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Integrins and cAMP mediate netrin-induced growth cone collapse

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ABSTRACT

Growth cones integrate a remarkably complex concert of chemical cues to guide axons to their appropriate destinations. Recent work suggests that integrins contribute to axon guidance by interacting with a wide range of extracellular molecules including axon guidance molecules, by mechanisms that are not fully understood. Here, we describe an interaction between integrins and netrin-1 in growth cones that contributes to growth cone collapse. Our data show that netrin-1 causes growth cone collapse in a substratum-specific manner and is integrin-dependent. Netrin-1 causes collapse of cultured chick dorsal root ganglion (DRG) growth cones extending on high levels of laminin-1 (LN) but not growth cones extending on low levels of LN or on fibronectin. Blocking integrin function significantly decreases netrin-induced growth cone collapse on high LN. Netrin-1 and integrins interact on growth cones; netrin-1 causes integrin activation, a conformational shift to a high ligand-affinity state. Netrin-1 directly binds to integrin α3 and α6 peptides, further suggesting a netrin–integrin interaction. Interestingly, our data reveal that netrin-1 increases growth cone levels of cAMP in a substratum-specific manner and that netrin-induced growth cone collapse requires increased cAMP in combination with integrin activation. Manipulations that either decrease cAMP levels or integrin activation block netrin-induced collapse. These results imply a common mechanism for growth cone collapse and novel interactions between integrins, netrin-1 and cAMP that contribute to growth cone guidance.

1. Introduction

During development, millions of neurons project axons over long distances with remarkably precise trajectories to reach their appropriate targets and form synapses. Growth cones at the tips of these extending axons integrate a concert of cues from their surrounding milieu to guide axon extension. The extraordinary ability of growth cones to navigate to their

Abbreviations: DRG, dorsal root ganglion; LN, laminin-1; FN, fibronectin

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precise target is essential for proper formation of the nervous system (Chen and Cheng, 2009; Schmid and Maness, 2008) and for axonal regeneration following injury (Lemons and Condic, 2008). Thus, it is essential to understand the extracellular ligands and neuronal receptors that direct growth cone guidance. While growth cone ligand–receptor complexes are beginning to be unraveled (reviewed in: Halloran and Wolman, 2006; Huber et al., 2003), significant gaps in our knowledge of axon guidance still exist. This study helps address these gaps by revealing the role of integrins in netrin-mediated growth cone repulsive behaviors.

As embryonic chick dorsal root ganglion (DRG) neurons extend axons, they encounter numerous extracellular molecules, including laminin-1 (LN), fibronectin (FN) (Guan et al., 2003), and netrin-1 (Guan and Condic, 2003; Masuda et al., 2008; Watanabe et al., 2006). Netrin-1 can serve as either a chemoattractant or a chemorepellent, depending on several variables, including specific netrin receptors (Bradford et al., 2009; Huber et al., 2003; Ly et al., 2008), levels of cyclic nucleotide signaling (Hopker et al., 1999; Ming et al., 1997; Nicol et al., 2011; Wu et al., 2006) and the presence of extracellular matrix molecules (Hopker et al., 1999; Ratcliffe et al., 2008). In this study we demonstrate that integrins are a critical component of how DRG neurons respond to netrin-1.

1.1. *Integrin receptors play an important role in axon guidance*

Integrins are a family of receptors well known for their ability to bind to various extracellular matrix proteins, including LN and FN. In addition to their well-documented roles in motility, cell cycle progression, cell survival and cell differentiation, recent evidence suggests that these receptors also play a role in growth cone steering by modulating the influence of extracellular guidance molecules. For example, integrin receptors influence growth cone behaviors mediated by semaphorin (Nakamoto et al., 2004; Pasterkamp et al., 2003), slit (Stevens and Jacobs, 2002), ephrins (Bourgin et al., 2007; Richter et al., 2007), Ig superfamily cell adhesion molecules, such as NCAM and LI (Schmid and Maness, 2008) and myelin-associated glycoprotein (Goh et al., 2008). In addition, integrins influence the growth inhibitory effects of specific proteoglycans on growth cones (Lemons et al., 2005; Tan et al., 2011). Integrins have not been previously shown to influence netrin-mediated growth cone behavior, however, they have been documented to play a role in netrin-induced epithelial cell adhesion (Yebra et al., 2003) and GABAergic neuronal cell body migration (Stanco et al., 2009). Our studies are the first to show integrins are involved in netrin-mediated growth cone behaviors.

1.2. *cAMP impacts growth cone behaviors*

Intracellular cAMP levels affect axon regeneration (Pearse et al., 2004), growth cone turning (Lohof et al., 1992) and response to guidance molecules, including netrin-1, semaphorins, slits and ephrins (Murray et al., 2009; Nicol et al., 2011; Song et al., 1998; Terman and Kolodkin, 2004; Wu et al., 2006). Growth cone speed can be increased by either integrin activation or elevation of cAMP signaling, yet when these two factors are simultaneously increased, growth cones collapse (Lemons and Condic, 2006). Integrins can become activated directly by binding to a ligand or indirectly through inside-out mechanisms (Calderwood, 2004; Takagi et al., 2002). Here we test the role of cAMP signaling in netrin-mediated integrin activation and growth cone collapse.

1.3. *Summary*

The goal of this study is to determine whether integrins, well-known to bind to extracellular matrix molecules, play a critical role in netrin-induced growth cone collapse of embryonic chick DRG neurons. Netrin-1 application causes transient, substrata specific growth cone collapse. Interestingly, select function-blocking integrin antibodies and competitive integrin peptides significantly decrease netrin-mediated growth cone collapse. Netrin and integrins interact on growth cones; netrin-1 application elevates integrin activation. Our results also show that netrin-1 induces growth cone collapse on the one substratum tested (high concentration of LN) where netrin-1 both activates integrins and elevates intracellular cAMP levels. When either integrin activation or cAMP activity is decreased, collapse does not occur, demonstrating the importance of these combined factors for growth cone repulsion (Lemons and Condic, 2006).

2. Results

2.1. *Netrin-1 causes growth cone collapse in a substratum-specific manner*

Timelapse analysis was performed to test the effects of widespread netrin-1 application on cultured chick sensory neurons grown on high concentrations of LN, low concentrations of LN, and also FN. To determine if global netrin-1 application induces collapse of chick embryonic sensory growth cones on high concentrations of LN, similar to previous reports of focal netrin-1 application causing repulsive *Xenopus* retinal ganglion growth cone turning (Hopker et al., 1999), dissociated chick DRG neurons were placed on high concentrations of LN and recombinant chick netrin-1 was applied globally. Growth cones were observed for 30 min prior to netrin-1 addition and for 30 min afterwards. Timelapse analysis revealed that netrin-1 induced collapse of growth cones that was often associated with significant retraction of the axon (Fig. 1A). Consistent with prior studies (Piper et al., 2005), netrin-1 induced collapse was rapid and transient, with most growth cones collapsing within 12 min and recovering within 30 min following collapse (Fig. 1B).

