

Cell-surface expression of both integrin $\alpha6\beta1$ and $\alpha3\beta1$ was significantly increased in neurons encountering laminin in the presence of aggrecan (Fig. 3B, Table 1). This increase in integrin cell-surface expression was associated with an increase in integrin mRNA (Fig. 3A) and total integrin protein (data not shown), all of which increase proportionately in neurons cultured on substrata containing laminin and aggrecan. These results suggest that aggrecan-associated increases in integrin expression are attributable to either transcriptional upregulation or an increase in the stability of the integrin message. Both of these mechanisms are distinct from the posttranslational regulation of integrin expression observed in neurons experiencing low availability of laminin (Condic and Letourneau, 1997), where neurons with increased surface expression of integrins show a concomitant decrease in the levels of integrin mRNA and total protein. The observation that integrin mRNA and protein levels both increase in the presence of aggrecan indicates that the response of neurons to aggrecan cannot be explained simply by aggrecan somehow "masking" laminin to generate a substrate that mimics the effects of low laminin.

Increased expression of integrin $\alpha3$ and $\alpha6$ in neurons encountering combined aggrecan and laminin suggests that upregulation of integrin expression by inhibitory CSPGs may underlie the adaptation of neurons to aggrecan. Interestingly, for cells cultured on substrata containing fibronectin and aggrecan (Fig. 3B, *FN/PG*, Table 1), integrin expression was not altered either for $\alpha6\beta1$ (a laminin receptor) or for $\alpha3\beta1$ (a receptor for both laminin and fibronectin), suggesting that the interactions of aggrecan with laminin and fibronectin are distinct. This conclusion is supported by the fact that neurons cultured on substrata con-
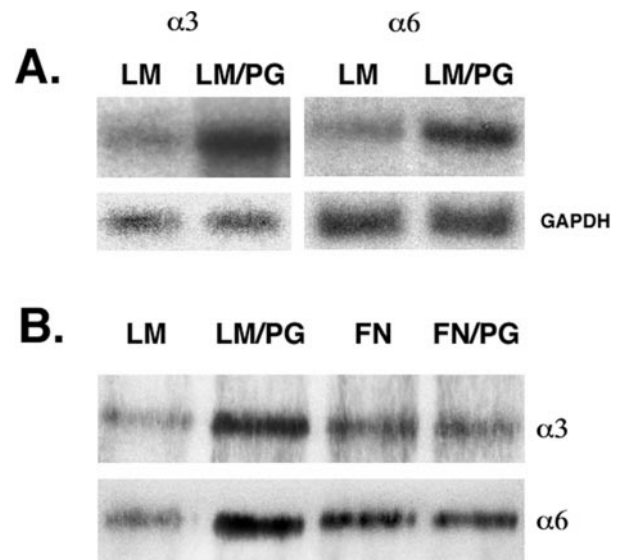

Figure 3. Increased expression of integrin $\alpha3$ and $\alpha6$ RNA and cell surface receptor protein in response to aggrecan. Quantifications of the results are given in Table 1. *A*, Northern analysis of integrin total RNA. The expression of RNA for $\alpha3$ and $\alpha6$ integrin is increased in neurons cultured on substrata containing both laminin and aggrecan (*LM/PG*) relative to those cultured on laminin alone (*LM*). The blots were stripped and reprobed for GAPDH as a loading control. *B*, Western analysis of biotinylated cell-surface integrin protein. Neurons cultured on substrata containing laminin and aggrecan (*LM/PG*) show an increase in the expression of laminin receptors integrin $\alpha3$ and $\alpha6$ at the cell surface relative to those cultured on laminin alone (*LM*), whereas the expression of these receptors does not increase in neurons cultured on substrata containing fibronectin and aggrecan (*FN/PG*) when compared with those cultured on fibronectin alone (*FN*).

