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Neural cell adhesion molecule NrCAM is expressed in the mammalian inner ear and modulates spiral ganglion neurite outgrowth in an *in vitro* alternate choice assay

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Abstract

Neuron-glial-related cell adhesion molecule (NrCAM) is a neuronal cell adhesion molecule involved in neuron-neuron and neuron-glial adhesion as well as directional signaling during axonal cone growth. NrCAM has been shown to be involved in several cellular processes in the central and peripheral nervous systems, including neurite outgrowth, axonal pathfinding and myelination, fasciculation of nerve fibers, and cell migration. This includes sensory systems such as the eye and olfactory system. However, there are no reports on the expression/function of NrCAM in the auditory system. The aim of the present study was to elucidate the occurrence of NrCAM in the mammalian cochlea and its role in innervation of the auditory end organ. Our work indicates that NrCAM is highly expressed in the developing mammalian cochlea (position consistent with innervation). Moreover, we found that NrCAM, presented in stripe micropatterns, provide directional cues to neonatal rat inner ear spiral ganglion neurites *in vitro*. Our results are consistent with a role for NrCAM in the pathfinding of spiral ganglion dendrites toward their hair cell targets in the sensory epithelium.

Keywords

growth cone guidance; neurite outgrowth; inner ear; NrCAM; spiral ganglion

Introduction

Cell adhesion molecules (CAMs) play critical roles in cell-cell interactions at many body sites and have been found to be important factors in the development of neuronal projections in both the peripheral and central nervous systems (reviewed in Brümmendorf and Rathjen

1996). Neuron-glia-related cell adhesion molecule (NrCAM) is a neuronal CAM with multiple immunoglobulin-like C2-type domains and fibronectin type-III domains and is a member of the L1 family. This ankyrin-binding protein is involved in neuron-neuron as well as neuron-glia adhesion and also promotes directional signaling during growth cone extension. Recently, several studies have indicated that mutations in the NrCAM gene results in neuropsychiatric disorders such as autism (Hutcheson et al. 2004; Bonora et al. 2005; Sakurai et al. 2006; Marui et al. 2009; Pinto et al. 2010), schizophrenia (Kim et al. 2009; Ayalew et al. 2012) and addiction-related behaviors (Ishiguro et al. 2006; Ishiguro et al. 2012). NrCAM is indicated in several cellular processes in the nervous system, including neurite outgrowth, axonal pathfinding and myelination, fasciculation of nerve fibers, and cell migration (Hortsch 1996). This includes a role in sensory systems such as the eye and olfactory system (Zelina et al. 2005; Heyden et al. 2008).

NrCAM has been shown to be a key component in retinal ganglion cells (RGC) axon pathfinding. NrCAM as an *in vitro* substrate affects growth cone shape and advance of RGC axons in the chick eye. Inhibition of NrCAM by function-blocking antibodies changes the dynamics of RGC axon growth *in vitro*, increasing the length of growth cone pauses and the frequency of neurite retraction. In the intact chick eye, lack of NrCAM results in defective RGC axon exit at the optic disc, so that the axons overshoot into the opposite side of the retina (Zelina et al. 2005). Demyanenko et al. (2011) showed striking mistargeting of motor and somatosensory thalamic axon contingents to the primary visual cortex in NrCAM knock-out mice. Moreover, it has been demonstrated that NrCAM promotes topographic retinocollicular mapping in mice (Dai et al. 2013). In the olfactory system, NrCAM is widely expressed, and NrCAM-null mice have alterations in olfactory nerve projections (Heyden et al. 2008).

In contrast to the situation in the retina and the olfactory system, the role of NrCAM in the auditory system is unknown. However, we have previously demonstrated that L1, like NrCAM also a member of the L1 family of ankyrin-binding proteins, can modulate spiral ganglion neurons (SGNs) *in vitro* (Brand et al. 2013).

