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Expression of $\alpha 5$ integrin rescues fibronectin responsiveness in NT2N CNS neuronal cells

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Abstract

The extracellular matrix protein fibronectin is implicated in neuronal regeneration in the peripheral nervous system. In the central nervous system (CNS), fibronectin is upregulated at sites of penetrating injuries and stroke; however, CNS neurons downregulate the fibronectin receptor, $\alpha 5 \beta 1$ integrin, during differentiation and generally respond poorly to fibronectin. NT2N CNS neuron-like cells (derived from NT2 precursor cells) have been used in pre-clinical and clinical studies for treatment of stroke and a variety of CNS injury and disease models. Here we show that, like primary CNS neurons, NT2N cells downregulate $\alpha 5 \beta 1$ integrin during differentiation and respond poorly to fibronectin. The poor neurite outgrowth by NT2N cells on fibronectin can be rescued by transducing NT2 precursors with a retroviral vector expressing $\alpha 5$ integrin under the control of the Murine Stem Cell Virus 5' long terminal repeat. Sustained $\alpha 5$ integrin expression is compatible with the CNS-like neuronal differentiation of NT2N cells and does not prevent robust neurite outgrowth on other integrin ligands. Thus, $\alpha 5$ integrin expression in CNS neuronal precursor cells may provide a strategy for enhancing the outgrowth and survival of implanted cells in cell replacement therapies for CNS injury and disease.

Keywords

NT2N; $\alpha 5$ integrin; fibronectin; neurite outgrowth

Introduction

Neurodegenerative diseases such as Parkinson's and Huntington's disease entail massive loss of distinct subsets of neurons. Traumatic brain injury or stroke also creates zones devoid of viable neurons. Cell replacement using neurons derived from cultured stem cells has been proposed as a potential therapy (Lindvall and Kokaia 2006; Lindvall et al. 2004), but many hurdles remain. Ideally, engrafted neurons would differentiate into the appropriate neuronal subtype, receive appropriate inputs, and make appropriate connections. Moreover, these events need to occur outside of the normal developmental context, in the terrain of the damaged central nervous system. The amenability of *in vitro*-cultured stem cells to gene transfer creates opportunities for addressing these obstacles. For example, expression of the Nurr1 transcription factor in embryonic or neuronal stem cells drives dopaminergic differentiation, potentially enhancing their utility in the treatment of Parkinson's disease (Kim et al. 2002).

The ability of engrafted neurons to survive and extend processes depends at least in part upon how they respond to competing extracellular cues at sites of brain injury. Central

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nervous system (CNS) injuries provoke a gliotic reaction, astrocyte hypertrophy, and the re-expression of extracellular matrix proteins. Many of these molecules, such as chondroitin sulfate proteoglycans, are inhibitors of neurite outgrowth (Fitch and Silver 2008; Silver and Miller 2004). Their presence may limit the ability of implanted neurons to make connections with the host brain and obtain target-derived trophic support. On the other hand, outgrowth-promoting proteins, such as laminin isoforms and fibronectin, are also upregulated at sites of CNS injury (McKeon et al. 1995; McKeon et al. 1991; Risling et al. 1993). Even the process of implantation itself, because it requires penetration of the brain parenchyma, triggers the expression of both outgrowth-promoting and inhibitory cues (Barker et al. 1996; McKeon et al. 1991). The intrinsic capacity of engrafted neurons to recognize and respond to growth-promoting cues may help determine the extent to which they are able to tolerate or overcome the presence of growth-inhibitory cues.

In transected peripheral nerve, fibronectin is upregulated at the transection site, and the major fibronectin receptor, $\alpha 5 \beta 1$ integrin, is re-expressed on the regenerating neuronal axons (Lefcort et al. 1992). Dorsal root ganglion (DRG) neurons seeded onto organotypic adult brain slices responded with robust outgrowth, which was strongly inhibited by anti-fibronectin antibodies (Tom et al. 2004). However, cortical neurons seeded onto the slices regenerated poorly, suggesting that their intrinsic capacity to respond to fibronectin is poor compared to that of the DRG neurons. In agreement with this view, isolated CNS neurons failed to extend stable neurites on purified, intact fibronectin in vitro, while peripheral nervous system (PNS) neurons responded with robust outgrowth (Rogers et al. 1983; Rogers et al. 1987; Rogers et al. 1985). One reason for the generally poor responsiveness of CNS neurons to fibronectin may be that the fibronectin receptor, $\alpha 5 \beta 1$ integrin, which is expressed by nestin-positive CNS neural precursors, is downregulated as the cells differentiate into nestin-negative neurons (Yoshida et al. 2003).

A previous study demonstrated that transgenic expression of the $\alpha 5$ integrin subunit in adult DRG neurons enhanced their capacity to respond to fibronectin (Condic 2001). The goals of our current study were to (i) introduce a constitutively-expressed $\alpha 5$ integrin construct into a proliferating CNS neural precursor cell type, (ii) determine whether enforced $\alpha 5$ integrin expression was compatible with neuronal differentiation, and (iii) assess the ability of transgenic $\alpha 5$ integrin to rescue neurite outgrowth on fibronectin. We selected the NT2/NT2N cell system for these studies. The human NT2 teratocarcinoma cell line is a model of CNS neuronal progenitors, which differentiate upon exposure to retinoic acid into CNS neuron-like NT2N cells (Trojanowski et al. 1997). NT2N neuron-like cells have been used extensively in pre-clinical neuronal cell replacement studies, and, more recently, in clinical trials (Hara et al. 2008; Kondziolka et al. 2005; Kondziolka and Wechsler 2008). Our results help to establish the feasibility of introducing $\alpha 5$ integrin into CNS precursor cells to enhance the capacity of their differentiated neuronal progeny to respond to fibronectin.

Materials and Methods

Antibodies and extracellular matrix proteins

Monoclonal antibodies recognizing $\beta 1$ integrin (clone M13) and $\alpha 3$ integrin antibody (clone A3-IIF5) were previously referenced (Stipp and Hemler 2000). Anti- $\alpha 5$ integrin monoclonal antibodies were PID6 (Covance) and JBS5 (Chemicon). Anti-neurofilament antibodies recognizing NF66/ α -internexin (clone 1D2) and peripherin (clone 7C5) were from Imgenex. Anti-MAP2 antibody (clone HM-2) was from Sigma. Affinity purified human laminin-5 (laminin-332) was prepared using the 6F12 anti-laminin-5 monoclonal antibody conjugated to Affigel-10 (BioRad), by a modification of the method of Burgeson and colleagues (Marinkovich et al. 1992), as previously described (Winterwood et al. 2006). Human plasma fibronectin and mouse laminin-1 (laminin-111) were from BD Biosciences.