Table 1. Relative amounts of surface protein and RNA on different culture substrata

	LM-CSPG/LM	FN-CSPG/FN
Total RNA		
$\alpha3$	1.91 (2.69–1.36)*	
$\alpha6$	2.24 (2.55–1.97)*	
Surface protein		
$\alpha3$	2.67 (4.62–1.55)**	0.57 (1.05–0.31)
$\alpha6$	2.61 (3.09–2.20)*	1.00 (1.26–0.79)

The ratios of surface integrin protein and integrin RNA were calculated from densitometry of films derived from at least three experiments (see Materials and Methods). Means and 95% confidence intervals are given. Statistically different from a ratio of 1 at **$p < 0.1$; *$p < 0.05$ (*t* test). Ratios of surface protein on FN-CSPG/FN are not statistically different from 1.

taining low levels of aggrecan and laminin adapt to inhibition (Fig. 1), whereas aggrecan continues to inhibit the outgrowth of neurons on fibronectin after 10 hr or more in culture (Snow et al., 1996).

Neurons with high levels of integrin expression are less inhibited by aggrecan

If alterations in integrin expression play a role in the adaptation of neurons to aggrecan, then increased integrin expression must be associated with changes in neuronal behaviors that are important to neurite outgrowth. Both neuronal adhesion and neurite outgrowth on laminin and on laminin/aggrecan substrata were examined in cells that had been previously cultured on different substrata to manipulate their levels of integrin expression. To
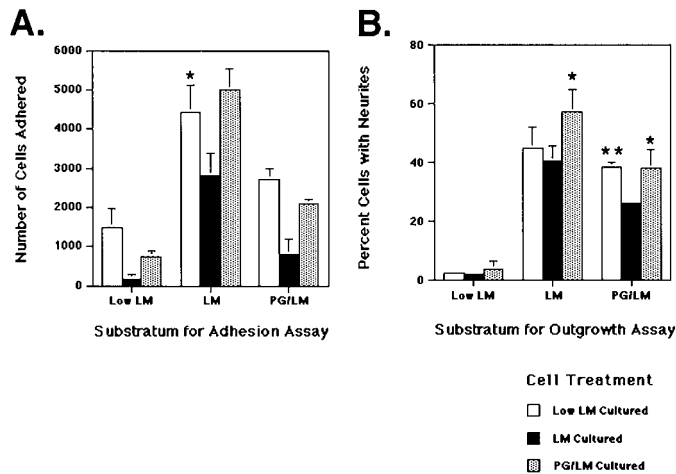
Figure 4. Increased expression of laminin receptors at the cell surface is associated with increased neuronal adhesion and outgrowth. Means + SEMs from three experiments are given. *A*, Neurons expressing high levels of integrin α3 and α6 at the cell surface (*Low LM Cultured* and *PG/LM Cultured*) show increased adhesion to laminin and to PG/LM substrata. *Different from LM cultured at *p* < 0.1 (*t* test). Values for all other low laminin-cultured and PG/LM-cultured conditions are significantly different from LM cultured at *p* < 0.05 or better. *B*, Neurons expressing high levels of integrin α3 and α6 at the cell surface extend neurites more readily on laminin both in the presence and absence of growth inhibitor aggrecan. *Different from LM cultured at *p* < 0.1. **Significantly different from LM cultured at *p* < 0.005. Where error bars do not appear, they are <1%.

