The cell adhesion molecule NrCAM is crucial for growth cone behaviour and pathfinding of retinal ganglion cell axons

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Summary

We investigated the role of the cell adhesion molecule NrCAM for axonal growth and pathfinding in the developing retina. Analysis of the distribution pattern of NrCAM in chick embryo retina sections and flat-mounts shows its presence during extension of retinal ganglion cell (RGC) axons; NrCAM is selectively present on RGC axons and is absent from the soma. Single cell cultures show an enrichment of NrCAM in the distal axon and growth cone. When offered as a substrate in addition to Laminin, NrCAM promotes RGC axon extension and the formation of growth cone protrusions. In substrate stripe assays, mimicking the NrCAM-displaying optic fibre layer and the Laminin-rich basal lamina, RGC axons preferentially grow on NrCAM lanes. The three-dimensional analysis of RGC growth cones in retina flat-mounts reveals that they are enlarged and form more protrusions extending away from the correct pathway under conditions of NrCAM-inhibition. Time-lapse analyses show that these growth cones pause longer to explore their environment, proceed for shorter time spans, and retract more often than under control conditions; in addition, they often deviate from the correct pathway towards the optic fissure. Inhibition of NrCAM in organ-cultured intact eyes causes RGC axons to misroute at the optic fissure; instead of diving into the optic nerve head, these axons cross onto the opposite side of the retina. Our results demonstrate a crucial role for NrCAM in the navigation of RGC axons in the developing retina towards the optic fissure, and also for pathfinding into the optic nerve.

Key words: NrCAM, Axonal CAMs, L1, Axon navigation, Embryonic retina, Central nervous system development, Time-lapse, 3D reconstruction

Introduction

Axonal pathfinding during development of the visual system is not yet well understood. Among some other proteins (see Discussion), three cell adhesion molecules (CAMs) of the immunoglobulin superfamily (IgSF) have been demonstrated to play a role in the navigation of retinal ganglion cell (RGC) axons: NCAM, L1 and DM-GRASP (Avci et al., 2004; Brittis et al., 1995; Pollerberg and Beck-Sickinger, 1993). The IgSF-CAM NrCAM, originally termed BRAVO, is an integral membrane protein present on retinal ganglion cell (RGC) axons (de la Rosa et al., 1990; Drenhaus et al., 2003; Grumet et al., 1991; Morales et al., 1996). A functional role of NrCAM in the navigation of RGC axons, however, had not been elucidated until now.

RGC axons are the first to be formed during retina development, and are the only ones to leave the eye and project to the optic tectum following stereotype pathways (Halfter and Deiss, 1986; Stahl et al., 1990). All RGC axons strictly extend towards the optic fissure (OF) in the central retina, fasciculating with other RGC axons and gradually building up the optic fibre layer (OFL). RGC axons then have to turn to grow towards the optic nerve head and into the optic nerve to leave the eye. Together with the differentiation wave spreading across the retina, RGC axonogenesis proceeds in a spatiotemporally controlled manner from the centre to the periphery.

Here, we report the impact of NrCAM on RGC axon functions. NrCAM as a substrate affects growth cone shape and axon advance, and is able to guide RGC axons when offered as substrate stripes. Three-dimensional (3D) reconstructions of RGC growth cones in the retina reveal the importance of NrCAM for growth cone size, complexity and directed shape. Time-lapse studies in retina flat-mounts show that NrCAM is required for straight and steady RGC axon routing to the optic fissure. In eyes organ cultured under NrCAM inhibition, RGC axons fail to leave the eye. Together, these findings demonstrate a crucial role of NrCAM for RGC axonal growth and pathfinding in the developing retina.

Materials and methods

Animals

Fertilized white Leghorn chicken eggs were obtained from a local provider and incubated at 38°C.

Antibodies

Monoclonal antibody (mAb) 2B3 and rabbit sera against NrCAM were produced as described (de la Rosa et al., 1990; Suter et al., 1995). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories; serum against Laminin (L9393) was purchased from Sigma. F(ab) fragments were generated according to Mage (Mage,
Affinity purification of NrCAM

NrCAM was immunoaffinity-purified from the brains of newly hatched chicks using a mAb 2B3 column as described before (de la Rosa et al., 1990). The purity of NrCAM was analysed by SDS-PAGE.

