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In vitro guidance of retinal axons by a tectal lamina-specific glycoprotein Nel

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Abstract

Nel is a glycoprotein containing five chordin-like and six epidermal growth factor-like domains and is strongly expressed in the nervous system. In this study, we have examined expression patterns and in vitro functions of Nel in the chicken retinotectal system. We have found that in the developing tectum, expression of Nel is localized in specific laminae that retinal axons normally do not enter, including the border between the retino-recipient and non-retinorecipient laminae. Nel-binding activity is detected on retinal axons both in vivo and in vitro, suggesting that retinal axons express a receptor for Nel. In vitro, Nel inhibits retinal axon outgrowth and induces growth cone collapse and axon retraction. These results indicate that Nel acts as an inhibitory guidance cue for retinal axons, and suggest its roles in the establishment of the lamina-specificity in the retinotectal projection.

INTRODUCTION

One of the major goals of neurobiology is identification of the molecules and mechanisms that regulate the establishment of precise patterns of neuronal connections. Over the last two decades, many families of axon guidance cues, which act as long- or short-range cues and as attractants or repellents, have been found and characterized. Considering the complexity of the vertebrate neuronal network, however, it seems likely that many of important molecules that control axon behavior during development have not yet been identified or characterized.

Nel (Neural epidermal growth factor-like) was first isolated from an embryonic chicken cDNA library and was so named because it contains six epidermal growth factor (EGF)-like domains and is expressed strongly in neural tissues (Matsuhashi et al., 1995, 1996). Subsequently, two related genes, NELL1 and NELL2 (Nel-like genes 1 and 2), were identified in mammals

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(Kuroda et al., 1999; Watanabe et al., 1996), and NELL2 is considered to be the mammalian ortholog of chicken Nel. The chicken Nel gene encodes a secreted protein that consists of 816 amino acids and has a deduced molecular weight (MW) of 91 kDa (Matsuhashi et al., 1995, 1996). Nel is a multimodular protein and contains, from N-terminus to C-terminus, a N-terminal thrombospondin 1 (N-TSP1) domain (Beckmann et al., 1998), five chordin-like/von Willebrand factor (vWF) C domains, and three Ca^{2+}-binding– and three non Ca^{2+}-binding–EGF-like domains. Previous studies have shown that Nel/NELL2 stimulates neuronal differentiation and proliferation in chick dorsal root ganglia in vivo (Nelson et al., 2004), and promotes survival of rat hippocampal and cortical neurons in vitro (Aihara et al., 2003). Furthermore, NELL2-deficient mice showed enhanced long-term potentiation in the dentate gyrus (Matsuyama et al., 2004) and impairment of spatial learning (Matsuyama et al., 2005). However, functions of Nel in neuronal network formation are still unknown.

In this study, we have explored the function of Nel in development of the chicken retinotectal system. We show that at the time of retinotectal projection, Nel is expressed in specific laminae of the tectum that retinal axons normally do not invade. Correspondingly, Nel binding activity is detected on retinal axons, suggesting that retinal axons express a receptor for Nel. In vitro, Nel significantly inhibits retinal axon outgrowth and induces growth cone collapse and axon retraction. These results indicate that Nel is a novel inhibitory guidance cue for retinal axons and suggest its roles in the establishment of the lamina-specificity of the retinotectal projection.

RESULTS

Nel expression is developmentally-regulated in the chicken tectum

We first examined expression patterns of Nel RNA and protein in the developing tectum. Since first retinal axons reach the tectum by embryonic day 6 (E6) and the initial projection pattern is established by E18, we focused on those developmental stages.

By Northern blot analyses (Fig. 1A), Nel RNA was detected as early as E5, and its expression persisted at least until E18. Expression level of Nel significantly increased between E8 and E12, and the high expression level was maintained until E18.

To determine Nel protein expression, we raised polyclonal antibody against chicken Nel. The quality of the anti-Nel antibody was verified by Western blot, using cell lysates of HEK293T cells transfected with an expression construct of the full length Nel fused with an alkaline phosphatase (AP) tag (Nel-AP) (Fig.1B). The antibody detected a single band whose molecular size is about 190 kDa. No band was detected in control lysates prepared from HEK293T cells expressing a control AP. When the developing tectum was analyzed for Nel protein expression by Western blot, the antibody recognized a specific band of 130 kDa (Fig. 1C). The size was similar to that previously reported for the rat ortholog NELL2 (140 kDa) (Kuroda et al., 1999;Nelson et al., 2004), and was higher than the molecular size deduced from its amino acid sequence (91 kDa). As previously shown for rat NELL2 (Kuroda et al., 1999), this is likely due to glycosylation, because chick Nel and rat NELL2 share many potential N-linked glycosylation sites (Kuroda et al., 1999). The protein expression in the tectum followed a pattern similar to that of RNA expression (Fig. 1C); the expression was low at E5, increased dramatically between E8 and E12, and maintained at least until E18. These results indicate that both RNA and protein of Nel are expressed in the tectum at the time of retinal axon innervation, and the expression is developmentally regulated.