manipulate surface integrin levels, cells were cultured overnight on laminin, low laminin, or laminin in combination with low levels of aggrecan (see Cell culture and substratum preparation). Cultures on both PG/LM (Fig. 3) and low LM (Condic and Letourneau, 1997) increase integrin expression, apparently by different molecular mechanisms. Cells expressing high levels of α6- and α3-integrin at the cell surface (i.e., cells cultured either on low laminin or on laminin in combination with aggrecan) were significantly more adherent both to laminin and to laminin/aggrecan-containing substrata (Fig. 4*A*). Aggrecan was also less inhibitory to neuronal attachment for cells expressing high levels of integrin α3 and α6 at the cell surface. For control cells (with low expression of integrin α3 and α6 at the cell surface (Fig. 4*A*, *black bars*), aggrecan in combination with laminin reduced neuronal attachment to 28% of the levels seen on laminin alone. In contrast, for neurons with high levels of integrin expression (*white* and *gray bars*), attachment to aggrecan-containing substrata was considerably closer to that seen on laminin alone; either 61% (*white bars*) or 42% (*gray bars*) of control adhesion. Similarly, neurons expressing high levels of integrin α3 and α6 at the cell surface extended neurites more readily on inhibitory (PG/LM-containing) substrata (Fig. 4*B*). These data indicate that increased cell surface integrin expression allows neurons to overcome inhibition by aggrecan and both attach and extend neurites, despite the presence of inhibitory matrix components in the extracellular environment.

Overexpression of integrin is sufficient for neuronal adaptation to aggrecan

To determine the role of increased integrin expression in neuronal adaptation to aggrecan, we tested the outgrowth of neurons overexpressing a single laminin receptor (integrin α1β1) in short-term assays (i.e., time points at which upregulation of endogenous
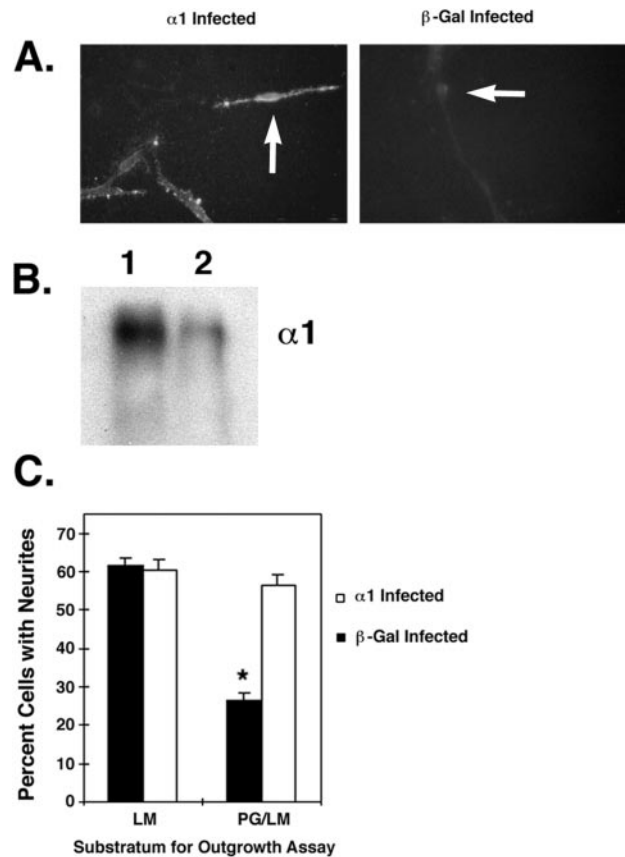

Figure 5. Increased expression of α1-integrin is sufficient to mediate adaptation of neurons to aggrecan. *A*, Neurons infected with adenoviral constructs expressing a control protein (*β-Gal*, *right*) or mouse α1-integrin (left) were stained live with an antibody that specifically recognizes the mouse α1 subunit. *Arrows* indicate the positions of neuronal cells. *B*, Overexpression of mouse α1-integrin in chick neurons results in an approximately twofold increase in total α1-integrin expressed at the cell surface (2.25 + 0.2). Infected neurons were cultured on LM substrata, where endogenous integrin expression is low (see Materials and Methods). Cell surface-biotinylated proteins from mouse α1-integrin-infected (*lane 1*) or control, β-galactosidase-infected (*lane 2*) neurons were immunoprecipitated with an antisera that recognizes both mouse and chick α1-integrin subunits. Total α1-integrin expressed at the surface is increased after infection with α1-expressing adenovirus, reflecting the contribution of the exogenous mouse protein to total α1 expression. Expression of endogenous α1 (control cells, *lane 2*) is quite low under these conditions. *C*, Control or α1-integrin-infected neurons were cultured on substrata containing laminin alone or laminin in combination with aggrecan as in Figure 4. Control neurons expressing β-galactosidase (*black bars*) are strongly inhibited in the presence of aggrecan after 3 hr in culture (Figs. 1, 4). At this time in culture, endogenous increases in integrin expression have not yet occurred. Neurons with increased laminin-receptor expression (α1-expressing; *white bars*) are not inhibited by aggrecan, showing outgrowth equivalent to that seen on laminin alone. Means + SEMs from at least three experiments are given. *Significantly different from all other conditions at *p* < 0.0001 (*t* test).