Cell culture and retroviral transduction

Standard growth medium for NT2 precursor cells was DMEM with 10% FBS (Invitrogen), penicillin-streptomycin, and 2 mM glutamine. The retroviral vector pMSCVpuro (Clontech), containing EGFP or $\alpha 5$ integrin cDNA inserts, was co-transfected along with a VSVG retroviral coat protein expression vector into GP2-293 packaging cells (Clontech). The resulting supernatants were used to transduce PT67 packaging cells (Clontech), and stably transduced, virus-producing PT67 cell populations were selected. PT67 cell supernatants were in turn used to transduce NT2 cells, and stable NT2 transductants were selected with 0.5 $\mu\text{g/ml}$ puromycin and maintained in 0.1 $\mu\text{g/ml}$ puromycin.

NT2N neuron-like cells were obtained by a modification of the method of Pleasure et al., 1992. NT2 precursor cells were treated with 10 μM all-trans retinoic acid (Sigma) for 4-5 weeks. Cells were then rinsed with PBS and treated for 20-30 min at 37°C with 30 U/ml papain (Worthington Biochemical Corp.) in Hanks balanced salt solution with 25 mM HEPES. Cells were then harvested into growth medium with 0.1 mg/ml DNase I (Worthington Biochemical Corp.), recovered by centrifugation, resuspended in fresh growth medium, and dissociated by trituration. Dissociated cells were split 1:6 into fresh flasks, refed after 2 days with growth medium containing mitotic inhibitors, and cultured an additional 10-14 days, as previously described (Pleasure et al. 1992). NT2N cells, growing as clusters over a monolayer of non-neuronal cells, were isolated by rinsing with phosphate-buffered saline (PBS) and treating for 2-3 minutes with 0.25% trypsin/EDTA diluted 30-fold in PBS. Purified neurons were maintained on plates coated with 50 $\mu\text{g/ml}$ poly-L-lysine followed by Matrigel (Becton Dickinson) diluted 1:30 in PBS. In our hands, the use of papain for the first replating produces smaller neuronal cell clumps that are more easily isolated from the non-neuronal cells in the second replating. For cells transduced with EGFP and $\alpha 5$ integrin retroviral vectors, puromycin selection was discontinued at the outset of the differentiation protocol.

Neurite outgrowth assays

Acid-washed glass coverslips were coated overnight at 4°C with human plasma fibronectin (50 $\mu\text{g/ml}$ in PBS), mouse laminin-1 (20 $\mu\text{g/ml}$ in PBS), or human laminin-5 (2 $\mu\text{g/ml}$ in PBS with 0.005% Tween-20). Coverslips were blocked with 10 mg/ml cell culture-grade BSA for 1 h at room temperature and rinsed. Purified NT2N cells prepared as described above were harvested with 0.25% trypsin/EDTA diluted 1:10 in PBS and collected into DMEM with 5 mg/ml BSA and 0.2 mg/ml soybean trypsin inhibitor (Worthington Biochemical Corp.). After centrifugation, cells were resuspended in a serum free medium (SFM) consisting of DMEM with B27 additives (Invitrogen) and 1 mM glutamine. Approximately 10,000 cells and clusters of cells were plated on substrate-coated coverslips in the presence or absence of function-blocking anti-integrin antibodies used at 10 $\mu\text{g/ml}$. Except where indicated, two coverslips per cell type per condition were plated in each experiment. At the end of the assay, coverslips were formalin-fixed, mounted, and 4 fields per coverslip were photographed, including edge and center fields. Neurite lengths were measured with NIH Image 1.63, and total outgrowth was calculated as the total length of all the neuritic material in each field divided by the total perimeter of all the clusters of neurons in the field (Stipp and Hemler 2000). In all cases, duplicate coverslips produced nearly identical results, so the data from the 2 coverslips in each set were pooled.

Cell surface labeling and immunoprecipitation

NT2 cells and NT2N cells were biotinylated 1 h at room temperature with 0.1 mg/ml Sulfo-NHS-LC-biotin (Thermo Scientific Pierce) in 20 mM HEPES, 0.15 M NaCl, and 2 mM MgCl_2 (HBSM). Cells were then lysed for 1 hour in HBSM with 1% Triton X-100, 1mM phenylmethylsulfonylfluoride, 20 $\mu\text{g/ml}$ aprotinin, and 10 $\mu\text{g/ml}$ leupeptin. Insoluble

material was removed by centrifugation, and protein concentrations were determined by amido black dye binding (Schaffner and Weissmann 1973). Lysates were immunoprecipitated overnight with 5 μ g/ml anti-integrin antibodies and protein-G Sepharose (Thermo Scientific Pierce). Samples were resolved by SDS-PAGE, transferred to nitrocellulose, and developed with 1 μ g/ml HRP-Extravidin (Sigma).

Immunostaining

Purified NT2N cells were plated in SFM on acid washed glass coverslips coated with 50 μ g/ml poly-L-lysine followed by Matrigel diluted 1:30 in PBS. Cells were cultured overnight and then fixed with 10% formalin in PBS with 2 mM $MgCl_2$ and 4% sucrose. For integrin staining, cells were blocked with 10% goat serum in PBS and then stained 1h at room temperature with 5 μ g/ml specific antibody diluted in blocking buffer. Coverslips were rinsed and stained 45 min at room temperature with 7.5 μ g/ml Cy2-goat anti-mouse secondary antibodies (Jackson ImmunoResearch) diluted in blocking buffer. Coverslips were rinsed and mounted on FluorSave (Calbiochem). For neurofilament and MAP2 staining, cells were permeabilized with 0.2% Triton X-100 during the blocking step, and, for MAP2 staining, Hoechst 33342 was added to 5 μ g/ml along with the secondary antibody. Samples were photographed on a Leica DMIRE2 inverted microscope using a Hamamatsu ORCA-285 CCD camera, controlled by OpenLab software (Improvision, Inc.). Positive staining and negative control staining were acquired with identical exposure parameters and processed identically using the Adjust Levels command of Adobe Photoshop 7.0.