Previous work has indicated that neurons cultured on high LN but not low LN are repelled by netrin-1 (Hopker et al., 1999; Ratcliffe et al., 2008). We tested the response of chick DRG neurons to netrin-1 on different concentrations of LN and also on FN. Netrin-1 induced robust collapse of growth cones extending on high levels of LN, but not on low levels of LN nor on FN substrata (Fig. 1C). This result is similar to the effect of netrin-1 on *Xenopus* growth cone steering; netrin repels growth cones extending on high LN, while attracting growth

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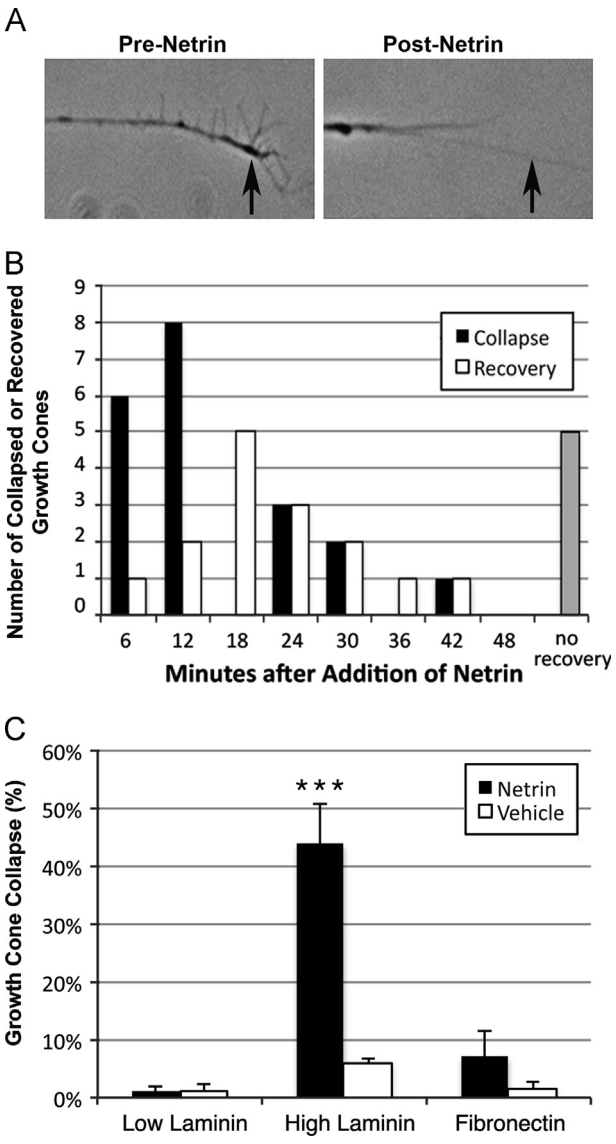

Fig. 1 – Netrin-1 induces transient growth cone collapse in a substratum-specific manner. (A) Photomicrographs of an embryonic chick DRG growth cone cultured on high LN shown before treatment and after a 15 min exposure to netrin-1. (B) Netrin-1 causes rapid and transient collapse of neurons plated on high LN. Most growth cones collapse in the first 12 min after exposure to netrin-1. Recovery peaks at approximately 20 min after exposure to netrin-1, with approximately 75% of collapsed growth cones recovering within 1 h. Twenty collapsed growth cones from a single experiment are shown, with similar results having been obtained in at least four independent experiments. (C) Netrin-induced growth cone collapse of embryonic chick DRG neurons is observed in growth cones extending on high levels of LN but not on FN or low levels of LN. Collapse for neurons during the pre-treatment was <1%. Vehicle-treated neurons did not show significant growth cone collapse. High LN/Netrin condition is different from all other conditions (*$p<0.001$; ANOVA), with all other conditions being statistically identical to each other. At least three independent experiments and at least 50 growth cones were analyzed for each condition. Error bars represent standard error of the mean.**

cones extending on FN. On low LN, *Xenopus* growth cones are neither attracted nor repelled by netrin-1 (Hopker et al., 1999).

2.2. *Specific integrin subunits are necessary for netrin-mediated growth cone collapse*

RT-PCR was done to confirm the presence of both integrin and netrin receptors in embryonic DRG neurons. Consistent with previous results (Guan and Condic, 2003; Guan et al., 2003; Hall et al., 1990; Tomaselli et al., 1993), DRG neurons express LN receptors containing integrin α3 and α6 subunits, as well as netrin-1 and netrin receptors containing neogeninin and Unc-5HA-D (Fig. 2A). The netrin receptor DCC is not present in the chick genome (Phan et al., 2011). Our data indicate that both LN-binding integrin subunits and netrin-1 receptors are expressed by DRG neurons and could therefore contribute to netrin-induced collapse.

The ability of netrin-1 to cause collapse on high LN but not on FN or low LN suggests the possibility that LN-binding integrins could be involved in collapse. Surface levels of integrins on these neurons have been previously shown to be regulated by the levels of integrin-ligands the growth cone encounters (Condic and Letourneau, 1997) as well as by non-integrin binding components of the extracellular environment (Lemons et al., 2005). For example, neurons extending on low levels of LN express five-fold higher levels of laminin receptor at the cell surface, compared to neurons extending on high levels of LN (Condic and Letourneau, 1997), yet a much greater proportion of these receptors are inactivated (Lemons and Condic, 2006).

To determine if integrins are involved in netrin-mediated collapse, specific integrin function-blocking antibodies were applied to cultured DRG neurons 5 min prior to netrin-1 application and the response to netrin was observed using timelapse imaging. Function-blocking anti-integrin antibodies decrease neuronal adhesion and reduce the rate of neurite extension on LN for both embryonic (Plantman et al., 2008) and regenerating adult sensory neurons (Tomaselli et al., 1993), suggesting that reducing integrin-mediated substratum adhesion should enhance netrin-induced collapse. Surprisingly, application of function-blocking antibodies against either integrin α3 or α6 prior to netrin-1 application significantly decreased netrin-mediated growth cone collapse compared to neurons that received only netrin-1 treatment or only integrin antibody treatment (Fig. 2B). In contrast, function-blocking antibodies against integrin α4, a subunit that does not bind to LN but is expressed by these neurons (Guan et al., 2003), did not have any effect on netrin-induced growth cone collapse (Fig. 2B). These results indicate that the LN-binding integrin subunits, α3 and α6, are necessary for netrin-mediated growth cone collapse.

To further test if α3 and α6 containing integrins are involved in netrin-mediated growth cone collapse, integrin peptides were used as competitive inhibitors. These 17 amino acid long peptide sequences are within the beta propellar region of the integrin α-subunit that binds to the urokinase receptor (uPAR; Wei et al., 2001). Interestingly, this region is distinct from the known LN-binding domain of these integrins. When integrin peptides were applied 5 min prior to netrin-1, growth cone collapse was significantly decreased compared to growth cones

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treated only with netrin-1 (Fig. 2C). Scrambled versions of the peptides had no effect on growth cone collapse. These findings suggest that α3 and α6 integrin subunits play a key role in netrin-mediated growth cone collapse of chick sensory neurons.

2.3. *Netrin-1 activates integrins*

The ability of LN-binding integrins to mediate netrin-induced growth cone collapse suggests that netrin and integrins interact, either directly or indirectly. To further investigate the nature of this interaction, we examined whether netrin-1 could activate integrins on sensory growth cones. Integrin activation is a conformational change in the integrin receptor that leads to increased ligand affinity (Calderwood, 2004; Takagi et al., 2002). Integrin activation was assessed using immunocytochemistry with TASC, a well-documented antibody that specifically labels activated β1 integrin subunits (Cruz et al., 1997; Lemons and Condic, 2006; Stanco et al., 2009). The majority of integrins on chick DRG neurons contain the β1 subunit, so TASC immunoreactivity provides an accurate assessment of growth cone integrin activation status.