Nel is expressed at the border between the retinorecipient and non-retinorecipient laminae in the tectum

The mature chicken tectum is divided into 16 laminae: stratum opticum (SO), stratum griseum et fibrosum superficiale (SGFS) (a-j), stratum griseum centrale (SGC), stratum album centrale (SAC), stratum griseum periventriculare (SGP), stratum fibrosum periventriculare (SFP) and ependima. Each lamina contains distinct populations of neurons that can be distinguished by their morphology and molecular properties (LaVail and Gowan, 1971). In addition, neurons in different laminae receive distinct extratectal inputs (Hunt and Brecha, 1984). Retinal axons, for example, enter the tectum through the most superficial lamina, the SO, and then project to the specific laminae (laminae a/b, d, and e/f) of the SGFS, which are collectively referred to as "retinorecipient laminae" (Crossland et al., 1974; Inoue and Sanes, 1997; LaVail and Gowan, 1971; McLoon, 1985; Rager and von Oeynhausen, 1979; Thanos and Bonhoeffer, 1987; Yamagata et al., 1995). Remarkably, during development, retinal axons do not invade lamina g of the SGSF or deeper (Inoue and Sanes, 1997; LaVail and Gowan, 1971; Yamagata et al., 1995), and thus the border between lamina f and lamina g represents the boundary between the superficial retinorecipient laminae and the deeper non-retinorecipient laminae in the tectum.

To determine whether Nel is expressed in lamina-specific patterns, we next performed RNA in situ hybridization using tectal sections. In this report, we use the nomenclature for developing tectal laminae by LaVail and Cowan (LaVail and Gowan, 1971), which numbers the tectal layers starting from the ventricular side. Whenever possible, we indicate their relationship to the lamina(e) in the mature tectum.

After entering the tectum, most retinal axons remain confined to the SO until E10 (Yamagata and Sanes, 1995). At this stage (E10), Nel RNA was expressed in four layers: layer VIII (future lamina g of the SGFS), layer VI (future lamina i of the SGFS), layer IV (future SGC), and layer II (future SGP) (Fig. 2A). At E14, by which time many retinal axons reach retinorecipient laminae, and at E18, Nel RNA expression was still detected in layers VIII, IV, II, whereas its expression in layer VI significantly decreased (Figs. 2 B and 2C).

Since Nel is a secreted protein, the Nel protein in principle could diffuse distances to be distributed in multiple laminae of the tectum. To determine the Nel protein distribution in the developing tectum, we examined tectal sections by immunohistochemistry using anti-Nel antibody. As shown in Fig. 2D, the distribution of Nel protein in the tectum showed a very similar pattern to that of Nel RNA expression, indicating that most of the secreted Nel protein remains in the laminae of its origin.

Our results indicate that Nel RNA and protein are expressed in specific tectal laminae that retinal axons do not invade. In particular, strong Nel expression was detected in layer VIII (future lamina g of the SGFS), which locates at the border between the retinorecipient and non-retinorecipient laminae.

Nel can bind to retinal axons both in vitro and in vivo

If Nel acts as a guidance cue in the retinotectal projection, Nel should bind to and be recognized by retinal axons. To test this, we next examined Nel-binding activity of retinal axons both in vitro and in vivo, using Nel-AP as a probe (affinity probe in situ (Flanagan et al., 2000)).

We prepared retinal explants from E6 chick and cultured them in vitro to allow retinal axons to grow out. Axons were then incubated with Nel-AP, and binding was detected by the AP enzyme reaction. As shown in Fig. 3A, retinal axons showed significant binding activity to Nel-AP. The binding was observed along the entire length of axons. A control AP or heat-inactivated Nel-AP (HI–Nel-AP) did not show detectable binding activity to retinal axons

(Figs. 3B and 3C). In contrast to retinal axons, axons from olfactory bulb explants did not appear to have significant Nel-AP binding activity (Fig. 3D).

To examine Nel-binding activity of retinal axons in vivo, coronal sections were prepared from E12 chick tecta and treated with Nel-AP. Strong Nel-AP binding activity was detected in the SO of the tectum (Fig. 3E), which mostly consists of innervating retinal axons. At higher magnifications, axon branches entering retinorecipient laminae were also detected by Nel-AP (Fig. 3F). No binding activity was observed for AP (Fig. 3G). These results indicate that Nel can bind to retinal axons both in vitro and in vivo, and suggest that Nel in the tectum can be recognized through a specific receptor on retinal axons. Although Nel has heparin-binding activity, it seems unlikely that the observed Nel-AP binding pattern represents non-specific binding to heparan sulfate proteoglycans (HSPGs), which exist on retinal axons and in the tectum with lamina-non-specific distribution patterns (Halfter, 1993).

Nel inhibits retinal axon outgrowth in vitro

Our results that Nel is expressed at the border of retinorecipient and non-retinorecipient laminae in the developing tectum (Fig. 2) raise the possibility that the Nel protein could act as an inhibitory cue that prevents retinal axons from invading deeper non-retinorecipient laminae. To test whether Nel could affect retinal axon behavior, we performed in vitro axon outgrowth assays using retinal explants. Retinal explants prepared from E6 chick were cultured for 48 hours on a Nucleopore filter containing laminin and either Nel-AP, AP, or HI–Nel-AP. Whereas robust axon outgrowth was observed on the control AP [n=69, score 1.83 ± 0.13 (s.e.m.)] and HI–Nel-AP substrata [n=34, score 1.91 ± 0.19 (s.e.m.)], axon outgrowth was significantly inhibited on the Nel-AP substratum [n=63, score 0.75 ± 0.13 (s.e.m.); $p<0.001$] (Figs. 4A-4C, and 4J).