Results

NT2N cells downregulate $\alpha 5\beta 1$ integrin and respond poorly to fibronectin

CNS neuronal precursors downregulate $\alpha 5$ integrin concomitantly with neuronal differentiation (Yoshida et al. 2003) and CNS neurons generally respond poorly to $\alpha 5\beta 1$ integrin ligand, fibronectin (Rogers et al. 1983; Rogers et al. 1987; Rogers et al. 1985). To determine if this pattern of $\alpha 5$ integrin expression and function is recapitulated in the NT2-NT2N cell system, we compared the outgrowth of NT2N neuronal cells on fibronectin to that on laminin-5 (laminin-332), a ligand for $\alpha 3\beta 1$ integrin. Within 5 hours after plating, clusters of purified NT2N cells extended copious neurites on laminin-5, but very few neurites were extended on fibronectin (Fig. 1 A,B). By 18 hours after plating, outgrowth on laminin-5 was even more extensive, while outgrowth on fibronectin remained poor (Fig. 1 C,D). Quantification revealed that total neurite outgrowth on laminin-5 after 5 hours was ~10-fold higher than outgrowth on fibronectin (Fig. 1 E). After 18 hours, total outgrowth on laminin-5 remained over 7-fold higher than on fibronectin (Fig. 1 E). To determine whether the poor responsiveness of NT2N cells to fibronectin corresponded to a downregulation of $\alpha 5$ integrin expression, we immunoprecipitated $\alpha 5\beta 1$ or $\alpha 3\beta 1$ integrin from extracts of cell surface-biotinylated NT2 and NT2N cells. In the NT2 precursor cells, $\alpha 5\beta 1$ integrin was strongly expressed, but little or no $\alpha 5$ expression was detected in the differentiated NT2N cells (Fig. 1 F, lanes 1&2). In contrast, $\alpha 3\beta 1$ integrin, which was modestly expressed in the NT2 precursor cells, was significantly upregulated in the NT2N neuronal cells (Fig. 1 F, lanes 3&4). Thus, the downregulation of $\alpha 5$ integrin that occurs during the differentiation of primary CNS neuronal precursors is recapitulated during NT2N cell neuronal differentiation.

The Murine Stem Cell Virus 5' LTR drives persistent gene expression in NT2N cells

Previous studies have reported successful transfection of NT2 precursor cells, but subcloning and screening for clones that both expressed the transgene and were capable of neuronal differentiation were involved (Pleasure et al. 1992). In our preliminary experiments, we were able to transduce the NT2 precursor cell line with retroviral constructs driving transgene expression from a Moloney Murine Leukemia Virus 5' LTR. However,

transgene expression from these constructs was unstable (C.S.S. unpublished results). As an alternative, we tested a retroviral vector, pMSCVpuro, in which transgene expression is driven by the Murine Stem Cell Virus 5' LTR. NT2 precursor cells were transduced with a pMSCVpuro-EGFP construct, and stable transductants were selected and maintained as an uncloned population. EGFP transgene expression was widespread throughout the transduced population (Fig. 2 A), was maintained throughout the differentiation process (in the absence of ongoing puromycin selection; Fig. 2 B), and continued in the purified NT2N neuronal cells obtained after the 6 week differentiation protocol (Fig. 2 C). These experiments identified pMSCVpuro as a vector that permits efficient and stable transduction of NT2 precursor cells and provides for continued transgene expression upon neuronal differentiation.

Expression of $\alpha 5\beta 1$ integrin is compatible with NT2N neuronal differentiation

Since $\alpha 5$ integrin is normally downregulated upon CNS neuronal differentiation, we first addressed whether enforced expression of $\alpha 5$ was compatible with NT2N cell differentiation. NT2 precursor cells were transduced with a pMSCVpuro- $\alpha 5$ integrin construct, and stable transductants were selected. When we subjected this uncloned population to the standard NT2N differentiation protocol, we obtained normal numbers of morphologically correct NT2N neurons (Fig. 3 A). We assessed $\alpha 5\beta 1$ integrin expression in these cells by immunostaining of neurites extended on a Matrigel substrate. All of the neurites were positive for the endogenous $\beta 1$ integrin subunit (Fig. 3 B,C), and ~80% of the neurites were positive for the $\alpha 5$ integrin subunit (Fig. 3 D,E), indicating a good overall level of transgene expression in this uncloned population. Thus, at a gross morphological level, sustained expression of $\alpha 5$ integrin appeared not to impair NT2N neuronal differentiation. We refer to these cells as NT2N- $\alpha 5$ cells hereafter.

To further confirm that $\alpha 5$ integrin expression did not interfere with NT2N cell differentiation, we stained at different stages for the neuronal microtubule-associated protein, MAP2, which reveals the progressive accumulation of neuronal cells in differentiating NT2N cell cultures (Guillemain et al. 2003). As previously reported (Guillemain et al. 2003), MAP2-positive cells could be detected after 1 week of retinoic acid-induced NT2N cell differentiation (Fig. 4 A,B). Similar numbers of MAP2-positive NT2N- $\alpha 5$ cells were also detected at 1 week (Fig. 4 G,H). After 2 weeks of retinoic acid differentiation, both parental NT2N and NT2N- $\alpha 5$ cell cultures contained large clusters of MAP2-positive cells (Fig. 4 C,D, & I,J). After 5 weeks of retinoic acid treatment, the NT2N cell differentiation protocol entails replating the cells at a lower density (replate #1; Pleasure et al. 1992). Similar numbers of MAP2-positive cells were obtained in both parental and NT2N- $\alpha 5$ cell cultures at this stage as well (Fig. 4 E,F & K,L). Quantification of MAP2-positive cells upon counterstaining with Hoechst dye confirmed that NT2N- $\alpha 5$ cell cultures generated just as many MAP2-positive cells at each stage as did parental NT2N cell cultures (Fig. 4 M-O).

NT2N cells expressing $\alpha 5\beta 1$ integrin appear to retain a CNS-like immunophenotype

NT2N cells were initially characterized as CNS neuron-like by demonstrating that they were positive for markers such as NF-66, a neurofilament that is predominately expressed in the CNS, and negative for markers such as peripherin, a neurofilament expressed by virtually all PNS neurons (Pleasure et al. 1992). Immunostaining of parental and NT2N- $\alpha 5$ cells revealed that the NT2N- $\alpha 5$ cells continued to express NF-66 at normal levels (Fig. 5, B,D), and remained peripherin-negative, just like NT2N cells derived from parental NT2 precursors (Fig. 5 F,H). Thus, at least as judged by these two markers, NT2N- $\alpha 5$ cells appear to retain the CNS-like immunophenotype of the parental NT2N cells.