integrins has not yet occurred). Neurons were cultured on laminin substrata (300 ng/cm^2). On this substrata, endogenous laminin receptors are expressed at low levels (Fig. 3, α3β1, α6β1; Fig. 5*B*, α1β1). Neurons were infected with adenoviral constructs expressing a full-length mouse α1-integrin subunit. In sensory neurons, α1β1-integrin functions as a laminin and collagen receptor and is thought to be the receptor primarily responsible for adhesion of dorsal root ganglion neurons to laminin (Tomaselli et al., 1993). Consequently, altering the expression of this receptor

would be predicted to have the greatest effect on neuronal out-growth on laminin. In most cells, the $\beta 1$ subunit is synthesized in considerable excess (De Strooper et al., 1991; Muller et al., 1993; Koivisto et al., 1994), and exogenously supplied α-subunits are efficiently expressed as α-$\beta 1$ heterodimers at the cell surface (Hayashi et al., 1991; Felsenfeld et al., 1996; Miyakawa et al., 1996). Infection with mouse integrin $\alpha 1$-expressing adenovirus resulted in expression of mouse $\alpha 1$-containing integrins at the surface of all sensory neurons in culture (Fig. 5*A*). The levels and time course of infection were manipulated to yield an approxi-mately twofold increase (2.25 + 0.2) in expression of $\alpha 1$-containing integrins at the cell surface (Fig. 5*B*). This increase was similar in magnitude to that which occurs naturally in neu-rons that have adapted to aggrecan (Fig. 3*B* to Fig. 5*B*). Similar to the "adapted" neurons with high levels of integrin expression shown in Figure 4, neurons directly manipulated to express high levels of $\alpha 1$-integrin also showed marked improvement in out-growth on substrata containing aggrecan (Fig. 5*C*). At 3 hr in culture, a time point at which upregulation of endogenous inte-grins has not yet occurred and control neurons are strongly inhibited, neurons expressing high levels of $\alpha 1$-integrin exhibited outgrowth in the presence of aggrecan that was equivalent to that seen on laminin alone. These results demonstrate a causal link between integrin modulation and neuronal adaptation, indicating that increased integrin expression is sufficient to promote the outgrowth of neurons in the presence of aggrecan.

DISCUSSION

We have shown that integrin expression at the surface of sensory neurons increases in response to low levels of the inhibitory proteoglycan aggrecan, an ECM molecule that is not itself a ligand for integrins. The aggrecan-associated increase in integrin expression occurs through a mechanism that is distinct from that seen in response to low availability of laminin (Fig. 3) (cf. Condic and Letourneau, 1997), indicating that the effects of aggrecan are unlikely to be caused by a simple "masking" of laminin by pro-teoglycan. Increased integrin expression is correlated with in-creased neuronal adhesion and neurite outgrowth in the contin-ued presence of the inhibitor. Manipulation of integrin expression directly with adenoviral constructs indicates that high levels of integrin expression are sufficient to mediate adaptation of neurons to aggrecan. The ability of embryonic neurons to adapt to aggrecan suggests that modulation of integrin expression contributes to the regenerative capability of embryonic neurons in the presence of inhibitory proteoglycans after injury. Changes in neuronal responsiveness to laminin as a consequence of expo-sure to an inhibitory ECM proteoglycan demonstrates that the "cellular history" of a neuron can be a significant factor in neuronal interactions with both positive and negative guidance molecules. The regulation of integrins by aggrecan is significant because it demonstrates that proteoglycans can alter expression of receptors that mediate neuronal interactions with other, non-proteoglycan components of the ECM and because it suggests a possible mechanism for promoting the outgrowth of adult neu-rons in the presence of inhibitory proteoglycans that are ex-pressed after injury to the nervous system.