In a separate set of experiments, retinal cells were dissociated and plated onto substratum containing either Nel-AP/laminin, AP/laminin, or HI–Nel-AP/laminin. After 24 hours, the dissociated retinal cells were stained for neurofilament (a neuronal marker) and Islet-1 (a retinal ganglion cell marker), and axon outgrowth from double positive cells was evaluated. Compared to the control substrata, axon outgrowth was significantly inhibited on the Nel-AP/laminin substratum (Figs. 4D-4I, and 4K).

Nel induces growth cone collapse and axon retraction

Our results have indicated that the substratum containing Nel suppresses retinal axon outgrowth, and suggested that Nel acts as an inhibitory guidance molecule for retinal axons. If that is the case, it is likely that Nel affects growth cone morphology, as previously shown for other guidance molecules, such as ephrins (Drescher et al., 1995), semaphorins (Luo et al., 1993), slits (Nguyen Ba-Charvet et al., 1999), and RGM (Monnier et al., 2002). We therefore examined effects of Nel on retinal growth cones using in vitro growth cone collapse assays.

Retinal explants were prepared from E6 chick and cultured for 18 hours on a laminin-coated substratum. Under this culture condition, the tip of almost all axons had growth cones that are well-defined by the presence of lamellipodia and filopodia. Then, various concentrations of Nel-AP, AP, or HI–Nel-AP were added to the culture, and the morphology of growth cones was observed after 30 minutes (Figs. 5A-5C). Nel-AP has a clear collapsing activity at a concentration as low as 20 μg/ml, and the percentage of collapsed growth cones increased in a dose-dependent manner (Fig. 5D). A control AP or HI–Nel-AP did not affect the growth cone morphology in this concentration range. Fig. 5E shows a time-lapse study on the collapsing activity of Nel-AP. During the control period, a retinal axon growth cone had extensive lamellipodia and many filopodia, and crawled forward at a steady pace. Addition of control AP had no effects on the morphology or forward movement of the growth cone for the 20

minutes period after the pulse. When exposed to Nel-AP, however, the growth cone collapsed completely within 1 minute, and the axon retracted. The growth cone recovered from the collapse and regained significant lamellipodia and filopodia after 40 minutes (data not shown). These results indicate that Nel can act as an inhibitory guidance cue for retinal axons, by modifying growth cone dynamics.

DISCUSSION

Lamina-specific tectal expression of Nel and its inhibitory functions in retinal axon guidance in vitro

To explore the potential roles of Nel in neuronal network formation, we have chosen the chicken retinotectal projection as a model system and examined its expression and binding patterns and in vitro functions in regulation of retinal axon behavior.

By Northern and Western blot analyses, Nel was detected in the developing chicken tectum at the time of retinotectal projection. The expression levels increased significantly between E8 and E12 (Figs. 1A and 1C), when retinal axons in the SO migrate into retinorecipient laminae. Remarkably, RNA in situ hybridization has shown that Nel expression is confined to specific laminae of the tectum that retinal axons normally avoid, including lamina g of the SGFS, which locates at the border between the retinorecipient and non-retinorecipient laminae (Figs. 2A-2C). Although Nel is a secreted protein, immunohistochemical studies using anti-Nel polyclonal antibody have indicated that most of the Nel protein remains in the laminae of its origin (Fig. 2D). This limited diffusion of Nel protein may be due to its haparin-binding activity, as HSPGs are abundant in the developing tectum (Halfter, 1993), and they may trap the Nel protein in situ soon after secretion from Nel-expressing cells.

Affinity probe in situ experiments using Nel-AP have revealed that Nel has binding activity to retinal axons both in vitro and in vivo (Fig. 3). These results indicate that retinal axon can specifically recognize Nel, presumably through a cognate receptor(s) on their surface.

Our in vitro assays have provided direct evidence for axon guidance functions of Nel. The Nel protein immobilized in the substratum exerted a strong inhibitory effect on retinal axon outgrowth (Fig. 4). In addition, soluble Nel induced rapid growth cone collapse and axon retraction (Fig. 5). These findings represent the first demonstration that Nel can regulate axon behavior and indicate that Nel acts as an inhibitory guidance cue for retinal axons.

Possible roles of Nel in the lamina-specific retinotectal projection

In the retinotectal system, all retinal axon terminals are confined to specific retinorecipient laminae in the superficial (pial) part of the tectum and never invade deeper non-retinorecipent laminae. At least two different types of guidance cues can be involved in this lamina-specific retinotectal projection. First, the retinorecipient laminae could contain positive guidance cues that attract or induce specific adhesion of retinal axons to those laminae. Previous studies have shown that N-cadherin (Miskevich et al., 1998; Yamagata et al., 1995) and glycoconjugates recognized by the plant lectin Vicia villosa agglutinin-B4 (VVA) are specifically expressed in retinorecipient laminae and can promote lamina-specific connectivity of retinal axons (Inoue and Sanes, 1997). In addition, several other molecules that could regulate axon behavior are expressed in retinorecipient laminae, such as SC1/DM-GRASP/BEN (Yamagata et al., 1995), neuropilin (Feiner et al., 1997; Takagi et al., 1995), and ephrin-B2 (Braisted et al., 1997), and they may also act as positive guidance cues in the lamina-specific retinal axon projection.