In this study, we evaluated the distribution of NrCAM in the developing mammalian cochlea and analyzed the effects of NrCAM on spiral ganglion (SG) neurites in an *in vitro* alternate choice assay.

Materials and Methods

Animals

Histology and Immunocytochemistry studies were performed in C57BL/6 mice. Animal care and use were conducted in conformity with the European Communities Council Directive of 24 November 1986 (86/609/EEC), and were reviewed and permitted by the Kantonales Veterinäramt, Basel, Switzerland. All *in vitro* experiments were performed in Sprague-Dawley rats. The local animal subjects committee of the VA San Diego Healthcare System approved the surgical procedures in accordance with the guidelines laid down by the National Institute of Health regarding the care and use of animals for experimental procedures. The rationale to use rats and not mice for our *in vitro* experiments was that the

larger cochlea of the rat allowed more tissue to be harvested, thus fewer animals needed to be sacrificed for the experiments.

Preparation of Paraffin Cochlear Sections

The expression of NrCAM during development was assessed on mouse cochleae from wild-type C57BL/6 mice of both sexes. Immunohistochemistry was performed on 4 mice per embryonic and postnatal day. Mice were killed with an overdose of sodium pentobarbital (100 mg/kg) and transcardially perfused with 50 ml of phosphate-buffered 4% paraformaldehyde (pH 7.4, at 4°C). The inner ear was carefully removed. Decalcification was carried out in a light-protected flask for 10 days in a solution of 120 mM EDTA (Merck, New Jersey, USA) in distilled water (pH 6.8). After decalcification, cochleae were prepared for paraffin embedding. Briefly, cochleae were dehydrated in graded ethanol solutions (at 70%, 80%, 95%, and 3 × 100%, each for 1 h; 3 × xylol for 1 h; 2 × paraplast at –60°C for 1 h; and paraplast at –60°C for 10 h), and embedded in paraffin at 56°C.

Histology and Immunocytochemistry

For histological evaluation, cochlear sections of 10 µm thickness were cut on a Leitz microtome and mounted on Superfrost plus slides (Menzel, Braunschweig, Germany). Sections were deparaffinized, rehydrated, washed in PBS for 5 min, and subjected to immunohistochemistry. Microtome sections of mouse cochlea were incubated for 1 h at room temperature in blocking solution (PBS-T) containing 5% Triton X-100 (pH 8) and 3% normal goat serum (NGS). The sections were incubated with primary antibody diluted in PBS-T with 1% NGS overnight at 4°C. The following primary antibodies were used: rabbit polyclonal anti-NrCAM antibodies (1:300, Santa Cruz Biotech, USA), mouse monoclonal anti-Hes5 antibody (1:300, AbCam, UK). After 3 washes in PBS-T, the sections were incubated for 1 h at room temperature with the appropriate secondary antibodies (1:250, Alexa-conjugated, Molecular Probes, Lubio Science, Switzerland) diluted in PBS-T with 1% NGS for 2 h at room temperature. After washing in PBS, the sections were counterstained with DAPI and mounted on glass slides with Mowiol. Slices were visualized on an Olympus AX-70 microscope equipped with a spot digital camera. Recorded images were adjusted for brightness and contrast using Image-Pro Plus and Photoshop image processing software.

Preparation of Tissue Culture Plates

To prepare stripe molds, templates were generated by photolithography to produce parallel stripes of photoresist compound 100 µm wide, 100 µm high, and 100 µm apart. Silicone rubber (Sylgard®184 Silicone Elastomer, Dow Corning Inc., Midland, MI USA) was then applied to the templates to generate stripe molds. After sterilization with 100% ethanol for 20 min and air drying inside a sterile hood (The Baker Company Inc., Sanford, ME USA), 4×7 mm pieces of the resultant silicone molds were placed groove side down into the center of individual wells of a 24-well cell culture plate (Costar®, Corning Inc., Acton, MA USA), as described previously by Evans et al. (2007). As an initial step, NrCAM protein solution (1 µg/ml, 5 µg/ml or 10 µg/ml in PBS; R&D Systems, Minneapolis, MN USA) was placed along the mold perpendicular to the channels, and drawn into the mold's channels by suction applied from the other side of the mold. The plate was then incubated overnight at 37°C. Each well was then washed twice with PBS with the molds in place as described above. The