Expression of $\alpha 5$ integrin in NT2N cells rescues outgrowth on fibronectin

We next tested whether outgrowth on fibronectin is restored for NT2N- $\alpha 5$ cells. In contrast to parental NT2N cells, which again responded poorly, NT2N- $\alpha 5$ cells extended numerous neurites on fibronectin (Fig. 6 A,B). All of this enhanced neurite outgrowth on fibronectin was $\alpha 5$ -integrin-dependent, because it could be blocked with an anti- $\alpha 5$ function-blocking antibody (Fig. 6 C). Quantification revealed an ~300% increase in the ability of NT2N- $\alpha 5$ cells to extend neurites on fibronectin, as compared to parental NT2N cells, and this increase was completely reversed upon treatment with the anti- $\alpha 5$ integrin antibody (Fig. 6 E). Treatment of parental cells with the anti- $\alpha 5$ antibody resulted in a modest inhibition of the already low level of outgrowth by these cells on fibronectin, suggesting a small contribution by a trace of $\alpha 5$ expression (Fig. 6 D,E). Collectively, these data revealed that $\alpha 5$ integrin expression in NT2N cells dramatically enhances their ability to extend neurites on fibronectin.

NT2N cells expressing $\alpha 5$ integrin retain neurite outgrowth on other ECM substrates

To assess whether the presence of the $\alpha 5$ integrin subunit might influence the function of other integrins in differentiated NT2N cells, we compared NT2N and NT2N- $\alpha 5$ cell neurite outgrowth on fibronectin, laminin-1 (laminin-111), and laminin-5. On fibronectin, NT2N- $\alpha 5$ cells again responded with abundant neurite outgrowth, while parental NT2N cells again responded poorly (Fig. 7 A,B). In contrast, on the laminin isoforms, both cell types extended abundant neurites (Fig. 7 C-F). Quantification revealed that in this set of experiments, NT2N- $\alpha 5$ cell neurite outgrowth on fibronectin was over 10-fold higher than that of the parental cells (Fig. 8A). In addition, while NT2N- $\alpha 5$ cell neurite outgrowth on laminin-1 appeared somewhat reduced compared to that of parental NT2N cells, outgrowth on laminin-5 was identical for both cell types. Outgrowth of NT2N- $\alpha 5$ cells on fibronectin was almost completely abolished by a function-blocking anti- $\alpha 5$ integrin antibody, as in the previous trial. A function-blocking anti- $\beta 1$ integrin antibody abolished outgrowth by both cell types on laminin-1, and an anti- $\alpha 3$ integrin antibody strongly inhibited outgrowth by both cell types on laminin-5 (Fig. 8A). In a second set of experiments performed several months later, NT2N- $\alpha 5$ cell neurite outgrowth on fibronectin was over 5-fold higher than that of the parental cells, and was again abolished by an anti- $\alpha 5$ integrin antibody (Fig. 8B). In contrast, both cell types displayed identical, robust outgrowth on a laminin-rich Matrigel substrate. The anti- $\alpha 5$ integrin antibody had no effect on outgrowth on Matrigel by either cell type, confirming that antibody ligation of $\alpha 5$ integrin on NT2N- $\alpha 5$ cells does not generate a signal that blocks outgrowth non-specifically (Fig. 8B). Overall, these data showed that NT2N- $\alpha 5$ cells use $\alpha 5$ integrin specifically for outgrowth on fibronectin, but are still able to respond to $\alpha 5$ -integrin-independent ligands with robust neurite outgrowth.

Discussion

Significant evidence implicates fibronectin in regenerative outgrowth of peripheral nerves. In transected peripheral nerve, fibronectin and its receptor, $\alpha 5\beta 1$ integrin, are both upregulated (Lefcort et al. 1992; Siironen et al. 1992). Fibronectin expression is highest near the transection site, and $\alpha 5\beta 1$ integrin is expressed on axons re-growing through the bridge region between proximal and distal nerve stumps (Lefcort et al. 1992). Fibronectin is also upregulated at the dorsal root entry zone after dorsal root injury (Golding et al. 1999) and in crushed sciatic nerve, where embryonic forms of fibronectin containing the $\alpha 4\beta 1$ integrin binding site are upregulated (Mathews and French-Constant 1995; Voegelzang et al. 1999). A pre-conditioning sciatic nerve crush triggers the redistribution of $\alpha 5\beta 1$ integrin to the axons and growth cones of DRG neurons and enhances their ability to extend neurites on fibronectin (Gardiner et al. 2007), and in human patients with peripheral neuropathy, fibronectin expression is associated with nerves undergoing regeneration (Previtali et al.

2008). Collectively, these data suggest that fibronectin may be an important substrate for the regenerative outgrowth of peripheral nerves.

The extent to which fibronectin plays role in CNS regeneration may be more of an open question. In favor of such a role, fibronectin is upregulated at sites of CNS injuries. For example, spinal cord contusion (Ma et al. 2001) or hemisection (Camand et al. 2004) or penetrating brain injury or implantation (Egan and Vijayan 1991; McKeon et al. 1991) all trigger upregulation of fibronectin at the injury site. Potential sources of fibronectin in CNS injuries include reactive astroglia and ingressing fibroblasts or Schwann cells, but plasma fibronectin is also an important source. Plasma fibronectin enters the brain parenchyma at sites of traumatic injury and ischemia, and in mice specifically deleted for plasma-borne fibronectin, lesion size and neuronal cell death associated with injury or ischemia were both significantly increased (Sakai et al. 2001; Tate et al. 2007). In addition, although fibronectin is downregulated during CNS development (Chun and Shatz 1988; Sheppard et al. 1995; Sheppard et al. 1991), it appears that residual fibronectin associated with the white matter tracts of adult brain slices may be sufficient to help promote the outgrowth of ectopic dorsal root ganglion neurons (Tom et al. 2004). Thus, it appears clear that fibronectin is often present at CNS injury sites, and at least has the potential to contribute to neuronal survival and regenerative outgrowth. What remains less clear is how well CNS neurons are actually able to respond to any fibronectin that may be present. Unlike PNS neurons, which generally respond well to fibronectin, outgrowth by CNS neurons on fibronectin is generally poor (Rogers et al. 1983; Rogers et al. 1987; Rogers et al. 1985), possibly because $\alpha 5$ integrin is downregulated when cortical precursors undergo neuronal differentiation (Yoshida et al. 2003).