Axon growth assay

Single-cell cultures of embryonic day (E) 6 retinal cells were prepared as described (Avci et al., 2004; Halfter et al., 1983). Poly-L-lysine (PLL)-coated glass coverslips were incubated with either Laminin (5 μg/ml; Invitrogen) or a NrCAM/Laminin mixture (1.5 and 5 μg/ml, respectively). Only unipolar neurons with a process longer than 10 μm and a proper growth cone were counted as axon-forming. RGCs were identified in phase-contrast microscopy by the following morphological criteria: large, elongated soma (13 μm length, 8 μm width) compared with other neurons/neuroblasts (round, 7 μm diameter); and a single, strong axonal process (this was confirmed by staining with mAb RA4; a kind gift of S. McLoon, Minneapolis). Growth cone area and perimeter were measured using ImageJ (NIH). As a measure for the formation of protrusions/indentations, the ratio of the perimeter and square root of area was calculated for each growth cone. Significance of differences between mean values was determined by t-tests.

Axon preference assay

Preference assays were performed as described (Avci et al., 2004; Vielmetter et al., 1990), except that NrCAM (3 μg/ml) was employed. A substrate lane was counted as exerting axonal preference when it contained axons/axon bundles and at the same time was neighboured by lanes containing no axons (with the exception of a very few). A retinal explant strip was considered to be showing preference if its RGC axons respected the borders of at least 50% of the lanes of a given substrate.

Eye organ culture

Eyes of E4.5 embryos were isolated (pigment epithelium removed) and cultured for 24 hours as described (Avci et al., 2004). Eyes were incubated with NrCAM F(ab) fragments or non-specific F(ab) fragments (1 mg/ml). Retinae were then flat-mounted and DiO crystals (Sigma) were placed at a distance of 400-500 μm away from the OF to visualise groups of RGC axons. Retinae were evaluated using an inverted microscope (Axiovert 200M, Zeiss) equipped with a digital camera (AxioCam, Zeiss).

Retina flat-mount culture

Retinae of E4.5 embryos were spread out flat on nitrocellulose filters and pre-cultured in 200 μl Neurobasal medium (Invitrogen) for 1 hour in presence of NrCAM F(ab) fragments or non-specific F(ab) fragments (1 mg/ml). For live imaging, RGC axons were labelled by small DiO crystals (placed on the vitreal side of the retina) and monitored for up to 8 hours using a climate-controlled inverted microscope (37°C, 5% CO2; Axiovert 200M, Zeiss) equipped with a digital camera (AxioCam, Zeiss). Growth cone kinetics were computed using the Track-function of ImageJ (NIH) and were statistically analysed by t-test.

For 3D analysis of growth cone morphology, flat-mounted retinae were fixed after 1 hour in culture in the presence or absence of F(ab) fragments and RGC axons were labelled by DiI crystals. Images of growth cones were captured using a laser scanning confocal microscope (TCS SP2, Leica). At least 10 growth cones per retina (in at least four retinae) were examined in each experiment. 3D reconstructions were performed and the volume/surface of growth cones quantified using the Volocity system (Improvision, USA). Two categories with respect to growth cone shape were employed (Mason and Wang, 1997). ‘Simple growth cones’ are defined by an elongated or torpedo-like shape, and only occasional formation of short lamellipodial or filopodial protrusions. ‘Complex growth cones’ have a form that is approximately as broad as it is long with an irregular outline formed by abundant lamellipodia and filopodia. Significance of difference between frequencies of the two growth cone forms was determined by Chi-squared test.

Results

NrCAM is selectively present on RGC axons and growth cones from axon formation onwards

To visualise the distribution of NrCAM, immunolabelling of retina sections and retinal single cell cultures were performed (Fig. 1). In the E6 retina, when the maximum number of RGC axons extend, NrCAM is exclusively present in the OFL and optic nerve, i.e. selectively on RGC axons (Fig. 1A). RGC somata, which form the ganglion cell layer (GCL), as well as all other cells (undifferentiated neuroepithelial cells, NECs) are devoid of NrCAM (Fig. 1B). In sparse single cell cultures of E6 retina, immunolabelling shows that NrCAM is enriched in the distal region of the RGC axon and in its growth cone (Fig. 1C). This enrichment of NrCAM is only observed if the RGC axons exceed a length of 100 μm; in shorter axons NrCAM is homogeneously distributed (not shown). NrCAM is prominently present in the central and peripheral growth cone domain, including the finest filopodia.

Fig. 1. Localisation of NrCAM on RGCs. (A,B) Double labelling of a retina section stained by (A) NrCAM serum and (B) the nuclear marker DAPI. In the E6 retina, NrCAM is exclusively present in the optic fibre layer (OFL) and the optic nerve (ON), both formed by RGC axons. The ganglion cell layer (GCL), containing the RGC somata, is clearly NrCAM negative. The as yet undifferentiated neuroepithelial cells (NEC) are almost entirely NrCAM negative, with only a minor staining in the ventricular region near the pigment epithelium. (C) Single-cell cultures stained for NrCAM show that NrCAM is present on the entire RGC axon and is highly concentrated in its distal region. (D) NrCAM is strongly present in the central part of the RGC growth cone, and to a lesser degree on the lamellipodia and filopodia. Scale bars: (A,B) 100 μm; (C) 50 μm; (D) 10 μm.
Laser scanning microscopy of flat-mounted retinas stained for NrCAM allows the detection of its subcellular distribution in RGCs in the histotypic environment over the entire retina (Fig. 2). Retina flat-mounts show the developmental gradient from the centre to periphery. At E4, only the central retina is covered by RGC axon bundles, converging towards the optic fissure and growing into the optic nerve head. These axons are NrCAM positive (Fig. 2A). In the peripheral retina, thin axon bundles and also single axons are NrCAM labelled (Fig. 2B).