molds were removed, followed by an additional wash with PBS. Wells were then filled with 300 μ l of poly-L-lysine solution (PLL; 5 μ g/ml; Sigma-Aldrich) and incubated at 37°C for 1h. The wells were washed twice with PBS. Since it is unlikely that NrCAM would have occupied all of the potential binding sites on the culture surface, it is therefore likely that NrCAM stripes contained PLL as well. To prepare uniformly coated 24-well cell culture plates (Costar®, Corning Inc., Corning, NY USA), wells were filled with different concentrations of NrCAM solution (1 μ g/ml, 5 μ g/ml or 10 μ g/ml in PBS; R&D Systems) and incubated overnight at 37°C. The wells were washed twice with PBS. Controls were filled with PLL solution (5 μ g/ml; Sigma-Aldrich) and incubated at 37°C for 1h and washed twice with PBS. Prepared wells were then filled with 170 μ l of primary attachment medium, containing DMEM, 10% fetal bovine serum (Sigma-Aldrich), 25 mM HEPES buffer (both Gibco) and 300 U/ml Penicillin (Sigma-Aldrich).

Spiral Ganglion Dissection

Neonatal (postnatal day 5 (P5)) Sprague–Dawley rats were euthanized. The temporal bones were removed and further dissected similar to the method described by Van de Water and Ruben (1971). Briefly, the cochlear capsule was opened and the membranous labyrinth was removed from the modiolus. The spiral lamina containing the SG was carefully separated from the modiolus and transferred immediately into primary cell culture medium, where it was then cut into equal portions of 300 to 500 μ m before transfer into the prepared culture plates.

Cell Culture

SG explants was placed in the center of each well. They were first incubated for 24h at 37°C in the primary attachment medium, before the culture medium was changed to serum-free maintenance media (DMEM (Gibco), 25 mM Hepes buffer (Gibco), 6 mg/ml glucose (Gibco), 300 U/ml penicillin (Sigma-Aldrich) and 30 μ l/ml N2-supplement (Gibco). For trophic support of SGN survival and optimization of neurite outgrowth in the stripe assays, maintenance medium was supplemented with 10 ng/ml of recombinant BDNF (R&D Systems, Minneapolis, MN USA), which we have found to be optimal dosages (Kondo et al. 2013). Cultures were kept in a humidified incubator at 5% CO₂ and 37°C for 72h.

Immunohistochemistry

After fixation with 4% paraformaldehyde for 20 min at room temperature and two washes with PBS (Gibco), the explants were permeabilized with 5% triton X-100 (Sigma-Aldrich) for 10 min, washed twice with PBS and blocked for nonspecific antibody binding with 5% donkey serum (Sigma-Aldrich). Neurites were labeled for neurofilament using a mouse polyclonal 200 kDa anti-neurofilament primary antibody (1:400; Sigma-Aldrich). NrCAM stripes were visualized using polyclonal rabbit antibodies against NrCAM (1:400; R&D Systems). After primary antibody incubation overnight at 4°C, followed by two PBS washes, the neurites and stripes were visualized by 2.5h of incubation with fluorescein isothiocyanate (FITC) or Texas red (TR) conjugated secondary antibodies (1:100; Jackson ImmunoResearch, West Grove, PA USA) against the species of the respective primary antibody. Specificity of staining was confirmed by a series of negative control staining without primary antibodies.