There have been conflicting reports regarding the role that CSPGs play in regulating neurite outgrowth. CSPG immuno-staining *in vivo* often localizes to regions of embryos that exclude axons (Snow et al., 1990b; Oakley and Tosney, 1991; Snow et al., 1991; Brittis et al., 1992; Landolt et al., 1995), and CSPGs *in vitro* can inhibit neurite outgrowth (Snow et al., 1990a; Fichard et al.,

1991; Snow and Letourneau, 1992; Braunewell et al., 1995; Maeda and Noda, 1996; Challacombe et al., 1997). In contrast, CSPG immunostaining *in vivo* is sometimes correlated with tissues that support the growth of axons (for review, see Pearlman and Sheppard, 1996), and CSPGs in combination with other matrix proteins can constitute a permissive substratum for axon out-growth under some conditions *in vitro* (Streit et al., 1993; Faissner et al., 1994) (also see Fig. 1*A*). One possible explanation for these conflicting results is that different studies have measured the response of neurons to different species of CSPG. Alternatively, it has been suggested that the growth-inhibiting or growth-promoting properties of CS-containing ECM preparations do not reflect CSPG function but are attributable to the presence of differing CS-binding proteins that in turn mediate the observed effects on neurite outgrowth (Emerling and Lander, 1996). This idea is supported by the fact that proteins known to be inhibitory to neurite outgrowth, including tenascins and thrombospondins, also bind to CS (Winnemoller et al., 1992; Barnea et al., 1994; Grumet et al., 1994). A third possibility is that CSPGs do affect neurite adhesion and outgrowth, but this effect is modulated by the precise combinations or concentrations of molecules present in the ECM (Snow and Letourneau, 1992). For example, the CSPGs versican and decorin inhibit adhesion of sensory neurons to fibronectin but not to laminin or collagen (Braunewell et al., 1995), and both chondroitin sulfate (Dou and Levine, 1995) and the cell surface proteoglycan NG2 (Dou and Levine, 1994) inhibit sensory neuronal outgrowth on laminin but not on the growth-promoting cell-surface molecule L1. A final possibility presented by the current observations is that because inhibitory CSPGs alter the neuronal response to growth-promoting matrix components over time, an individual neuron's response to CSPG can change depending on the length of time it has been in contact with CSPG-containing matrix and the availability of alternative (and perhaps more permissive) substrata for neurite outgrowth.

The adaptive response of neurons to inhibitory matrix compo-nents demonstrated here somewhat complicates the definition of "inhibitory." Clearly, aggrecan can arrest the outgrowth of neu-rons in acute assays when neurons encounter aggrecan in a discrete location and have the option of stopping and/or turning to avoid aggrecan-containing regions (Snow et al., 1990a, 1991; Snow and Letourneau, 1992; Challacombe et al., 1997). Our results demonstrate that under conditions in which neurons do not have the option of extending onto more favorable substrata, they can adjust their surface expression of integrins to compen-sate for their environment and extend neurites, in agreement with previous reports of limited neurite outgrowth on substrata containing either high amounts of CSPG (Snow et al., 1990a) or increasing steps of CSPG (Snow and Letourneau, 1992). In short-term assays, aggrecan inhibits neuronal attachment (Figs. 2, 4*A*), neurite initiation (Fig. 1*B*), and rate of neurite outgrowth (Fig. 1*C*). If only 20 hr cultures had been examined in isolation, none of these effects would have been evident because of the adaptive response of the neurons, and quite different conclusions might have been drawn regarding the properties of aggrecan in this system. The adaptive ability of neurons introduces the concept of cellular "history" into our thinking about how a neuron responds to inhibitory components of the ECM. The past experience of a growth cone may significantly alter the strength and even the nature of a neuron's response to what is currently present in the extracellular environment.