For growth cones extending on both high and low levels of LN, the fluorescence intensity of activated β1 integrin staining was significantly higher following application of netrin-1 (Fig. 3A and B). This demonstrates that integrin activation alone is not sufficient to cause growth cone collapse (Figs. 1C and 3A and B). While increased integrin activation has been demonstrated in response to netrin-1 in forebrain extracts (Stanco et al., 2009), this study is the first to show a netrin–integrin interaction in neuronal growth cones.

To determine if netrin-induced integrin activation is necessary for collapse, we tested if the function-blocking integrin antibodies and competitive integrin peptides that decreased netrin-induced growth cone collapse (Fig. 2B and C) also diminished netrin-induced integrin activation. Quantitative immunocytochemistry for integrin activation was performed on neurons treated with integrin function-blocking antibodies or peptides prior to netrin-1 application. Growth cones pre-treated with integrin α3 and α6 function-blocking antibodies or with competitive peptides had a significant decrease in integrin activation compared to growth cones treated with netrin-1 alone (Fig. 3C). These results suggest that netrin-induced integrin activation is a required component of netrin-induced collapse.

2.4. *Netrin-1 binds to integrin peptides*

The ability of netrin-1 to activate integrins suggests that netrin-1 either binds to integrins and/or activates them indirectly through intracellular signaling pathways. Sensory neurons

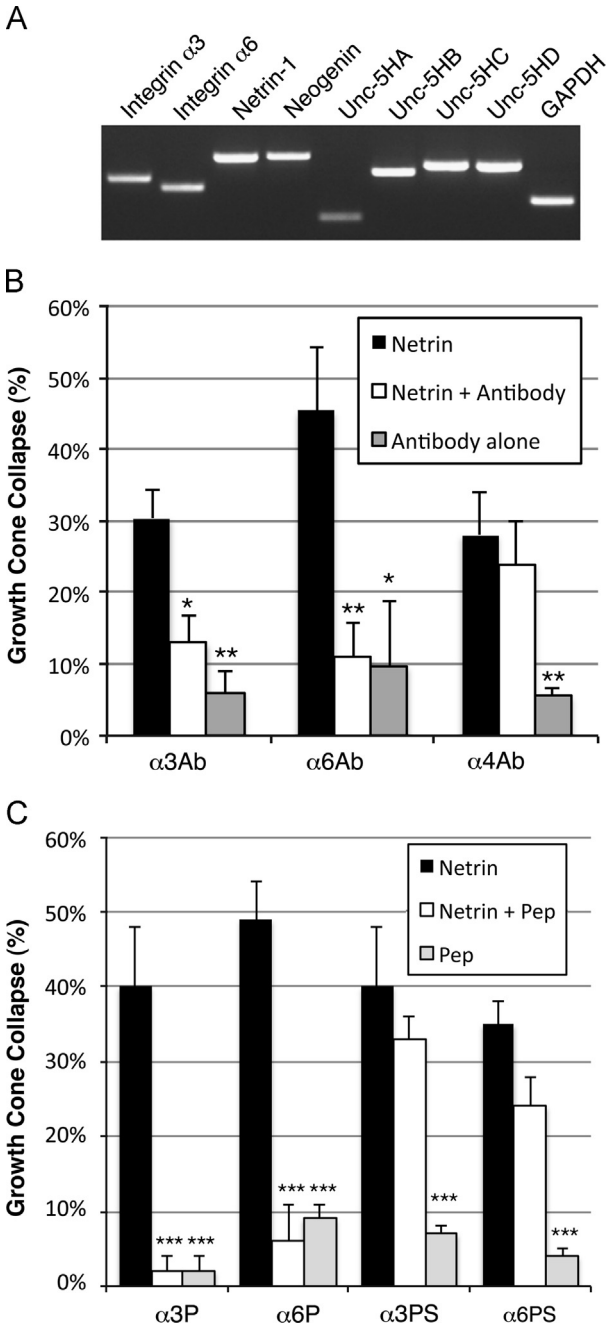

Fig. 2 – LN-binding integrins α3 and α6 mediate netrin-induced growth cone collapse on laminin-1. (A) RT-PCR reveals that embryonic chick DRGs express transcripts for integrin α3, α6 in addition to netrin-1 and netrin receptors; neogenin, and Unc-5HA-D integrin α4 is also expressed at this time (Guan and Condic, 2003). (B) Pre-treating LN-plated neurons with function-blocking antibodies against integrin α3 and α6 prior to netrin-1 application eliminates netrin-mediated collapse. Integrin function-blocking antibodies against integrin α4 do not effect collapse. * $p<0.05$, ** $p<0.01$ compared to netrin-1 treatment; ANOVA, Tukey post hoc test. At least three independent experiments and at least 60 growth cones were analyzed for each condition. (C) Pre-treating laminin-plated neurons with integrin α3 or α6 peptides significantly decreases netrin-mediated collapse on laminin-1. Scrambled integrin α3 or α6 peptides do not effect collapse. *** $p<0.001$ compared to netrin-1 treatment; ANOVA, Tukey post hoc test. At least three independent experiments and at least 50 growth cones were analyzed for each condition. Ab, function-blocking antibody; P, peptide; PS, scrambled peptide; α3, integrin α3; α6, integrin α6. Error bars represent standard error of the mean.