Second, negative guidance cues may exist in lamina g of the SGFS (and deeper laminae) and inhibit retinal axon invasion into the non-retinorecipient laminae (Yamagata et al., 1995). Several previous studies have suggested the existence of inhibitory guidance cues in lamina g

of the SGFS. For example, during development of the retinotectal projection, the boundary between laminae f and g of the SGFS is well respected, and few if any retinal axons cross this border to extend to deeper non-retinorecipient laminae (Yamagata and Sanes, 1995). In addition, in a co-culture system of a retinal explant and a transverse tectal section, where retinal axons have simultaneous access to all the tectal laminae, only poor axon growth was observed on lamina g of the SGFS (Yamagata and Sanes, 1995). Finally, when lamina g of the SGFS is disrupted by overexpression of the transcription factor Grg4, retinal axons abnormally extend beyond lamina g in vivo (Sugiyama and Nakamura, 2003). The properties of Nel that have been revealed in this study, its expression patterns in the tectum and in vitro functions in retinal axon guidance, are therefore well suited to a role of an inhibitory cue in lamina g of the SGFS that has been predicted by previous studies. To reveal the function of Nel in the lamina-specific retinotectal projection directly in vivo, both gain-of-function and loss-of-function analyses will be required.

Signaling mechanisms of Nel-induced inhibition of axon behavior

At present, no specific receptor has been identified for Nel, and the intracellular signaling pathway by which Nel exerts its functions is still unknown. Nel could bind and activate other secreted factors, which in turn bind to receptors on the axonal surface. Nel could also directly interact with a cognate cell surface receptor and induce intracellular signaling. Our results that purified Nel-AP can bind to retinal axons both in vitro and in tectal sections (Fig. 3) suggest that Nel can be recognized by a specific receptor expressed on axons. Since Nel has been shown to have a heparin-binding activity in vitro (Kuroda et al., 1999), a cognate receptor may be a cell surface HSPG or may take a form of a peptide receptor-heparin complex. Furthermore, since the Nel protein consists of different domains, it seems plausible that different domain interact with distinct receptors and exert different functions, as is the case for thrombospondin-1 (Chen et al., 2000). Interestingly, rat Nell2 promotes survival of hippocampal and cortical neurons in vitro at concentrations lower than those required for retinal axon inhibition (Aihara et al., 2003). This may suggest that Nel/Nell2 exerts inhibitory axon guidance functions and neurotrophic effects through distinct receptors that have different affinities for the ligand. Alternatively, the same receptor may mediate both positive and negative signals in response to different concentrations of the ligand, as shown for the Eph receptor-ephrin interaction (Hansen et al., 2004;Matsuoka et al., 2005). Further studies, particularly identification of the cognate receptors, will be required for understanding molecular mechanisms by which Nel exerts its functions.

Functions of Nel in development of neural and other tissues

Nel is expressed in many different regions of the developing nervous system, including the cerebral cortex, hippocampus, amygdala, retina, and spinal cord (Matsuhashi et al., 1995; Nelson et al., 2004; Nelson et al., 2002; Oyasu et al., 2000). In view of the properties of Nel in the retinotectal system, it seems plausible that Nel has guidance functions in other neuronal projections. Nel may also have other analogous functions in neural development, including neuronal proliferation, differentiation (Nelson et al., 2004), and migration. Within the tectum, for example, in addition to lamina g of the SGSF, Nel is also expressed in deeper laminae (e.g. SGC, SGP) (Fig. 2). Although its function in those laminae is not addressed in this study, Nel may be involved in development of the tectal system, such as formation of the tectal layer structure and establishment of tectofugal and intratectal neuronal pathways. Furthermore, Nel (Nell2) and its related gene Nell1 are expressed in non-neuronal tissues with distinct but overlapping patterns (Kim et al., 2002; Kuroda et al., 1999; Luce and Burrows, 1999; Oyasu et al., 2000; Watanabe et al., 1996). Interestingly, overexpression of NELL1 has been implicated in human craniosynostosis (Ting et al., 1999; Zhang et al., 2002). Taken together with our findings presented in this report, Nel family genes therefore could have diverse

functions in the formation of the precise and complex spatial order of different systems during vertebrate development.

EXPERIMENTAL METHODS

Chick embryos

Fertilized White Leghorn chicken eggs were purchased from Charles River SPAFAS (North Franklin, CT) and Henry Stewart (England), and incubated at 38°C until use.

Northern blot analyses

Total RNA was prepared from embryonic chicken tecta by using Trizol reagent (Invitrogen, Carlsbad, CA), separated in 1% formaldehyde agarose gels, and transferred to nylon membranes. The membranes were hybridized with a digoxigenin-labeled DNA probe prepared from the full coding region of chicken Nel cDNA. Chemiluminescence detection using alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Applied Science, Indianapolis, IN) was performed according to the manufacture's instructions.

Construction, expression and purification of Nel-AP

For preparation of a fusion protein of Nel with an alkaline phosphatase tag (Nel-AP), chicken Nel cDNA (from nucleotide 103 to 2565 (Matsuhashi et al., 1996); GenBank accession NM001030740) was amplified from chicken embryo cDNA by polymerase chain reaction, and inserted between the BamHI and MluI sites of the pCMV-AP vector. Nel-AP and AP expression constructs were transfected into HEK293T cells using the FuGENE transfection reagent (Roche Applied Science). After 3-4 days, the supernatants containing Nel-AP or AP were collected, and applied to an anti-AP antibody column. After washing, Nel-AP and AP was eluted with 0.1M glycine-HCl (pH 2.5), neutralized with 0.75M Tris-HCl (pH 8.8), and dialyzed against PBS. Concentrations of Nel-AP and AP were evaluated by the Bradford assay, SDS-PAGE, Western blot using anti-AP antibody, and by measuring AP activity (Flanagan et al., 2000), and comparable concentrations were used. Heat inactivation of Nel-AP (HI–Nel-AP) was performed at 65°C for 30 min. Since the human placental alkaline phosphatase used as a tag is heat stable, this treatment does not affect the enzyme activity (Flanagan et al., 2000).