Perhaps as a result of this developmental downregulation, immunohistochemical analysis of $\alpha 5$ integrin expression in the adult brain has produced mixed results. Some studies have reported virtually no parenchymal $\alpha 5$ expression (Paulus et al. 1993; Pesheva et al. 1988). Others have reported widespread $\alpha 5$ expression in brain neurons that was generally diffuse and cytoplasmic, but with occasional punctate cell surface staining (King et al. 2001). Prominent staining of $\alpha 5$ integrin in apical dendrites in the hippocampus and deep neocortex appeared spiral in nature, possibly indicating localization to a spiral shaped endoplasmic reticulum found in dendritic shafts (Bi et al. 2001). Expression of $\alpha 5$ was also detected in adult brain by RT-PCR and by immunoblotting of synaptosomal extracts, and compound heterozygote mice with reduced expression of $\alpha 5$ in combination with $\alpha 3$ and $\alpha 8$ integrin, displayed deficits in hippocampal long term potentiation and spatial memory (Chan et al. 2003). Lastly, unlike the upregulation of $\alpha 5$ integrin reported for damaged peripheral nerve (Lefcort et al. 1992), the low to moderate $\alpha 5$ expression reported for CNS neurons in one study was maintained, but not increased, upon ischemic insult (Sakai et al. 2001).

Collectively, the studies summarized above support the view that subsets of CNS neurons may express low to moderate amounts of $\alpha 5$ integrin, which may participate in synaptic function, but which may not be expressed broadly or highly enough to support robust, long-range process outgrowth on fibronectin. Thus, our new data that enforced $\alpha 5$ expression is both compatible with a CNS neuron-like differentiated phenotype and capable of restoring robust outgrowth on fibronectin is of potential significance. Neurons more fully capable of responding to the fibronectin present at CNS injury sites may display improved survival and process outgrowth in cell replacement strategies to treat CNS trauma, stroke, or degenerative disease.

We selected NT2N cells for our study because they have been used extensively for cell replacement studies in rodent models of stroke, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and spinal cord injury (Hara et al. 2008). In each model, at

least some of the studies have indicated functional improvement in the engrafted animals. NT2N cells have also been used in Phase I and Phase II clinical trials for the treatment of cerebral stroke (Kondziolka et al. 2005; Kondziolka and Wechsler 2008). In the Phase II trials, although there was no significant motor improvement, some improved activities of daily living were noted, together with improved performance on some cognitive tests. In the pre-clinical stroke models, the survival rate of NT2N cells engrafted into the penumbra zone has been reported to be ~15% (Hara et al. 2008), underscoring the potential benefit of modifications that could improve survival post-implantation. The survival of NT2N cell grafts in human patients has not been rigorously assessed, although some engrafted cells were detected at autopsy in one patient, 27 months after they had been implanted (Nelson et al. 2002).

A potential advantage of neuronal precursor cell lines such as NT2 is the possibility of creating stable, transgene-expressing sublines to provide a renewable, well-characterized source of genetically modified neurons. However, standard transfection protocols, although technically feasible (Pleasure et al. 1992), are poorly efficient with NT2 cells. Relatively few examples exist where stable NT2 subpopulations have been created for the purpose of studying transgene function in differentiated NT2N cells. Expression of transgenes introduced into NT2 cells and maintained throughout differentiation into NT2N neuronal cells has been reported for an SV40-based viral vector using an immediate early CMV promoter (Cordelier et al. 2003) and for the MMLV retroviral vector, pBabe (Hara et al. 2007). In our hands, the MMLV 5' LTR has not provided stable transgene expression in NT2 cells (C.S.S. unpublished data). However, we show in our current study that the MSCV 5' LTR is capable of driving stable transgene expression during and after NT2N cell differentiation. Thus, the MSCV promoter may provide a useful additional avenue for gene expression in this system.

In conclusion, our data provide evidence that sustained $\alpha 5$ integrin expression is compatible with CNS neuronal differentiation, confers robust outgrowth on fibronectin, and does not strongly affect outgrowth on other integrin ligands. Expression of $\alpha 5$ integrin by implanted CNS neuronal cells could provide an advantage for survival and process outgrowth. Further testing of $\alpha 5$ -expressing NT2N cells (or a next generation neuronal cell type) in pre-clinical models of CNS injury or disease is warranted.

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References

- Barker RA, Dunnett SB, Faissner A, Fawcett JW. The time course of loss of dopaminergic neurons and the gliotic reaction surrounding grafts of embryonic mesencephalon to the striatum. *Exp Neurol*. 1996; 141(1):79–93. [PubMed: 8797670]
- Bi X, Lynch G, Zhou J, Gall CM. Polarized distribution of $\alpha 5$ integrin in dendrites of hippocampal and cortical neurons. *J Comp Neurol*. 2001:184–193. [PubMed: 11391640]
- Camand E, Morel MP, Faissner A, Sotelo C, Dusart I. Long-term changes in the molecular composition of the glial scar and progressive increase of serotonergic fibre sprouting after hemisection of the mouse spinal cord. *Eur J Neurosci*. 2004:1161–1176. [PubMed: 15341588]
- Chan CS, Weeber EJ, Kurup S, Sweatt JD, Davis RL. Integrin requirement for hippocampal synaptic plasticity and spatial memory. *J Neurosci*. 2003:7107–7116. [PubMed: 12904471]