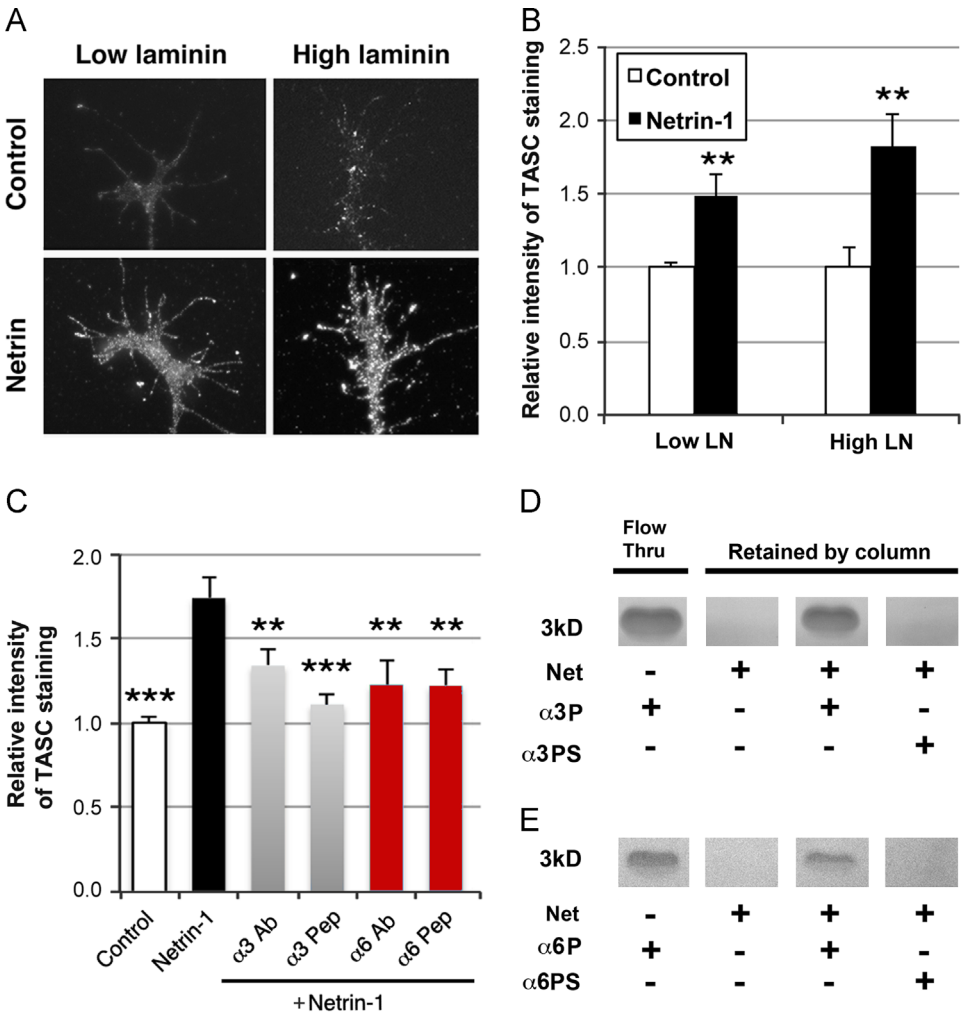

Fig. 3 – Netrin-1 activates integrins on chick DRG growth cones and binds to integrin peptides. (A) Photomicrographs of growth cones plated on either low or high concentrations of LN and immunostained for activated β1 containing integrins (labeled by TASC antibodies) after either vehicle or netrin-1 treatment. (B) Intensity of activated β1 integrin staining (shown relative to vehicle control on same substratum) is significantly increased after netrin-1 treatment (black bars) compared to vehicle (white bars) for neurons plated on either low or high concentrations of LN. Activated integrins are immunolabeled with TASC antibodies. Results from three independent experiments of at least 10 growth cones each are shown. $^{**}p<0.01$ compared to control; ANOVA, error bars represent standard error of the mean. (C) Application of integrin function-blocking antibodies or peptides derived from integrins α3 and α6 5 min prior to netrin application blocked netrin-induced increase in integrin activation on high LN. $^{**}p<0.01$, $^{***}p<0.001$ compared to netrin-1 treatment; ANOVA. At least three independent experiments, with a total of 15–55 growth cones per condition were analyzed. Ab, function-blocking antibody; Pep, peptide, error bars represent standard error of the mean. (D) Netrin-1 binds to chick integrin α3 peptide. Netrin-1 (Net) was incubated with either integrin α3 peptide (α3P) or integrin α3 scrambled peptide (α3PS) and subjected to 30 kD size exclusion columns. In the first lane, vehicle was incubated with α3P and the flow through from the size exclusion column reveals a 3 kD integrin peptide. The peptides retained by the column are shown in all subsequent lanes. (E) Netrin-1 binds to chick integrin α6 peptide (α6P). Experiments for α6 peptide and scrambled α6 peptide (α6PS) were performed as described for α3.

express both netrin-1 and integrins (Fig. 2A). To test whether there is a direct interaction between integrins and netrin, we used size exclusion columns and the same integrin α3 and α6 peptides that blocked netrin-induced growth cone collapse (Fig. 2C). The 17 amino acid integrin peptides run as approximately 3 kD bands (Fig. 3D and E). Recombinant netrin-1 was incubated with either: (1) a specific chick integrin peptide, (2) a scrambled integrin peptide or (3) vehicle. These solutions were separately subjected to a column with a 30 kD size exclusion, and both the flow through and the material retained on the column were analyzed by gel electrophoresis under non-reducing conditions. Any integrin peptide that did not bind to netrin-1 was not retained by the 30 kD size exclusion column, and was detected only in the flow-through (Fig. 3D and E; "Flow Thru").

When netrin-1 was incubated with integrin α3 peptide, the small integrin peptide band was retained by the column, indicating that the integrin α3 peptide binds to recombinant netrin-1 (Fig. 3D). In contrast, when netrin-1 was incubated with the scrambled integrin α3 peptide, the scrambled

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integrin peptide was not retained by the column (Fig. 3D). These results indicate that netrin-1 can bind directly to integrin α3 peptide. Similar results were seen when netrin-1 was incubated with integrin α6 peptide (Fig. 3E). Interestingly, these peptides correspond to a region within integrins that binds to uPAR and not to LN (Zhang et al., 2003). These results suggest a direct netrin–integrin interaction could contribute to netrin-mediated collapse.

2.5. *Netrin-1 elevates intracellular cAMP in a substratum-specific and integrin-dependent manner*

Previous studies have shown that integrin activation enhances neurite outgrowth (Ivins et al., 2000; Lemons and Condic, 2006; Tan et al., 2011). In contrast, our current results indicate that netrin-mediated integrin activation (Fig. 3A) is associated with growth cone collapse on high concentrations of LN, but not on low levels of LN. Previous work from our lab has shown that integrin activation in combination with elevated intracellular cAMP levels leads to collapse by inducing rapid removal of integrin from the cell surface (Lemons and Condic, 2006). To determine the mechanism by which netrin-1 induces growth cone collapse, we examined the effect of netrin-1 on intracellular cAMP levels.

Data from whole-cell cAMP enzyme immunoassays show that netrin-1 application led to an increase in cAMP levels within neurons plated on high LN but not on low LN or FN (Fig. 4A). This result indicates that netrin-1 affects intracellular cAMP levels in a substratum-specific manner that is correlated with growth cone collapse. Collapse was observed on the one condition where both integrin activation and cAMP levels were elevated (e.g. netrin-1 on high LN).

To determine whether netrin-1 elevates intracellular cAMP levels *specifically* in growth cones, we performed quantitative cAMP immunocytochemistry on growth cones treated with netrin-1 or vehicle (Fig. 4B). We focused our investigation on high LN plated neurons because this is the condition where netrin-1 caused growth cone collapse (Fig. 1D). In agreement with the results from whole-cell cAMP enzyme immunoassays, growth cone cAMP levels also increased by netrin-1 application (Fig. 4B and C.)

To determine if integrins are involved in netrin-induced elevation of intracellular cAMP levels, neurons plated on high LN were pre-treated with integrin function-blocking antibodies (Fig. 2B) prior to netrin-1. Quantitative immunofluorescent staining of cAMP in growth cones revealed that function-blocking antibodies against integrin α3 and α6, but not β1, significantly blocked netrin-induced increase in cAMP (Fig. 4C). These results suggest that LN-binding integrin subunits involved in netrin-induced collapse are also either directly or indirectly involved in netrin-mediated increase in cAMP.