Data Analysis

Digital images were obtained on an inverted fluorescence microscope (Olympus FSX100, Center Valley, PA USA) equipped with appropriate excitation and emission filters for FITC and TR. For the stripe assay, stripes were directly visualized using the NrCAM antibody. For publication in this manuscript, images were optimized to achieve uniform brightness and contrast using Adobe Photoshop. The number of neurites terminating on each individual stripe substrate was counted. Data for each experiment showed normal distribution (in a Kolmogorow–Smirnow test) and homogenous variances so that data were compared by Mann–Whitney U test using Statview 5.0 (StatSoft Inc., Tulsa, OK USA). Neurite outgrowth from the SG was evaluated by measuring the number and lengths of the processes. Images of the immunostained cultures were analyzed by using NIH ImageJ software (NIH, Bethesda, MD USA). Each neurite was traced and the number of neurites and average lengths of neurites per explant were analyzed using a one-way analysis of variance followed by a Bonferroni-corrected least-significant-difference post hoc test. Data presented in the text and figures are means and standard deviations. Results were considered to be significant when the likelihood for a type I error was less than 5% ($p < 0.05$); 16–20 SG explants were analyzed per experimental condition.

Results

Embryonic Expression of NrCAM

At embryonic day 14 (E14), NrCAM appeared to be associated with the thickened epithelial cells located in the Kollikers' organ (KIO), the nascent cochlear sensory epithelium (Fig. 1D, 1F). NrCAM immunoreactivity was detected at E17 in the region of the sensory epithelium with the hair cells (HCs) and supporting cells along the length of the cochlear duct, which in this stage have become arranged into the characteristic pattern for the organ of Corti (OC) (Fig. 1E, 1G). Some weaker staining was observed opposite the area of the thickened epithelia cells located in the KIO in the developing Reissner's membrane (Fig. G). During postnatal development, NrCAM staining became restricted to the area of the sensory epithelium. Antibody against Hes5 was used as a marker for supporting cells in the OC.

Postnatal NrCAM Expression

At birth (P0), within the OC, NrCAM labeling included the region of the inner HC (IHC), outer HC (OHC), Deiter's cells, and inner sulcus cells (Fig. 2C). At P5, NrCAM labeling was slightly decreased but still present near the IHC (Fig. 2D). By P10, there is no staining in the IHC, but in the area of the OHC (Fig. 2E). At P14, NrCAM labeling was strongest in the region of the OHC while at P21 NrCAM labeling was strong in the region surrounding the IHC and OHC (Fig. 2F, 2G). Antibody against myosin 7A was used as a HC-specific marker.

Dose-dependent Preferential Termination of Neonatal Rat SG Neurites on NrCAM versus PLL Stripes, and Neurite Extension Along NrCAM Stripes

When spiral ganglion explants were cultured on stripes of NrCAM alternating with PLL, more neurites terminated upon NrCAM stripes at the two highest concentrations tested (5

µg/ml and 10 µg/ml, respectively) than on PLL stripes (5 µg/ml) (Fig. 3, Mann–Whitney U test, $p < 0.05$). In addition, many (but not all) neurites turned onto and elongated along NrCAM stripes, especially at 10 µg/ml (Fig. 4). At the lowest NrCAM concentration used (1 µg/ml), all explants showed a radial outgrowth pattern, typical of neurite growth on uniform substrates, unlike the stripe tracking seen at the higher NrCAM concentrations (Fig. 4). At the intermediate NrCAM concentration used (5 µg/ml), most neurites showed a radial outgrowth pattern, but significantly more neurites terminated on NrCAM stripes.

NrCAM Does Not Influence SG Neurite Number or Length

Because NrCAM influences neonatal rat neurite direction and termination preference, we evaluated whether it might also affect neuritogenesis, neurite growth rate or branching, since substrate-specific differences in these variables could influence the probability of observed termination on one substrate versus another. When SG explants were cultured on a uniform NrCAM-coated surface (1, 5 or 10 µg/ml, respectively), the number of neurites per explant did not differ from the number on control explants grown on 5 µg/ml PLL (Fig. 5, ANOVA, $p > 0.05$, for all conditions). Figure 5 shows representative images of explants cultured on increasing coating concentrations of NrCAM (1, 5 or 10 µg/ml, respectively) and of an explant cultured on 5 µg/ml PLL. Similarly, the length of neurites did not differ between growth on NrCAM, as above, and control explants grown only on 5 µg/ml PLL (Fig. 5, ANOVA, $p > 0.05$, for all concentrations). Finally, no differences in branching of neurites was observed. (Fig. 5).