- Chun JJ, Shatz CJ. A fibronectin-like molecule is present in the developing cat cerebral cortex and is correlated with subplate neurons. *J Cell Biol.* 1988;857–872. [PubMed: 3346327]
- Condic ML. Adult neuronal regeneration induced by transgenic integrin expression. *J Neurosci.* 2001; 21(13):4782–4788. [PubMed: 11425905]
- Cordelier P, Van Bockstaele E, Calarota SA, Strayer DS. Inhibiting AIDS in the central nervous system: gene delivery to protect neurons from HIV. *Mol Ther.* 2003;801–810. [PubMed: 12788654]
- Egan RA, Vijayan VK. Fibronectin immunoreactivity in neural trauma. *Brain Res.* 1991;330–334. [PubMed: 1814578]
- Fitch MT, Silver J. CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. *Exp Neurol.* 2008; 209(2):294–301. [PubMed: 17617407]
- Gardiner NJ, Moffatt S, Fernyhough P, Humphries MJ, Streuli CH, Tomlinson DR. Preconditioning injury-induced neurite outgrowth of adult rat sensory neurons on fibronectin is mediated by mobilisation of axonal alpha5 integrin. *Mol Cell Neurosci.* 2007;249–260. [PubMed: 17433878]
- Golding JP, Bird C, McMahon S, Cohen J. Behaviour of DRG sensory neurites at the intact and injured adult rat dorsal root entry zone: postnatal neurites become paralysed, whilst injury improves the growth of embryonic neurites. *Glia.* 1999;309–323. [PubMed: 10383050]
- Guillemain I, Fontes G, Privat A, Chaudieu I. Early programmed cell death in human NT2 cell cultures during differentiation induced by all-trans-retinoic acid. *J Neurosci Res.* 2003; 71(1):38–45. [PubMed: 12478612]
- Hara K, Matsukawa N, Yasuhara T, Xu L, Yu G, Maki M, Kawase T, Hess DC, Kim SU, Borlongan CV. Transplantation of post-mitotic human neuroteratocarcinoma-overexpressing Nurr1 cells provides therapeutic benefits in experimental stroke: in vitro evidence of expedited neuronal differentiation and GDNF secretion. *J Neurosci Res.* 2007;1240–1251. [PubMed: 17335085]
- Hara K, Yasuhara T, Maki M, Matsukawa N, Masuda T, Yu SJ, Ali M, Yu G, Xu L, Kim SU, Hess DC, Borlongan CV. Neural progenitor NT2N cell lines from teratocarcinoma for transplantation therapy in stroke. *Prog Neurobiol.* 2008;318–334. [PubMed: 18514379]
- Kim JH, Auerbach JM, Rodriguez-Gomez JA, Velasco I, Gavin D, Lumelsky N, Lee SH, Nguyen J, Sanchez-Pernaute R, Bankiewicz K, McKay R. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature.* 2002; 418(6893):50–56. [PubMed: 12077607]
- King VR, McBride A, Priestley JV. Immunohistochemical expression of the alpha5 integrin subunit in the normal adult rat central nervous system. *J Neurocytol.* 2001;243–252. [PubMed: 11709630]
- Kondziolka D, Steinberg GK, Wechsler L, Meltzer CC, Elder E, Gebel J, Decesare S, Jovin T, Zafonte R, Lebowitz J, Flickinger JC, Tong D, Marks MP, Jamieson C, Luu D, Bell-Stephens T, Teraoka J. Neurotransplantation for patients with subcortical motor stroke: a phase 2 randomized trial. *J Neurosurg.* 2005;38–45. [PubMed: 16121971]
- Kondziolka D, Wechsler L. Stroke repair with cell transplantation: neuronal cells, neuroprogenitor cells, and stem cells. *Neurosurgical focus.* 2008;E13. [PubMed: 18341389]
- Lefcort F, Venstrom K, McDonald JA, Reichardt LF. Regulation of expression of fibronectin and its receptor, alpha 5 beta 1, during development and regeneration of peripheral nerve. *Development.* 1992;767–782. [PubMed: 1289065]
- Lindvall O, Kokaia Z. Stem cells for the treatment of neurological disorders. *Nature.* 2006; 441(7097): 1094–1096. [PubMed: 16810245]
- Lindvall O, Kokaia Z, Martinez-Serrano A. Stem cell therapy for human neurodegenerative disorders-how to make it work. *Nat Med.* 2004; 10(Suppl):S42–50. [PubMed: 15272269]
- Ma M, Basso DM, Walters P, Stokes BT, Jakeman LB. Behavioral and histological outcomes following graded spinal cord contusion injury in the C57Bl/6 mouse. *Exp Neurol.* 2001;239–254. [PubMed: 11358439]
- Marinkovich MP, Lunstrum GP, Burgeson RE. The anchoring filament protein kalinin is synthesized and secreted as a high molecular weight precursor. *J Biol Chem.* 1992; 267(25):17900–17906. [PubMed: 1517226]
- Mathews GA, Ffrench-Constant C. Embryonic fibronectins are up-regulated following peripheral nerve injury in rats. *J Neurobiol.* 1995;171–188. [PubMed: 7707041]