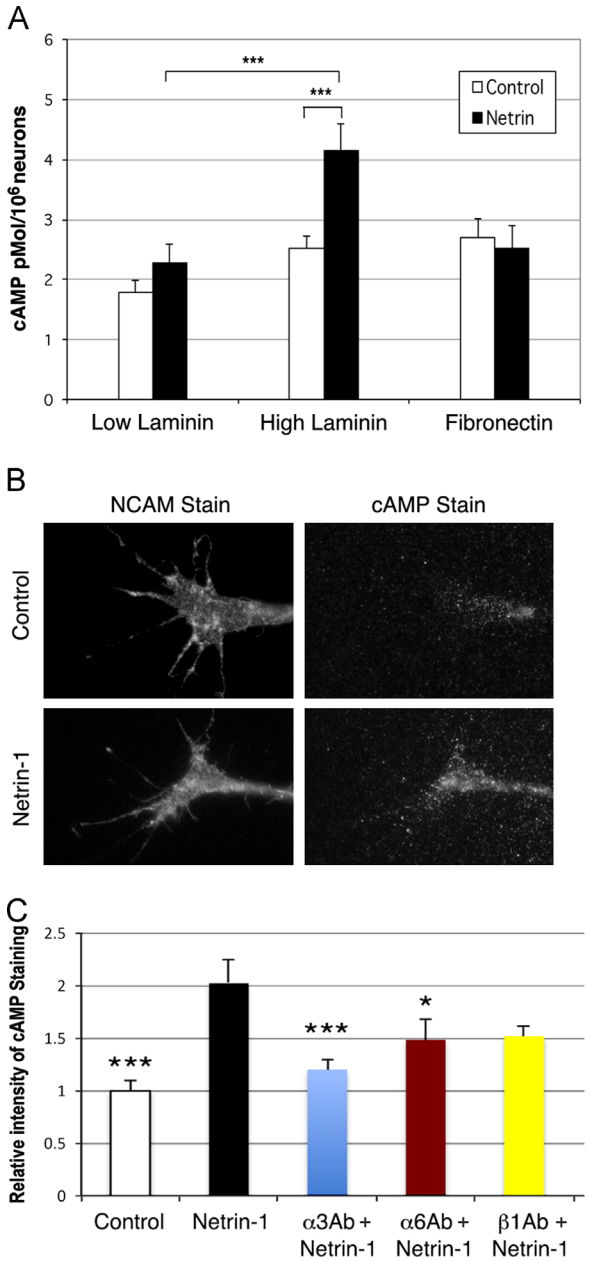

Fig. 4 – Netrin-1 causes a substratum-specific increase in intracellular cAMP levels within cultured chick DRG neurons. (A) Results from a cAMP immunoassay reveal that netrin-1 application causes an increase in cAMP levels within neurons plated on high concentrations of LN but not on FN or low concentrations of LN. Results are from five independent experiments. ***$p<0.001$; ANOVA. (B) Immunocytochemistry confirms that cAMP is elevated within netrin-treated growth cones plated on high LN compared to vehicle-treated growth cones on the same substrate. NCAM is used to identify the entire growth cone. (C) Quantitative immnocytochemistry of growth cones stained as in (B) reveals that netrin-mediated increase of intracellular cAMP levels is blocked by applying function-blocking antibodies against integrin α3 and α6 prior to netrin-1 treatment. *$p<0.05$, ***$p<0.001$, compared to netrin-1 treatment; ANOVA. The trend towards decreased cAMP levels with the function-blocking β1 antibody was not statistically significant by ANOVA (although it was significant at $p<0.05$ by *t*-test). At least four independent experiments with a total of 15–54 growth cones per condition were analyzed. Ab, antibody application 5 min prior to netrin-1 application. Error bars represent standard error of the mean.

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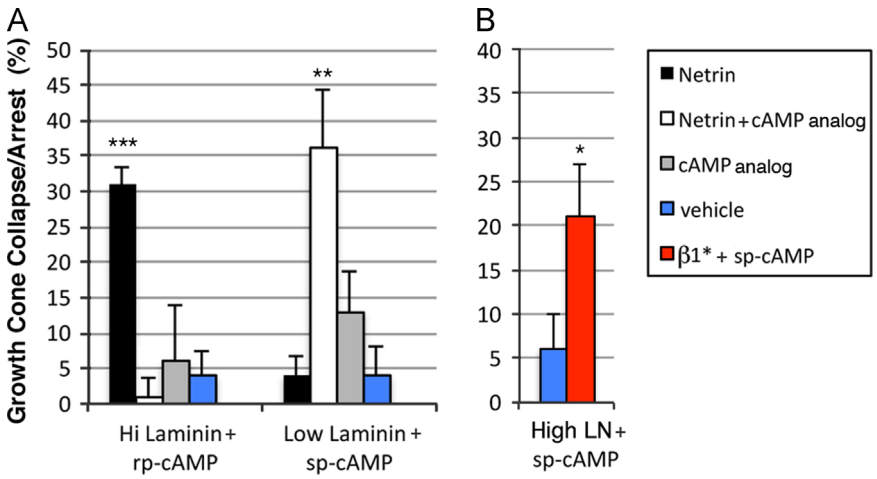

Fig. 5 – Increased cAMP combined with integrin activation is both necessary and sufficient for growth cone arrest and collapse. (A) Netrin-induced growth cone collapse and arrest on high LN can be blocked by application of rp-cAMP prior to netrin-1 application, showing elevated cAMP is required. In contrast, growth cones extending on a low LN can be induced to collapse or arrest in response to netrin-1 if cAMP signaling is concurrently enhanced by co-application with sp-cAMP, showing that elevated cAMP signaling is sufficient for netrin-induced collapse in these conditions. For all conditions, at least three independent experiments and at least 30 growth cones were analyzed. $^{***}p<0.001$, $^{**}p<0.01$ compared to all other treatments on the same substrata; ANOVA. (B) Integrin activation in combination with high cAMP signaling is sufficient to induce growth cone arrest and collapse, independent of netrin-1. Integrin activation is elevated via integrin activating antibodies and in combination with elevating cAMP activity with sp-cAMP application. Growth cone arrest and collapse was determined by timelapse analysis. $^{*}p<0.05$; ANOVA. Error bars represent standard error of the mean.

2.6. *Elevation of cAMP signaling contributes to netrin-mediated growth cone collapse*

To determine if netrin-induced elevation of intracellular cAMP contributes to collapse on high LN, neurons were pre-treated with the cAMP analog Rp-cAMP to decrease cAMP activity before netrin-1 application. When cAMP activity was experimentally suppressed, netrin-1 did not induce growth cone collapse (Fig. 5A). This result indicates that elevated cAMP is necessary for netrin-induced collapse on high LN.

Netrin-1 increases integrin activation in growth cones extending on low LN (Fig. 3A and B), but on this substrata, netrin-1 does not increase cAMP levels (Fig. 4A and B) nor induce collapse (Fig. 1D). To test if netrin-1 induced integrin activation combined with increased cAMP levels is sufficient to induce growth cone collapse, we treated low LN plated neurons with both netrin-1 and sp-cAMP, a specific activator of cAMP-dependent protein kinases. Netrin-1 induced significant growth cone collapse and arrest on low LN substrata only when cAMP signaling was experimentally increased (Fig. 5A), suggesting that netrin-induced integrin activation and elevation of cAMP signaling are sufficient for collapse.

To confirm that integrin activation combined with the elevation of cAMP signaling is sufficient to cause collapse in the absence of any other down-stream effects netrin-1 may have on the growth cone, we treated neurons plated on high LN with an integrin activating antibody (Vijayakumar et al., 2008) in combination with sp-cAMP to increase cAMP signaling. When integrin activation and cAMP activity were simultaneously enhanced, growth cone collapse occurred (Fig. 5B). Together, these results suggest that integrin activation and elevated intracellular cAMP levels are both necessary and sufficient for netrin-induced growth cone collapse of chick sensory neurons.