Discussion

To date, despite its importance at other central and peripheral nervous system sites, there have been no investigations of the expression or function of NrCAM in the inner ear. This motivated the present study. We documented the anatomical distribution of NrCAM in the mammalian inner ear from E14 until P21, and found that the molecule is strongly expressed in the target zones of spiral ganglion neuron afferent dendrites. Second, we determined whether NrCAM can modulate SG neurite outgrowth in an *in vitro* alternate choice assay. Finally, we analyzed the effect of NrCAM on neurite extension, neurite branching and neuritogenesis by quantifying the number of neurites extending from SGN and the effect on NrCAM on SG neurite growth rate *in vitro*.

During the past decades, the development of SGNs and their connectivity with sensory HCs has been extensively studied. A variety of axonal guidance molecules are expressed in the developing cochlea. Among them are Ephrins, Netrins, Semaphorins and Slit/Robos (reviewed in Coate and Kelly 2013). Moreover, patterns of extracellular matrix (ECM) molecules such as laminin, L1, fibronectin or collagen, which may be components of extracellular structures, be bound to their surfaces, or be expressed on the cell surface, can provide cues (e.g. Gillespie 2003; Gallo and Letourneau 2004; Hari et al. 2004; Oster et al. 2004; Yoshikawa and Thomas 2004). A wide variety of other molecules that reside on the surfaces of cells can provide directional signals at the cellular and the subcellular level (Marquardt et al. 2005). In the present study, we focused on NrCAM: a member of L1

family of immunoglobulin-class cell adhesion molecules like L1, NrCAM, CHL1 and Neurofascin.

We focused on the anatomical distribution on NrCAM on E14, E17 and the first 3 postnatal weeks, when important steps of cochlear and neural maturation occur (Coate and Kelly 2013, Pirvola et al. 1991). We found NrCAM to be expressed in the embryonic and postnatal mammalian cochlea. Interestingly, At E14, NrCAM is expressed in the thickened epithelial cells located in the KIO, a target of innervation by acoustic ganglion dendrites. At E17, the region of the sensory epithelium with the HCs and the supporting cells along the length of the cochlear duct have become arranged into the characteristic pattern of the OC. At this stage of development, NrCAM is preferentially expressed in the sensory cell region of the sensory epithelium, and again is the target zone for the innervation of the cochlea. At birth (P0), NrCAM is even more restricted, and is expressed preferentially around and underneath the IHC and OHC. At P5, NrCAM labeling is slightly decreased. However at P10, the levels of NrCAM is again strong in the target zone of the IHC and OHC (Fig. 2) and remains expressed until P21. It should be noted that hearing in the rodent cochlea begins on about P10 (Henley et al. 1989; Rybak et al. 1992), and reaches mature levels by P20.

The expression pattern of NrCAM is different from other molecules known to influence innervation of the HCs, unlike the ECM molecules laminin and fibronectin. NrCAM staining was not identified in the path of the SG dendrites, but only in the OC. Its effects are thus likely to be limited to neurite termination within the sensory epithelium. The lack of NrCAM expression in between the SG and the OC is consistent to its lack of influence in SG neurite length. NrCAM is expressed in the developing OC during embryonic development and becomes restricted around and underneath the IHC and OHC at birth. This is unlike L1 – another member of the family of immunoglobulin-cell adhesion molecules. In the newborn mouse cochlea, L1 was localized on nerve fibers in the SG and along the spiral lamina. Interestingly, in the OC, L1 labeling was observed only in the IHC region. There was no labeling in the region of the OHCs (Whitlon et al. 1999).