- McKeon RJ, Hoke A, Silver J. Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars. *Exp Neurol*. 1995; 136(1):32–43. [PubMed: 7589332]
- McKeon RJ, Schreiber RC, Rudge JS, Silver J. Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. *J Neurosci*. 1991:3398–3411. [PubMed: 1719160]
- Nelson PT, Kondziolka D, Wechsler L, Goldstein S, Gebel J, DeCesare S, Elder EM, Zhang PJ, Jacobs A, McGrogan M, Lee VM, Trojanowski JQ. Clonal human (hNT) neuron grafts for stroke therapy: neuropathology in a patient 27 months after implantation. *Am J Pathol*. 2002:1201–1206. [PubMed: 11943704]
- Paulus W, Baur I, Schuppan D, Roggendorf W. Characterization of integrin receptors in normal and neoplastic human brain. *Am J Pathol*. 1993:154–163. [PubMed: 8317546]
- Pesheva P, Juliano RL, Schachner M. Expression and localization of the fibronectin receptor in the mouse nervous system. *J Neurosci Res*. 1988:420–430. [PubMed: 2460633]
- Pleasure SJ, Page C, Lee VM. Pure, postmitotic, polarized human neurons derived from NTera 2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. *J Neurosci*. 1992; 12(5):1802–1815. [PubMed: 1578271]
- Previtali SC, Malaguti MC, Riva N, Scarlato M, Dacci P, Dina G, Triolo D, Porrello E, Lorenzetti I, Fazio R, Comi G, Bolino A, Quattrini A. The extracellular matrix affects axonal regeneration in peripheral neuropathies. *Neurology*. 2008:322–331. [PubMed: 18663178]
- Risling M, Fried K, Linda H, Carlstedt T, Cullheim S. Regrowth of motor axons following spinal cord lesions: distribution of laminin and collagen in the CNS scar tissue. *Brain Res Bull*. 1993; 30(3-4): 405–414. [PubMed: 8457890]
- Rogers SL, Letourneau PC, Palm SL, McCarthy J, Furcht LT. Neurite extension by peripheral and central nervous system neurons in response to substratum-bound fibronectin and laminin. *Dev Biol*. 1983:212–220. [PubMed: 6862106]
- Rogers SL, Letourneau PC, Peterson BA, Furcht LT, McCarthy JB. Selective interaction of peripheral and central nervous system cells with two distinct cell-binding domains of fibronectin. *J Cell Biol*. 1987:1435–1442. [PubMed: 2958484]
- Rogers SL, McCarthy JB, Palm SL, Furcht LT, Letourneau PC. Neuron-specific interactions with two neurite-promoting fragments of fibronectin. *J Neurosci*. 1985:369–378. [PubMed: 3973671]
- Sakai T, Johnson KJ, Murozono M, Sakai K, Magnuson MA, Wieloch T, Cronberg T, Isshiki A, Erickson HP, Fässler R. Plasma fibronectin supports neuronal survival and reduces brain injury following transient focal cerebral ischemia but is not essential for skin-wound healing and hemostasis. *Nat Med*. 2001:324–330. [PubMed: 11231631]
- Schaffner W, Weissmann C. A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal Biochem*. 1973; 56(2):502–514. [PubMed: 4128882]
- Sheppard AM, Brunstrom JE, Thornton TN, Gerfen RW, Broekelmann TJ, McDonald JA, Pearlman AL. Neuronal production of fibronectin in the cerebral cortex during migration and layer formation is unique to specific cortical domains. *Dev Biol*. 1995:504–518. [PubMed: 8612967]
- Sheppard AM, Hamilton SK, Pearlman AL. Changes in the distribution of extracellular matrix components accompany early morphogenetic events of mammalian cortical development. *J Neurosci*. 1991:3928–3942. [PubMed: 1720815]
- Siironen J, Sandberg M, Vuorinen V, Røyttä M. Expression of type I and III collagens and fibronectin after transection of rat sciatic nerve. Reinnervation compared with denervation. *Lab Invest*. 1992:80–87. [PubMed: 1625449]
- Silver J, Miller JH. Regeneration beyond the glial scar. *Nat Rev Neurosci*. 2004; 5(2):146–156. [PubMed: 14735117]
- Stipp CS, Hemler ME. Transmembrane-4-superfamily proteins CD151 and CD81 associate with $\alpha\beta 1$ integrin, and selectively contribute to $\alpha\beta 1$ -dependent neurite outgrowth. *J Cell Sci*. 2000; 113(Pt 11):1871–1882. [PubMed: 10806098]
- Tate CC, García AJ, LaPlaca MC. Plasma fibronectin is neuroprotective following traumatic brain injury. *Exp Neurol*. 2007:13–22. [PubMed: 17599836]
- Tom VJ, Doller CM, Malouf AT, Silver J. Astrocyte-associated fibronectin is critical for axonal regeneration in adult white matter. *J Neurosci*. 2004:9282–9290. [PubMed: 15496664]

- Trojanowski JQ, Kleppner SR, Hartley RS, Miyazono M, Fraser NW, Kesari S, Lee VM. Transfectable and transplantable postmitotic human neurons: a potential “platform” for gene therapy of nervous system diseases. *Exp Neurol.* 1997; 144(1):92–97. [PubMed: 9126157]
- Vogelezang MG, Scherer SS, Fawcett JW, French-Constant C. Regulation of fibronectin alternative splicing during peripheral nerve repair. *J Neurosci Res.* 1999:323–333. [PubMed: 10340741]
- Winterwood NE, Varzavand A, Meland MN, Ashman LK, Stipp CS. A critical role for tetraspanin CD151 in alpha3beta1 and alpha6beta4 integrin-dependent tumor cell functions on laminin-5. *Mol Biol Cell.* 2006; 17(6):2707–2721. [PubMed: 16571677]
- Yoshida N, Hishiyama S, Yamaguchi M, Hashiguchi M, Miyamoto Y, Kaminogawa S, Hisatsune T. Decrease in expression of alpha 5 beta 1 integrin during neuronal differentiation of cortical progenitor cells. *Exp Cell Res.* 2003:262–271. [PubMed: 12837282]

Fig. 1.

NT2N cells downregulate $\alpha 5$ integrin and respond poorly to fibronectin. NT2N cells were plated in serum-free medium on purified laminin-5 or fibronectin and cultured for 5 h (**A & B**) or 18 h (**C & D**). Laminin-5 promoted robust outgrowth, while little outgrowth was observed on fibronectin. Bar indicates 100 μ m. (**E**) Total neurite outgrowth on laminin-5 and fibronectin was quantified as described in Materials and Methods. Bar graph shows mean $\pm$ S.E.M. for n coverslips per condition; 4 fields per coverslip were quantified. Outgrowth on fibronectin was significantly reduced compared to laminin-5 at both time points (* $p < 0.0001$, unpaired t test). (**F**) NT2 precursor cells or purified NT2N neuronal cells were cell surface biotinylated and extracted with 1% Triton X-100. Either $\alpha 5$ or $\alpha 3$ integrin was immunoprecipitated and analyzed by SDS-PAGE followed by blotting with HRP-streptavidin. Lysate containing 100 μ g total protein was used for each immunoprecipitation. Arrows indicate the locations of α and β integrin subunits.

Fig. 2.

The Murine Stem Cell Virus (MSCV) promoter drives transgene expression throughout NT2N cell differentiation. NT2 precursor cells were transduced with an MSCV-EGFP retroviral vector. After selection, the stably transduced, uncloned population was differentiated into NT2N neurons. **(A)** After 1 week of retinoic acid-induced differentiation, patches of EGFP-expressing cells are widespread. **(B)** After 4 weeks, cells were re-plated (replate #1); clusters of EGFP-expressing NT2N cells sorted out from an underlying monolayer of non-neuronal cells. **(C)** After replate #2, clusters of highly purified NT2N cells were obtained, still expressing the EGFP transgene. No drug selection was used during the differentiation protocol. Bars indicate 50 μ m. The fields in A and B were photographed with a 20X objective, while the field in C was photographed with a 40X objective.

Fig. 3.