3. Discussion

In this study, we provide evidence that integrin activation is necessary for netrin-induced growth cone collapse in cultured chick sensory neurons. Netrin-induced collapse of chick sensory neurons is observed only in cells extending on high LN (Fig. 1), where netrin-1 increases both integrin activation and cAMP within growth cones. Blocking increases in integrin activation (Fig. 2) or blocking increases in cAMP (Fig. 5A) significantly decreases netrin-induced growth cone collapse. Conversely, elevated integrin activation in combination with elevated cAMP signaling is sufficient for collapse (Fig. 5B; Lemons and Condic, 2006), even on low LN substrata (Figs. 5A and 6).

3.1. *Integrins and netrin-1 interact in growth cones*

Our data show that netrin-1 activates integrins in growth cones (Fig. 3A and B) and can bind to integrin α3 and α6 peptides (Fig. 3D and E). Previous studies have shown that select integrins can associate with netrin-1 and are involved in netrin-mediated epithelial cell attachment (Yebra et al., 2003) as well as GABAergic neuronal migration (Stanco et al., 2009), yet the data shown here are the first to document a netrin–integrin interaction in growth cones that impacts growth cone behavior. Together, these results suggest that LN-binding integrins can function as netrin receptors in a range of cell types. Our data also indicate that although netrin-1 is a member of the laminin superfamily (Rajasekharan and Kennedy, 2009), it may interact with integrin

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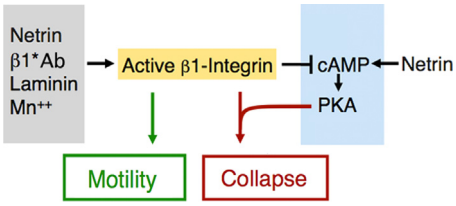

Fig. 6 – A working model for growth cone collapse induced by simultaneous integrin activation and elevated cAMP. Integrins can be activated by extracellular molecules (gray box) including netrin-1 (Fig. 3), activating β1 integrin antibodies (β1*Ab) (Vijayakumar et al., 2008), laminin and Mn^{++} (Lemons and Condic, 2006). This promotes motility (green arrow and green box). Normally, neuronal cAMP levels (blue box) are suppressed by integrin activation (Fleming et al., 2004; Lemons and Condic, 2006). However, netrin-1 elevates intracellular cAMP (Fig. 5), possibly via canonical netrin receptors. Consequently, netrin-1 leads to simultaneous integrin activation and elevated cAMP (red arrow). Previous work has shown that integrin activation in combination with pharmacologic elevation of cAMP signaling by sp-cAMP (an activator of PKA-dependent pathways) results in rapid endocytosis of integrins and growth cone collapse (red box; Lemons and Condic, 2006).

peptide sequences corresponding to regions that are distinct from the documented LN-binding domain (Wei et al., 2001).

Our data do not exclude possible indirect roles of integrins in netrin-induced collapse; e.g. through netrin–neogenin, netrin–Unc5 or netrin–DSCAM mediated signaling (the DCC gene is not present in chick.) Future experiments will determine if canonical netrin receptors expressed on chick neurons play a role in netrin-induced integrin activation and collapse (Fig. 6).

3.2. *Netrin-1 activates integrins and elevates cAMP independently*

Netrin's ability to attract or repel growth cones is determined by several factors, including intracellular cAMP signaling (Hopker et al., 1999; Ming et al., 1997). Using two different methods, we show that netrin-1 increases cAMP levels within neurons plated on high LN, but not low LN (Fig. 4). Previous studies have suggested that netrin-1 elevates cAMP levels in *Xenopus* retinal growth cones, and that this increase is suppressed by LN (Hopker et al., 1999). In contrast, our results show that netrin-1 elevates cAMP only on high LN (Fig. 4A). While the signaling cascade linking netrin-1 to cAMP is currently not clear, it should be noted that DCC is not present in the chick genome (Phan et al., 2011), suggesting that in avian neurons, netrin's effect on cAMP could be distinct from non-avian neurons due to the absence of this netrin receptor or to other species-specific differences. Interestingly studies on *Xenopus* spinal neurons cultured in the absence of LN show that netrin-1 induces DCC-dependent cAMP transients in filopodia with a slower and more sustained elevation in the growth cone (Nicol et al., 2011), again suggesting a link between netrin-1 and enhanced cAMP signaling.

Using a pharmacological approach, we have previously demonstrated that a combination of elevated cAMP and integrin activation induces growth cone collapse in chick sensory neurons (Lemons and Condic, 2006). Similarly in the current study, growth cones extending on high LN, the one condition where netrin-1 causes an increase in both integrin activation and cAMP, collapse in response to netrin-1 (Figs. 1, 3, and 4). However, on low LN, netrin-1 causes integrin activation but does not increase cAMP levels. In this condition, netrin does not cause collapse (Figs. 1, 3 and 4). The observation that netrin-1 activates integrins on both concentrations of LN (Fig. 3) while elevating cAMP only on high LN (Fig. 4) suggests that netrin-1 regulates cAMP at least in part through an integrin-independent pathway that could include canonical netrin receptors (Fig. 6).

In these studies, the cAMP analog used (sp-cAMP) activates PKA. However, cAMP influences axon growth via PKA dependent and independent pathways. Furthermore, cAMP has been demonstrated to influence transcription of genes such as arginase I that are critical for axon outgrowth (reviewed in: Hannila and Filbin, 2008), indicating that cAMP has a complex effect on growth cone migration and regeneration.

Previous studies show that cAMP can promote axonal regrowth in vivo following spinal cord injury in adult rodents (Pearse et al., 2004.) Both netrin-1 (Manitt et al., 2006) and laminin (Risling et al., 1993) are present in the adult injured spinal cord, however, the levels of these molecules are not clear. Therefore, it is challenging to directly compare these results to our culture experiments. However, previous studies suggest that the extracellular environment (high or low LN) can influence cAMP levels, integrin activation levels and growth cone response to netrin-1 (Lemons and Condic, 2006). While elevating cAMP signaling in vivo increases growth under some conditions (Pearse, et al., 2004; Lemons and Condic, 2006), the same treatment may not have an effect if injured neurons are surrounded by high LN, or could even decrease axon regrowth in a microenvironment containing both LN and netrin-1. The complex environment of the injured spinal cord may result in a wide range of axon responses to cAMP treatment.

3.3. *A substratum-specific balance between integrin function and cAMP maintains growth cone motility*

Our previous results have shown that both sensory neurons (Condic and Letourneau, 1997; Lemons and Condic, 2006) and neural crest cells (from which sensory neurons are developmentally derived; Strachan and Condic, 2004) maintain a very specific balance of integrin protein at the cell surface, depending on the concentration of integrin ligand they encounter. Moreover, the extent of integrin activation in growth cones is also modulated in a substratum-dependent manner. This regulation of integrins promotes consistent growth cone motility across a 10-fold change in ligand concentrations (Lemons and Condic, 2006) and is sufficient to enable growth cone migration under weakly growth promoting and inhibitory conditions (Condic, 2001; Condic et al., 1999; Lemons et al., 2005).