Affinity probe in situ

Affinity probe in situ using Nel-AP was performed essentially as previously described (Flanagan et al., 2000). Briefly, unfixed tissues and explants were incubated with Nel-AP, AP, or HI–Nel-AP for 30 minutes at room temperature. After washing, cells were fixed wit 8% formaldehyde for 3 minutes, washed, and heated for 60 minutes at 65°C to inactivate endogenous APs. Nel-AP binding was detected by incubation with 5-bromo-4-choloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) or with Cy3-conjugated donkey anti-AP antibody (1:200; Jackson Immuno Research Laboratories, West Grove, PA). In some experiments, cell nuclei were counter-stained with 4',6'-diamidino-2-phenylindole (DAPI).

Production and purification of anti-Nel polyclonal antibodies

An adult New Zealand white rabbit was immunized with Nel-AP, and antisera were collected, according to the standard immunization protocol of the Hybridoma Core Facility of Lerner Research Institute. Anti-sera were first applied to an AP column to remove the antibodies against AP, and the flow-through fraction was applied to a Nel-AP column for affinity purification of anti-Nel antibodies.

In situ RNA hybridization, histology, and immunohistochemistry

For in situ RNA hybridization, two separate anti-sense probes for chicken Nel were used. Both probes gave similar results. One fragment extends from a PstI site at nucleotide 203 to a HindIII site at nucleotide 1028, and the other was from the HindIII site to a KpnI site at nucleotide 1854. In situ hybridization was performed as described previously (Nishida et al., 2002), using digoxigenin-labelled RNA probes. For immunohistochemistry, sections were treated with rabbit anti-Nel antibody (0.1 μg/ml) and then with biotin-conjugated donkey anti-rabbit IgG (1:500; Jackson Immuno Research). Detection was performed using the standard ABC method (Vector Laboratories, Burlingame, CA). 30 μm cryosections of embryonic chicken tectum were used for both RNA in situ hybridization and immunohistochemistry.

Immunoblot analyses

Embryonic chicken tecta were homogenized in cold lysis buffer (50mM HEPES (pH 7.4), 50mM NaCl, 1% Triton X-100, 5mM EDTA, 1x protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO), 2mM sodium vanadate, 25mM sodium fluoride), and centrifuged for 10 minutes at 15,000 × g to remove insoluble material. The lysates were separated by SDS-PAGE (7.5% polyacrylamide gel), and the proteins were transferred onto Immobilon-P PVDF membrane (Millipore, Billerica, MA). The filters were blocked with 5% skim milk in PBS containing 0.05% Triton X-100, and treated with rabbit anti-Nel antibody (0.1μg/ml) and then with HRP-conjugated donkey anti-rabbit IgG (1:40,000; Jackson Immuno Research Laboratories). As a control, anti-β-actin antibody (1:10,000; Sigma) and HRP-conjugated donkey anti-mouse IgG (1:40,000; Jackson Immuno Research Laboratories) were used. The ECL plus system (Amersham, Piscataway, NJ) was used for detection.

Tissue culture and axon outgrowth assays

Retinal explants were prepared as described previously (Nakamoto et al., 1996). Briefly, retinae were dissected from E6 chick and flattened onto cellulose nitrate filters (0.45 μm pore size, Sartorius). The filters with retinae were cut into 300 μm wide strips using a McIllwain tissue chopper. Olfactory bulbs were dissected out from E10 chick embryos and cut into small pieces. Retinal strips and olfactory bulb tissues were put on a Nucleopore membrane (Whatman, Clifton, NJ) that was first coated with 50 μg/ml of laminin and then with 25 μg/ml of Nel-AP, AP, or HI–Nel-AP. Explants were cultured in the retinal culture medium (DMEM:F12=1:1, 10% fetal bovine serum, 5% calf serum, 1% penicillin/streptomycin, 0.6% glucose). After 48 hours, axons were labeled by incubating the cultures in 33 μM carboxylfluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, OR) for 5 minutes. Axon outgrowth for each explant was scored on a graded 0 to 3 scale, in which 0 was no or very sparse outgrowth from a living explant and 3 was very robust outgrowth.

Axon outgrowth assays of dissociated retinal ganglion cells were performed according to the protocol of Stepanek et al (Stepanek et al., 2001), except that we analyzed only neurofilament and Islet-1 double positive cells. 35 mm dishes (MatTek Corporation, Ashland, MA) were coated with 10 μg/ml of laminin for 1 hour and then with 25 μg/ml of Nel-AP, AP, or HI–Nel-AP for 30 minutes. The dishes were rinsed and blocked with the retinal culture medium. Dissociated retinal cells were plated at 1×10^5 cells/dish and allowed to grow overnight before fixation. After antigen retrieval, the cells were incubated with rabbit anti-neurofilament antibody (1:200, Millipore, Billerica, MA) and anti-Islet 1 antibody (4D5, 1:100, Developmental Studies Hybridoma Bank at the University of Iowa), and then with Alexa Fluor 594-conjugated donkey anti-rabbit IgG (2 μg/ml, Invitrogen, Paisley, Scotland) and Alexa Fluor 488-conjugated donkey anti-mouse IgG (5 μg/ml, Invitrogen). In each dish, the lengths of at least 15 axons from double stained cells were measured. The assay was repeated three times.