We evaluated the effects of NrCAM on SG neurites, beginning with an alternate choice stripe assay. We found dose-dependent, preferential termination of P5 neonatal rat SG neurites on NrCAM versus PLL. These findings are in line with the expression pattern of NrCAM by immunohistochemistry where NrCAM is expressed in the dendrite target regions of the sensory epithelium in a progressively restricted manner that focuses on the IHC and OHC. Taken together, these observations strongly suggest that NrCAM may serve to attract SG dendrites toward the HCs.

In our stripe assay experiments, we used organotypic explants from the cochlear SG. Besides neurons, the explants contained supporting cells, including fibroblasts and Schwann cells, which reside between the neurons *in vivo*. Both cells are known to provide guidance cues to advancing neurites and growth cones, and therefore might have influenced the observed neurite patterns in our experiments. However, these supporting cells grew out in a homogenous, radial way from the explant (Fig. 4). This suggests that the response of SG neurites to NrCAM is more likely to have been mediated directly, rather than via Schwann cells or fibroblasts, although this possibility must still be considered.

The mechanism by which NrCAM influences type I SG neurite growth is less clear from our study. It might be argued that the apparent preference of NrCAM versus PLL is due to a slower growth rate of type I SG neurites on the NrCAM surface compared with PLL. Therefore, the type I SG neurites would spend more time on the NrCAM surface and be found more often on the NrCAM substrate. However, the fact that a range of coating concentrations of NrCAM on a uniform surface did not influence type I neurite growth rate compared with PLL controls argues against this (Fig. 5). Another potential explanation for a different distribution of the type I SG neurites on the stripes is branching. If the substrate on one stripe increases branching of the neurites in response to the substrate, more type I SG neurites might be found on that particular stripe. However, again, increasing coating concentrations of NrCAM on a uniform surface did not influence type I neurite number compared with PLL controls (Fig. 5). In addition, differences in neuronal survival on different substrates could also influence the number of fibers found to terminate on them. However, there was no difference in neurite number on various NrCAM concentrations compared to PLL. Finally, the observation of preferential stripe tracking along NrCAM (Fig. 3, 4) strongly suggests preferential targeting and is inconsistent with any of these alternative mechanisms.

Hearing in the rat cochlea approximately begins on P10 (Henley et al. 1989; Rybak et al. 1992) and P5 prehearing neurons were studied since older neurons are more difficult to culture and neurite development is ongoing at this age (Ernfors et al. 1995; Echteler and Nofsinger 2000). It should be noted that we could not distinguish between the dendrites and axons of SGNs, since we have not found markers that distinguish between the two in explants.

There are two subtypes of SG neurons, the types I and II neurons (e.g., Reid et al 2004, Nayagam et al. 2011) – which have different cellular targets and functions. The dendrites of type I cells form afferent synapses exclusively with the IHCs, while the dendrites of type II cells exclusively receive synapses from the OHCs (Reid et al. 2004). Type II SGN innervate the OHC as early as E16.5 (Koudakjian et al. 2007), while type I SGN are rather believed to project terminals toward both IHCs and OHCs at birth (Echteler 1992; Huang et al. 2007; Simmons et al. 1994). Interestingly, we observed that NrCAM was expressed in the region of the developing OC at E17 and around and underneath the IHC and OHC at birth, suggesting a possible role of NrCAM in the innervation and synapse formation of both the IHC and OHC. The distribution of NrCAM did not suggest a potential for differential guidance between the two SGN types. Unfortunately, we could not distinguish between type I and type II SGN neurites, since peripherin labeling in the rat does not distinguish between these two classes of neurons, due to up-regulation of peripherin in type I neurons *in vitro* (Lallemend et al. 2007). However, since 95% of SGNs are type I cells, it seems likely that this class of neuron dominated our results.