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Previous results (Lemons and Condic, 2006) indicate that chick sensory neurons maintain a substratum-specific balance between integrin expression, integrin activation and cAMP. Compared to neurons on high levels of LN, cells extending on low LN have roughly four-fold higher levels of LN-binding integrin present at the cell surface, although a much higher proportion of the receptor pool is inactivated. Neurons cultured under these two conditions also have substratum-specific levels of cAMP, with higher levels seen on high LN substrata (Lemons and Condic, 2006). Importantly, this balance between integrin expression, integrin activation and intracellular cAMP levels is required for growth cone motility. When both integrin activation and cAMP levels are experimentally elevated, either by application of netrin-1 (Fig. 1C) or by pharmacologic methods (Lemons and Condic, 2006) (Fig. 5), growth cone collapse occurs.

3.4. *Integrin activation and integrin uptake in growth cone guidance*

It is somewhat counterintuitive that netrin-induced integrin activation (a manipulation that should increase substratum adhesion) results in collapse, whereas reducing integrin-based adhesion using function-blocking antibodies against LN-binding integrin subunits (Fig. 2B) prevents collapse. However, our previous results show integrins are rapidly removed from the cell surface and growth cones collapse when integrins are activated while cAMP signaling is high (Lemons and Condic, 2006), the same conditions we observe following netrin-1 application. Uptake of activated integrin has been seen in other cell types (Chao and Kunz, 2009) and is believed to contribute to cell motility by allowing the release of the trailing edge of the cell from the substratum and establishment of new adhesions at the leading edge. In neural crest, internalized integrins are recycled to the cell surface, and pharmacologically disrupting endocytotic trafficking significantly affects cell motility (Strachan and Condic, 2004).

Uptake of activated integrins would temporarily reduce substratum adhesion, resulting in growth cone collapse until integrin levels can be restored at the cell surface, either by insertion of newly synthesized protein into the plasma membrane or by recycling of receptors through the endocytotic pathway. In non-neuronal cell types, newly synthesized integrins are inserted at the cell surface in an inactive, low-energy conformation, thereby allowing the level of integrin function to be set by the extracellular environment and internal cell signaling pathways (Tiwari et al., 2011). Whether integrins are recycled in growth cones is not currently known, but either integrin recycling or restoration of integrin surface levels by newly synthesized protein would be consistent with the transient nature of growth cone collapse that we (Fig. 1B) and others (Piper et al., 2005) have observed in response to chemorepellants such as netrin-1. Intrestingly, in *Xenopus* retinal neurons, growth cone collapse in response to netrin-1 or sema3A, involve endocytosis, a process that is critically regulated by integrins (Wickstrom and Fassler, 2011), and growth cone recovery following collapse requires protein synthesis (Piper et al., 2005).

The ability of growth-repulsive guidance cues such as netrin-1 to disrupt the optimal balance of integrin activation and cAMP enables a variety of responses to the same molecule in different environments. The working model in Fig. 6 illustrates that netrin-1 could act through separate signaling pathways to both activate integrins and elevate cAMP (perhaps through canonical netrin receptors).

Our findings suggest that combined integrin activation and elevation of cAMP signaling may be a general mechanism underlying repulsive growth cone guidance under conditions where integrin-mediated substratum attachment must be reduced to enable either growth cone collapse or growth cone turning.

4. Experimental procedures

4.1. *Reagents and antibodies*

Recombinant chick netrin-1 (RnD systems, Minneapolis, MN) was used at 250 ng/ml, as used in other studies (Leung et al., 2006; Park et al., 2007; Petit et al., 2007). Commercially available function-blocking antibodies against integrin α6 (Millipore, Billerica, MA, MAB1378, Plantman et al., 2008), integrin α3 (Millipore, MAB1952Z, Wayner and Carter, 1987), and integrin α4 (Millipore, CBL1304, Miyake et al., 1991) were used for functional assays. To label activated β1 integrins, antibodies against the activated form of integrin β1 (Millipore, MAB19294) and NCAM (Millipore, AB5032) were used. To analyze cAMP levels using immunocytochemistry, antibodies against cAMP (Millipore, AB306) and NCAM (monoclonal antibody developed by Urs Rutishauser, obtained from the Developmental Studies Hybridoma Bank, 5eS) were used. All primary antibodies, including function-blocking antibodies, were used at 10 μg/ml except CBL1304 which was used at 5 μg/ml. Secondary antibodies (goat anti-mouse ALEXA 488 and goat anti-rabbit ALEXA 568) from Invitrogen were used at 2 μg/ml. Custom peptides were purchased from Anaspec (Freemont, CA). Peptides were used at 50 μM. The sequences were as follows: integrin α3peptide=PRYQHTGAVYLLSASPQ; integrin α3 scrambled peptide=SLQYHPTGAPVYRQALS, integrin α6 peptide=PRANHSGAVVLLKKEKN and integrin α6 scrambled peptide=AKELRSVPANKVHGLNK. Integrin peptides were designed based on previous studies (Wei et al., 1996). The cAMP analogs, rp-cAMP and sp-cAMP (Calbiochem, La Jolla, CA) were used at 50 μM. The activating integrin β1 antibody, MAB1951, was from Millipore (Vijayakumar et al., 2008).

4.2. *Dissociated chick DRG cultures*

DRGs were extracted from embryonic day 7–10 White Leghorn chicken eggs (supplied by Utah State University, Logan, UT). All studies were approved by the University of Utah Institutional Review Board. At this stage of development, the sex of the chick embryos is not determined. DRGs were dissociated with trypsin and pre-plated for 3 h in plastic culture dishes at 37 °C with F12 media (Invitrogen) containing 10% fetal bovine serum. DRG neurons (~95% purity) were selected from the pre-plate as previously described (Barres et al., 1988; Condic et al., 1999) and cultured for 16–24 h on glass coverslips coated

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Table 1 – Primers used for RT-PCR.

Gene	Sense	Anti-sense
Integrin α3	TCACAGTGACCGTCTGCTTC	GTGGTTCTGCTCAGTGACGA
Integrin α6	TTAAATGGGACCGCTGACTC	ACTGGGGTCTTCAGGGTTCT
Netrin	CGCTTCAACATGGAGCTGTA	TTCTTCATGGGCTTCACCTT
Neogenin	GCAACATGCTTCTTGTCATCA	AACGGATTCTGTGGAATTGG
Unc5HA	GACAGCTAGGGAGGTGTCCA	ATGGAGGAGTCTGCCACATC
Unc5HB	CTACCACAACCTGCGTCTCTC	GCTCGGCTTCCCATAAGTC
Unc5HC	GCATTTGGGACCTTCAACTC	AGGGCATCCTGTGTGTCATC
Unc5HD	CAAAGTCAAAGTTCAGAGTTCGTTC	AGCAGGCAGTAGCAGGAAGTAG
GAPDH	AGTCGGAGTCAACGGATTTG	TCTCCATGGTGGTGAAGACA

with either high LN, low LN or FN. Neurons were grown at 37 °C in HEPES-buffered F12H serum free media supplemented with 10 ng/ml NGF (R&D Systems, Minneapolis, MN), 10 ng/ml NT3 (Millipore), and N2 (Invitrogen, Carlsbad, CA) as done previously (Lemons and Condic, 2006).