Growth cone collapse assays

Growth cone collapse assays were performed essentially as described previously (Goshima et al., 1995). Retinal explants were prepared from E6 chick and cultured for 18 hours on glass coverslips coated with 100 μg/ml of laminin in 24-well plates in the retinal culture medium. Then Nel-AP or control AP or HI–Nel-AP was added to the culture. The cells were incubated at 37°C for up to 30 minutes, fixed, and stained with Alexa Fluor 488 Phalloidin (Invitrogen). In each experiment, at least 30 growth cones were scored for each treatment as collapsed or not collapsed, and three independent experiments were performed. In a separate set of experiments, growth cone behavior was directly observed under phase contrast microscopy during treatment with AP and Nel-AP.

Statistical analyses

Statistical significance of the data was determined using Student's t-test.

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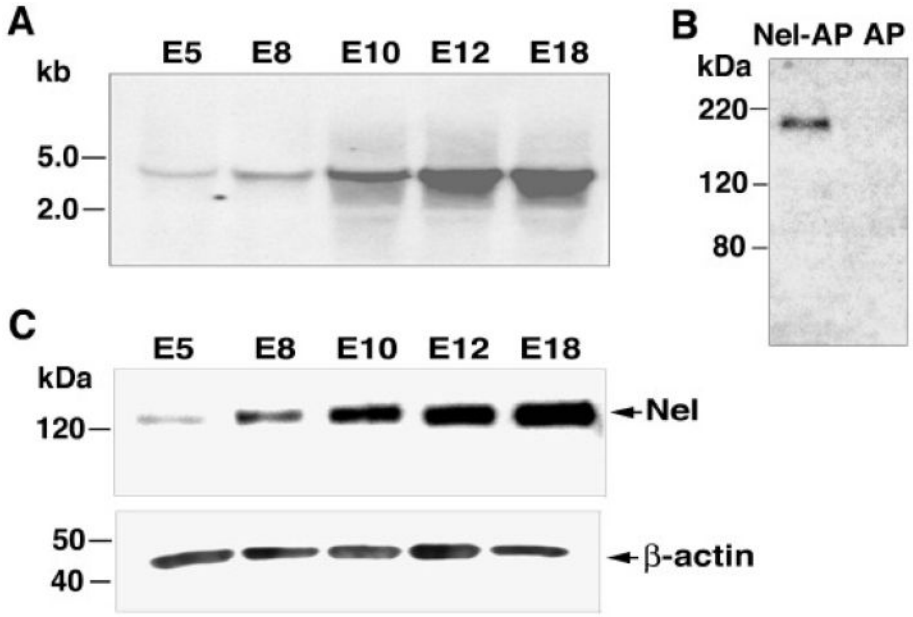

FIG. 1. Nel expression in the developing chick tectum and transfected cells in culture

(A) Nel RNA expression in the developing chicken tectum. 10 μg of total RNA prepared from E5, 8, 10, 12, and 18 chick embryos were analyzed by Northern blot using a probe for Nel.

(B) Detection of Nel-AP expression in transfected cells using affinity purified anti-Nel polyclonal antibody. Lysates of HEK-293T cells expressing either Nel-AP or AP (40 μg of proteins) were analyzed by Western blot.

(C) Nel protein expression in the developing tectum. Homogenates of embryonic tecta (40 μg of proteins) were subjected to Western blot analysis using anti-Nel antibody. Anti-β–actin antibody was used as a control.

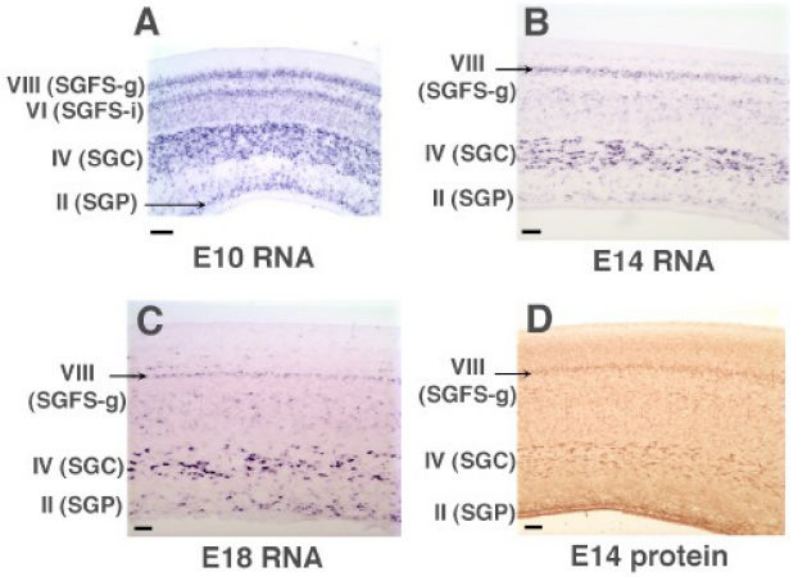

FIG. 2. Lamina-specific expression of Nel RNA and protein in the developing tectum

Sections of the E10 (A), 14 (B, D), and 18 (C) chick tectum were hybridized with an RNA probe for Nel (A-C) or treated with anti-Nel antibody (D). Roman numerals (VIII, VI, IV, II) indicate names of tectal layers by LaVail and Cowan (1971). In all the developmental stages examined, Nel is expressed in layers VIII (future lamina g of the SGFS), IV (future SGC), and II (future SGP). Expression in layer VI (future lamina i of the SGFS) is clearly detected at E10 (A), but becomes weaker at later stages (B-D). Scale bars, 100 μm.