Sensory neurons do not compensate for the inhibitory effects of aggrecan under all circumstances. When cells are cultured on

substrata containing both fibronectin and aggrecan, the rate of neurite extension remains quite low relative to cells grown on fibronectin alone after 10 hr or more in culture (Snow et al., 1996). The inability of sensory neurons to adapt to aggrecan when cultured on fibronectin is consistent with our observation that expression of the fibronectin receptor $\alpha3\beta1$ is not increased in cells cultured on aggrecan–fibronectin-containing substrata (Fig. 3, Table 1). Fibronectin is known to interact with CSPGs including aggrecan. The CSPG-binding domains of fibronectin are localized to the IIIcs (or second cell-binding) region of the molecule and to the adjoining type III repeats (no. 12–14) (Barkalow and Schwarzbauer, 1994; Iida et al., 1995). The fibronectin IIIcs domain also contains the binding site for integrin $\alpha4\beta1$ (Iida et al., 1995). Integrins $\alpha3\beta1$ and $\alpha5\beta1$ bind to a different region of the fibronectin molecule, the first cell-binding (or RGD-containing) domain. It is possible that binding of $\alpha3\beta1$ to fibronectin is not affected by the interactions of fibronectin with aggrecan at a remote site in the molecule and that the inhibition of neurite outgrowth observed on substrata containing both fibronectin and aggrecan is largely caused by alterations in integrin $\alpha4\beta1$ activity that are not compensated for by either integrin $\alpha3\beta1$ or $\alpha5\beta1$.

Inhibitory proteoglycans, including aggrecan, are prominent components of the neural extracellular matrix during development, and their expression generally declines postnatally to relatively low levels in the adult (Meyer-Puttlitz et al., 1995; Lebaron, 1996; Levine and Nishiyama, 1996; Margolis et al., 1996; Schwartz et al., 1996). After injury to the CNS, there is a pronounced increase in the expression of both CSPGs and growth-promoting matrix molecules in regions of damage (Hoke and Silver, 1996). In "atraumatic" injury models that prevent the increased expression of proteoglycans normally associated with CNS damage, adult neurons extend considerable distances through the white matter of the brain (Davies et al., 1997), suggesting that inhibitory proteoglycans play a pivotal role in suppressing regeneration. In the spinal cord, many axons near the site of injury do not retract but rather exhibit significant sprouting in the initial weeks after injury. These early sprouts, however, do not continue to extend, and their arrested progress is coincident with the time course for increased CSPG accumulation in and around the site of injury (Li and Raisman, 1994, 1995). In an *in vitro* model system, McKeon et al. (1995) have shown that CSPG expression associated with cortical injury inhibits axon outgrowth. Treatment with chondroitinase to remove GAG chains from CSPG molecules results in a significant increase in neurite extension over scar tissue derived from CNS injury. Interestingly, co-treatment with both chondroitinase and function-blocking anti-laminin antibodies reverses the growth-promoting effect of chondroitinase alone, suggesting that interactions of neurons with laminin can support regeneration once the inhibitory activity of CSPGs has been reduced (McKeon et al., 1995). Consistent with these results are the findings that inhibitory factors in CNS myelin can be overcome by the addition of laminin (David et al., 1995).

The results presented here indicate that aggrecan is inhibitory to the attachment and outgrowth of neurons in culture. Embryonic neurons are able to compensate for the inhibitory action of aggrecan over time by increasing their expression of integrins and their responsiveness to laminin. Increased integrin expression is sufficient to mediate adaptation of neurons to aggrecan. These results suggest that manipulation of integrin expression may provide a mechanism for increasing the regenerative potential of adult neurons after CNS injury.

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