4.3. *Laminin and fibronectin substrata*

Acid-cleaned glass coverslips were rinsed 10 times with distilled water and then baked at 350 °C for at least 3 h. On the same day that neurons were plated, coverslips were coated for 1 h at room temperature under sterile conditions with either: (1) laminin-1 (Invitrogen) at 20 μg/ml in PBS, referred to as high LN, (2) laminin-1 at 1–5 μg/ml in PBS, referred to as low LN, or (3) fibronectin (Invitrogen) at 20 μg/ml in PBS, referred to as FN. These conditions result in a 10-fold difference in LN adsorbed to the coverslip (Lemons and Condic, 2006; Strachan and Condic, 2004). The coverslips were then washed three times with PBS to remove any unbound protein. Neurons were immediately plated after the final PBS rinse. For timelapse analysis (see below), neurons were plated on coated glass coverslips that were attached with aquarium sealant to the bottoms of pre-drilled culture dishes.

4.4. *Timelapse and growth cone collapse*

Growth cone velocities were evaluated using a computer-controlled microscope (Nikon Eclipse 300) within a warmed chamber (37 °C). MetaMorph software (Molecular Devices, Sunnyvale, CA) was used to take images of growth cones every 5 min for 32-min sessions and to calculate growth cone velocity. Baseline velocity was established during the first 30 min recording session. Function-blocking antibodies, peptides or vehicle solutions were added. The velocity of the same growth cone was re-evaluated for an additional 30 min. Growth cone collapse was defined morphologically by a retraction of filopodia with subsequent decrease in size of the growth cone palm and a post-treatment velocity of either zero or a negative value (which represented growth cone retraction). Growth cone arrest was defined as failure of the growth cone to advance for 30 min, with or without decrease in size, but without retraction. For Fig. 1B, images of neurons plated on high LN were taken every 2 min after netrin-1 application for a total of 48 min, and data were binned in 6 min increments.

Timelapse studies were done in internally controlled sets. Neurons from one DRG dissection were plated in parallel dishes that were used for all conditions of a single experiment. All dishes were treated and recorded in 1 day and all analyses were done in a single session. Percent collapse was compared between each condition and between the 30 min pre-treatment period and the 30 min post-treatment period for each growth cone.

4.5. *RT-PCR*

mRNA was extracted from DRGs stage 35 chick embryos (Hamburger and Hamilton, 1951) using Oligotex Direct (Qiagen, Hilden, Germany) and reverse transcribed into cDNA using Superscript First-Strand Synthesis kit (Invitrogen). Specific primers for chick integrin α3, α6, netrin-1 were designed using Primer 3 and Ensembl, based on published mRNA sequences. For neogenin and Unc5, primers were designed based on the published mouse sequence, and compared to the chick genome to confirm the sequences were unique. Control primers were designed to amplify chick glyceraldehyde-3-phophate dehydrase (GAPDH) (Dugaiczyk et al., 1983) as a positive control. See Table 1 for a list of primers.

RT-PCR was performed under the following parameters: 96 °C for 2 min, 94 °C 45 s, 60 °C 45 s, 72 °C 45 s for 35 cycles and completed with a final extension for 10 min at 72 °C. PCR products were visualized on a 2.5% agarose gel stained with ethidium bromide. Images were taken using Geldoc software (Bio-Rad, Hercules, CA.)

4.6. *Immunocytochemistry and analysis of activated integrins*

DRG neurons grown on LN were treated with 250 ng/ml netrin-1 or vehicle for 5 min at 37 °C. Neurons were fixed with a 2% paraformaldehyde and 15% sucrose solution for 10 min at room temperature. Cells were rinsed four times with PBS and blocked with 5% normal goat serum in PBS for 1 h. Primary antibodies were applied for 1 h at room temperature. Monoclonal antibodies against activated β1 integrin and polyclonal antibodies against NCAM were diluted 1:500 in blocking solution. Neurons were rinsed three times with PBS. Secondary antibodies (Invitrogen) diluted 1:1000 in blocking solution were applied for 1 h. Cells were rinsed with PBS and mounted on glass microscope slides using fluoromount

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(Southern biotech, Birmingham, AL). Slides were stored horizontally in the dark at 4 °C.

Growth cones were visualized on a Nikon Eclipse 300 microscope using a 60X Plan Apo oil immersion objective. A cooled CCD camera (Spot RT monochrome camera) and spot acquisition software (Diagnostic Instruments, Inc.) were used to acquire digital images. Images were acquired and analyzed by an observer blinded to the experimental condition. All images from one experiment were captured on the same day. Fluorescent images of activated β1 integrin and NCAM were acquired for randomly selected growth cones. All activated β1 integrin images were taken at identical exposure times for all conditions. Using MetaMorph software, the distal 20 μm region of each growth cone was selected as a region of interest, using the thresholding option in MetaMorph for the ubiquitous NCAM stain. MetaMorph software was used to calculate the fluorescent intensity of activated β1 integrin staining in arbitrary units per μm^2 within each region of interest. Within each experiment, measurements for individual growth cones were normalized to the average value for the vehicle-treated controls, so that multiple experiments could be compared to each other.

4.7. *Use of size exclusion columns to test for direct netrin–integrin binding*

Recombinant chick netrin-1 was incubated with a chick integrin peptide (or a scrambled peptide as a negative control) for 1 h at 4 °C with gentle rocking. The netrin/peptide solution was placed into an Amicon ultra-0.5 filter device with a 30 kD molecular weight cut-off, centrifuged for 10 min at 4 °C at 14,000*g*, and flow through was collected. The column was then washed with 100 μl of PBS and re-spun under the same conditions. The column was inverted, placed into a new tube and spun for 2 min at 4 °C at 1000*g* to collect molecules larger than 30 kD retained by the column. The flow through and the samples retained on the column were run separately on a 4–12% Bis Tris gel (Invitrogen) at 125 V for 1 h. The gel was stained with a Simply Blue stain (Invitrogen) and rinsed with distilled water for over 24 h. Digital images of the gels were obtained using a Kodak Gel Dock Imager.

4.8. *cAMP analysis*

Intracellular cAMP levels were determined by using a cAMP enzyme immunoassay (Biomol) following manufacturer's protocols for the acetylated version. Chick sensory neurons were plated for 16–20 h, treated with recombinant chick netrin-1 or vehicle for 5 min and then treated as directed by Biomol. The cAMP concentration was calculated by a software package, AssayZap (Biosoft, Cambridge, United Kingdom) and expressed in pMol per plated neuron.

Intracellular cAMP was also analyzed using quantitative immunocytochemistry. DRG neurons were immunostained for NCAM (Developmental Studies Hybridoma Bank) and cAMP (Millipore). Quantitative immunocytochemistry was done in the same manner as described for activated integrin staining (see *Immunocytochemistry and analysis of activated integrins*, above.)

4.9. *Statistical analysis*

We performed analysis of variance (ANOVA) tests using the R statistical language (R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/). Results were also confirmed by student's *t*-test, the Benjamini–Hochberg procedure to determine false discovery rate and (for collapse data) a Generalized Linear Binomial Model (GLM) analysis, with a statistical significance of $p < 0.05$ or better being obtained by all four analyses for all data points reported here as statistically significant (analyses not shown). Due to the large number of comparisons in Fig. 2B and C, a Tukey post hoc test was also performed.

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