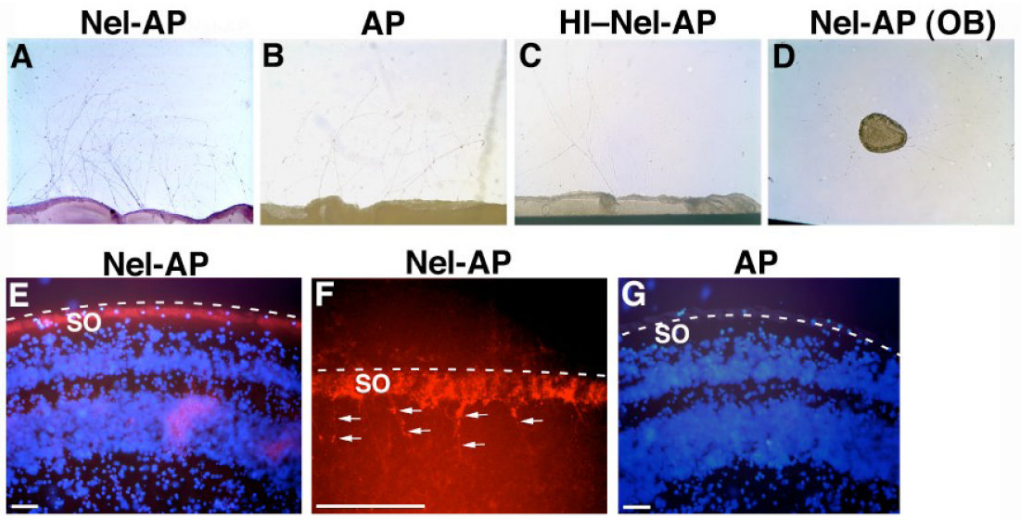

FIG. 3. Specific Nel binding activity of retinal axons detected by affinity probe in situ

(A-D) Axons from retinal explants were incubated with Nel-AP (A), AP (B), or heat-inactivated Nel-AP (HI–Nel-AP) (C). Retinal axons show strong binding activity for Nel-AP, but not for a control AP or HI–Nel-AP. No significant binding activity was detected for axons from olfactory bulb (OB) explants (D).

(E-G) Coronal sections of E12 chick tecta were treated with Nel-AP (E, F) or AP (G). Strong Nel-AP binding activity (red) is detected in the SO (E, F). In a higher magnification view (F), axon branches entering retinorecipient laminae (arrows) also show Nel-AP binding. AP did not show binding activity (G). The white dotted lines indicate the pial surface of the tectum. In (E) and (G), cell nuclei were counter-stained with DAPI (blue). Scale bars, 100 μm.

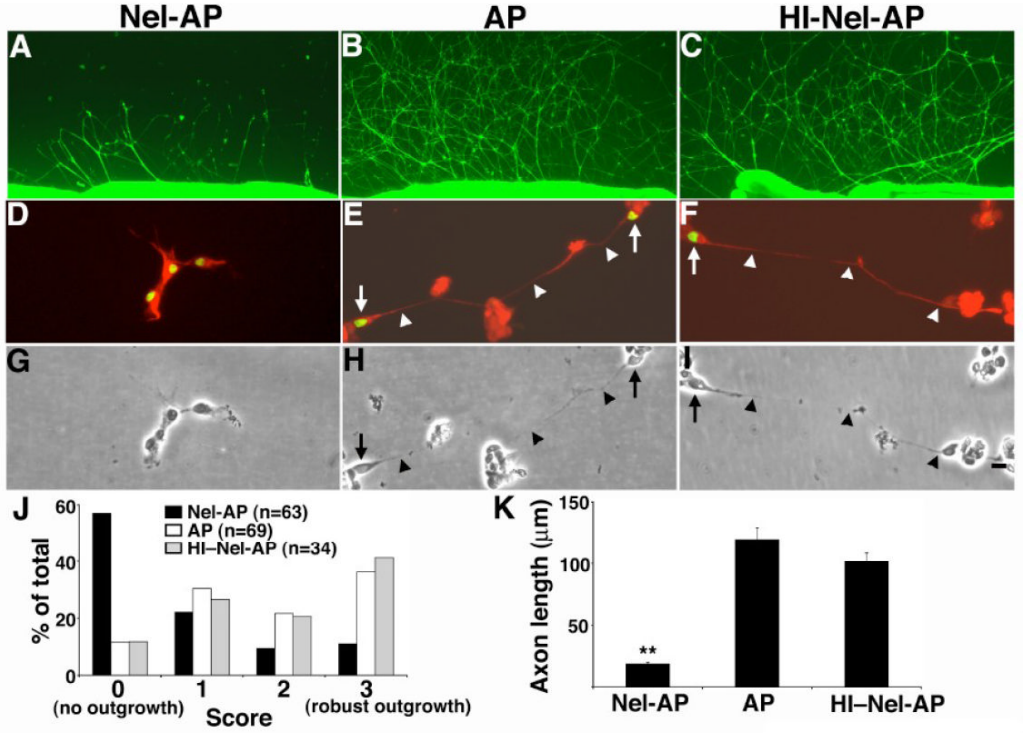

FIG. 4. Inhibition of retinal axon outgrowth on Nel-containing substratum

Retinal explants (A-C) or dissociated retinal ganglion cells (D-I) were prepared from E6 chick and cultured on a substratum coated with 50 μg/ml of laminin and 25 μg/ml of either Nel-AP (A, D, G), AP (B, E, H), or HI–Nel-AP (C, F, I).