Sustained expression of $\alpha 5$ integrin in differentiated NT2N cells. NT2 cells harboring the MSCV- $\alpha 5$ integrin expression construct were subjected to the retinoic acid differentiation protocol. (A) After replate #2, clusters of neuronal cells extended neurites in Matrigel-coated flasks. Bar = 50 μ m. (B & C) DIC and fluorescence images of individual NT2N- $\alpha 5$ cell neurites stained for $\beta 1$ integrin. (D & E) DIC and fluorescence images of NT2N- $\alpha 5$ cell neurites stained for $\alpha 5$ integrin. Most neurites were positive for $\alpha 5$ integrin, but the asterisk indicates an unstained neurite in this field. (F & G) DIC and fluorescence images of neurites stained with isotype non-immune IgG. For B-G, cells were plated on Matrigel-coated glass coverslips and cultured for 5 h. Bar = 50 μ m.

Fig. 4.

Expression of $\alpha 5$ integrin does not alter the efficiency of NT2N cell neuronal differentiation. Parental NT2N cells (NT2N-P) and NT2N- $\alpha 5$ cells were stained for the neuronal microtubule-associated protein, MAP2, at 1 week, 2 weeks, and 5 weeks (replate 1) after the beginning of retinoic acid-induced neuronal differentiation. **(A-F)** NT2N-P cells. **(G-L)** NT2N- $\alpha 5$ cells. MAP2 staining is shown in red in A,C,E,G,I, & K. Hoechst staining is overlaid with MAP2 in the adjacent panels. Bar = 50 μ m. **(M & N)** The percentage of MAP2-positive cells was counted in 5 fields per cell type at the 1 week and 2 week time points. For the 2 week time point, the values are reported as % MAP2-associated cells because the dense clustering within the MAP2-positive zones made it difficult to score individual cells as positive or negative with absolute certainty. **(O)** At the 5 week time point, the lower cell density following replat 1 (see Materials & Methods) facilitated unambiguous scoring of MAP2-positive cells. The percentage of MAP2-positive cells in 10 fields per cell type was determined. No significant differences in the percentage of MAP2-positive cells were observed at any time point ($p = 0.59, 0.32$, and 0.10 at 1 week, 2 week, and replat 1 stages, respectively, unpaired t test).

Fig. 5. NT2N- $\alpha 5$ cells retain a CNS neuron-like immunophenotype. Purified parental NT2N cells (NT2N-P) and NT2N- $\alpha 5$ cells were plated on Matrigel-coated coverslips and cultured for 1 week before immunostaining for the neurofilament proteins, NF-66 and peripherin. Both cell types were NF-66 positive and peripherin-negative, as originally reported for differentiated NT2N cells (Pleasure et al. 1992). A,C,E, & G show the DIC images for the fields stained with the indicated antibodies in B,D,F, & H. Bar = 50 μ m.

Fig. 6.

Expression of $\alpha 5$ integrin in NT2N cells confers neurite outgrowth on fibronectin. NT2N- $\alpha 5$ and parental NT2N cells (NT2N-P) were plated in serum-free medium on fibronectin in the presence or absence of function-blocking anti- $\alpha 5$ integrin antibody, P1D6. After 5 h, the cultures were fixed for analysis. (**A & C**) NT2N- $\alpha 5$ cells displayed robust outgrowth on fibronectin, almost all of which could be blocked by P1D6. (**B & D**) NT2N-P cells displayed poor outgrowth on fibronectin, which was further diminished by treatment with P1D6. Bar = 100 μ m. (**E**) The bar graph shows the mean $\pm$ S.E.M. of the total neurite outgrowth in 8 fields per condition (4 fields on each of 2 coverslips). NT2N- $\alpha 5$ cell outgrowth on fibronectin was strongly enhanced compared to NT2N-P cells or NT2N- $\alpha 5$ cells treated with P1D6 (* $p < 0.001$, ANOVA with Bonferroni t test). In this set of experiments, P1D6 did produce a modest additional reduction in the already poor outgrowth of the NT2N-P cells (** $p < 0.05$, ANOVA with Bonferroni t test), suggesting a trace amount of $\alpha 5$ expression and function in the parental NT2N cell population.

Fig. 7.

NT2N- $\alpha 5$ cells retain robust neurite outgrowth on laminin isoforms. NT2N- $\alpha 5$ cells and parental NT2N cells (NT2N-P) were plated in serum-free medium on fibronectin (FN), laminin-1 (LN1) or laminin-5 (LN5). After 5 h of outgrowth, the cultures were fixed for analysis. Compared to NT2N-P cells, NT2N- $\alpha 5$ cells displayed dramatically enhanced outgrowth on fibronectin and similar outgrowth on the laminin isoforms. Bar = 100 μ m.

Fig. 8.

Quantification of neurite outgrowth. (A) NT2N-α5 (light gray) and parental NT2N cells (NT2N-P; dark gray) were plated in serum-free medium plated on the indicated substrates, in the presence or absence of specific function-blocking antibodies. Total outgrowth after 5 h was quantified as described in Materials and Methods. Bar graph shows mean $\pm$ S.E.M. for 8 fields per condition (4 fields on each of 2 coverslips). (B) An independent experiment, performed as in (A) except that outgrowth was for 8 h. Statistical comparisons (all by ANOVA with Bonferroni t test) are as follows: (a) NT2N-α5 displayed dramatically enhanced outgrowth on fibronectin compared to either NT2N-P cells, or NT2N-α5 cells treated with the JBS5 anti-α5 integrin function-blocking antibody ($p < 0.001$); (b) the poor outgrowth of NT2N-P cells on fibronectin was reduced slightly further by treatment with JBS5, but the difference was not statistically significant in this set of experiments; (c & d) NT2N-α5 cell neurite outgrowth on laminin-1 was ~45% reduced compared to NT2N-P cells ($p < 0.001$) and outgrowth by both cell types was almost completely abolished by the M13 anti-β1 integrin antibody ($p < 0.001$ vs. untreated cells); (e & f) both cell types performed identically well on laminin-5, and outgrowth on laminin-5 was virtually abolished by the A3-IIF5 anti-α3 integrin antibody ($p < 0.001$ vs. untreated cells); (g) NT2N-α5 cell neurite outgrowth was dramatically enhanced compared to NT2N-P cells or NT2N-α5 cells treated with P1D6 anti-α5 integrin function-blocking antibody ($p < 0.001$).