Previously, we found that control conditions where the protein from the solution used to generate stripe patterns was omitted showed no SG neurites preferential termination (Brand et al. 2013). This raises the possibility that the mere presence of any protein might provide guidance signaling in comparison to PLL. However, we have previously found that stripes

of the neutral protein bovine serum albumin do not influence either SG neurite termination or direction (Evans et al. 2007).

Further studies will be needed to characterize auditory function in NrCAM knock-out animals and determine whether this molecule is necessary for the normal development of hearing. Similarly, the cellular mechanism by which NrCAM influences type I SG neurite growth could be probed using cell signaling inhibitors, as we have used for other guidance factors (e.g., Aletsee et al. 2001; Mullen et al. 2012; Euteneuer et al. 2013).

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Figure 1. Immunolabeling of NrCAM in the mouse cochlea at E14 and E17

Cell nuclei are in blue, NrCAM in red, Hes5 in green. (A, C) Overview of sagittal section of mouse head at the embryonic stage E14 and E17, stained with Hematoxylin/Eosine. Boxed is the cochlea. (B) The diagram of the embryonic OC. Source: <http://syllabus.med.unc.edu>. (D, E) Overview, NrCAM can be found in the KIO. In (D, F) NrCAM can be detected in epithelial cells located in the embryonic cochlear duct at E14. (E, G) At E17 immunofluorescence images, NrCAM can be observed in the OC. Hes5 staining served as marker for embryonic cochlea. (A, C) Image by light microscopy, (D–G) Images by immunofluorescence microscopy. 4 mice per embryonic day were analyzed. Scale bar = 50 μ m.

Figure 2. The protein expression pattern of NrCAM in the mouse cochlea from P0 through P21 Cell nuclei are in blue, NrCAM in red, Myosin 7A in green served as marker for HCs. (A) Diagram of the OC. Source: <http://syllabus.med.unc.edu>. (B) Negative control. (C) NrCAM labeling included the region of the IHC, OHC, and DC. (D) NrCAM labeling was slightly decreased. (E) Levels of NrCAM labeling is strong in the target zone of the IHC and OHC. (F) NrCAM labeling is strongest in the region of the OHC. (G) NrCAM labeling is strong at the IHC and OHC region. (B–G) Immunofluorescence microscopy images. 4 mice per postnatal day were analyzed. Scale bar = 100 μ m.

Figure 3. Quantitative analysis of termination by P5 rat SG neurites on alternating stripes of NrCAM (1 µg/ml, 5 µg/ml or 10 µg/ml, respectively) versus PLL (5 µg/ml)
Neurites showed a dose dependent preference of NrCAM. Bars represent mean and SD of neurites/explants. * $p < 0.05$, Mann-Whitney U test. 16–20 SG explants were analyzed per experimental condition.

Figure 4. Representative example of a P5 rat SG explant

Explants were cultured on an NrCAM (1 µg/ml, 5 µg/ml or 10 µg/ml, respectively) versus PLL (5 µg/ml) alternating stripe pattern for 72h and stained with an anti-neurofilament antibody (red) to visualize all SG neurites, and with an anti-NrCAM antibody (green) to visualize NrCAM. There was a tendency for neurites to terminate upon and/or grow along the NrCAM substrate at NrCAM concentrations of 5 µg/ml and 10 µg/ml. Stripe width 100 µm.

Figure 5.

(A) Effects of NrCAM on the number of neurites extending from SG explants cultivated on increasing concentration of NrCAM. Number of neurites did not differ from control explants grown on PLL 5 µg/ml. Mean of neurites/explant ± SD. **(B) Effects of NrCAM on the average length of neurites per SG explant cultivated on increasing concentration of NrCAM.** The average length of neurites did not differ from control explants grown on PLL 5 µg/ml. Mean length of neurites/explant ± SD. 16–20 SG explants were analyzed per experimental condition. **(C) Representative example for P5 rat SG explants grown on uniform NrCAM or uniform PLL surface.** Explants were cultured for 72h and stained with an anti-neurofilament primary antibody to visualize all SG neurons.