(A-C) Compared with the AP/laminin (B) and HI–Nel-AP/laminin (C) substrata, axon outgrowth from retinal explants was significantly suppressed on the Nel-AP/laminin substratum (A).

(D-I) Dissociated retinal cells were double labeled for neurofilament (red) and Islet-1 (green) (D-F). While retinal ganglion cells (neurofilament/Islet-1 double positive cells) extend long axons on AP/laminin (E) and HI–Nel-AP/laminin (F), axons were significantly shorter on Nel-AP/laminin (D). Corresponding phase contrast images are shown in (G-I). Arrows indicate retinal ganglion cell body, and arrowheads show their axons. Scale bar, 10 μm.

(J) Quantification of axon outgrowth from retinal explants (data shown in (A-C)). Axon outgrowth was scored on a graded 0 (no or very sparse outgrowth) to 3 (very robust outgrowth) scale.

(K) Quantification of axon outgrowth from dissociated retinal ganglion cells (data shown in (D-I)). Axon lengths from retinal ganglion cells were platted as mean ± s.e.m. **, $p<0.001$.

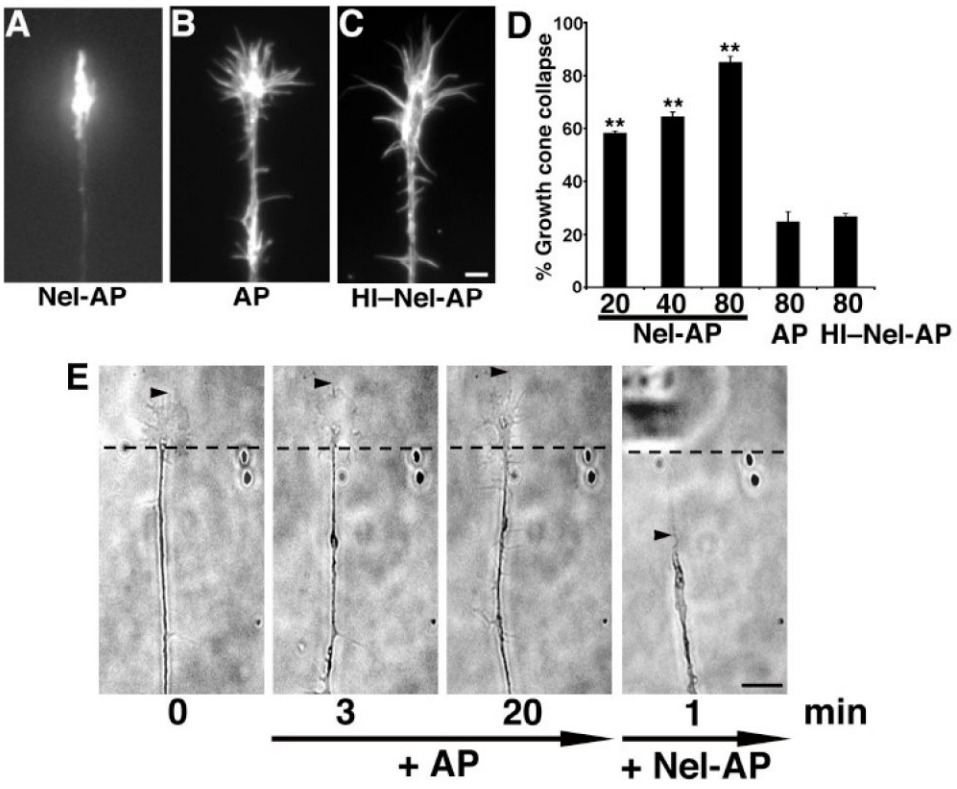

FIG. 5. Nel-induced growth cone collapse in retinal axons

Retinal explants prepared from E6 chick embryos were cultured on laminin-coated glass coverslips and incubated for 18 hours. Nel-AP, AP, or HI–Nel-AP was added to the culture, and the growth cone morphology was observed.

(A-C) Representative growth cone morphology after treatment with 80 μg/ml of Nel-AP (A), AP (B) or HI–Nel-AP (C). The explants were fixed and stained with Alexa Fluor 488 Phalloidin (Invitrogen). The growth cone treated with Nel-AP shows a collapsed morphology, whereas the AP- or HI–Nel-AP–treated control axon has a well-spread growth cone. Scale bar, 10 μm.

(D) Quantification of the growth cone collapsing activity. The percentage of collapsed growth cones was platted as mean ± s.e.m. Concentrations of Nel-AP, AP, and HI–Nel-AP are given in μg/ml. ** indicates $p<0.001$.

(E) Time-lapse analysis of retinal axon response to Nel-AP and AP in vitro. Retinal explants were first treated with AP for 20 minutes, and then Nel-AP was added to the culture. AP did not affect the growth cone behavior, and the growth cone with well-developed lamellipodia and filopodia moved forward at a steady pace. In contrast, Nel-AP induced a rapid growth cone collapse and axon retraction. Dotted lines indicate the corresponding position in each column. Scale bar, 50 μm.