In addition, very young axons with a length of 10 to 20 μm are NrCAM positive, suggesting a role of NrCAM from an early phase of RGC axon growth onwards. Both RGCs in the peripheral retina just sending out their axon (Fig. 2B) and more mature RGCs in the central retina (Fig. 2C) carry NrCAM selectively on the axon and not on the soma, indicating an axon-specific role.

Together, the localisation studies show that NrCAM is present at the right time (phase of axon extension) and place (axon and growth cone) to play a role for growth and navigation of RGC axons.

**NrCAM enhances axon advance and the formation of growth cone protrusions**

Because in vivo RGC axons extend in contact with both the NrCAM-containing OFL and the Laminin-rich basal lamina, we investigated the impact of NrCAM, offered as substrate, on RGC axons in retinal single-cell cultures (Fig. 3). On glass coverslips coated with poly-L-lysine (PLL) only, RGCs merely form short axons (26±13 μm, n=94) within 1 day in vitro (div): Fig. 3A). Coating coverslips with PLL and NrCAM increases the overall axon length by 27% (33±7 μm, n=187, P<0.0006; Fig. 3A). The proportion of axons longer than 50 μm is increased threefold (Fig. 3B). Laminin-coating results in a high overall axon length (75±47 μm, n=236); if, in addition, coverslips are coated with NrCAM, overall axon length is increased by 18% (88±68 μm; n=298, P<0.017; Fig. 3A). The proportion of RGC axons longer than 100 μm is increased by 44% on NrCAM/Laminin when compared with axons on Laminin (Fig. 3B).

The data show that NrCAM is capable of enhancing axon extension by itself, causing an average increase in length of 7 μm. Moreover, axons extending on Laminin, which are considerably longer than those on PLL, gain an extra 13 μm in length if NrCAM is also present.

In contrast to its impact on axon extension, NrCAM does not have the capacity to promote axon formation. In E6 retinal single cells cultured on coverslips coated with PLL alone or with PLL and NrCAM, identical proportions of cells (6.6% (n=1309) and 6.4% (n=2868), respectively) form an axon within 1 div. Coating with Laminin in addition to PLL increases the number of axon-forming cells considerably; this proportion is not affected by additional coating with NrCAM [15% (n=1521) and 16% (n=1812), respectively]. These data are in concordance with RGC axon outgrowth in the retina taking place on the basal lamina, which provides Laminin but not NrCAM.

We also evaluated whether NrCAM as a substrate affects the morphology of growth cones. The average RGC growth cone size (area covered) is not significantly changed whether retinal cells are cultured on NrCAM/Laminin or on Laminin (111±45 μm² and 106±39 μm², respectively; Fig. 3C). By contrast, the growth cone perimeter is increased by 14% on NrCAM/Laminin when compared with Laminin [79±22 μm (n=121) and 69±22 μm (n=110), respectively; P<0.001; Fig. 3D]. The ratio of perimeter and square root of area as a measure of protrusion formation is significantly increased on NrCAM/Laminin compared with Laminin, by 16% (7.9±2.2 and 6.8±1.4, respectively; P<0.0002; Fig. 3E).

Together, these data show that NrCAM enhances RGC axon length and the formation of protrusions, indicating that...
NrCAM as a substrate not only has an impact on axon advance but also on form and explorative behaviour of growth cones.

**NrCAM lanes are capable of guiding RGC axons**

To address the question whether the restricted occurrence of NrCAM on RGC axons/axon bundles might be involved in axonal guidance, we employed an in vitro assay offering RGC axons alternating lanes coated with NrCAM/Laminin or Laminin only (Fig. 4). This assay reflects the in vivo situation of RGC axons in the retina, where RGC axons have the choice between a NrCAM/Laminin combination (in the OFL) and Laminin without NrCAM (in the neighbouring GCL and basal lamina). From almost all retinal explants (95%, n=56) axons extended with a clear preference for the NrCAM/Laminin lanes (Fig. 4A). When immunoglobulins, which like NrCAM are members of the IgSF, are coated as a control instead of NrCAM, RGC axons display random growth patterns (with 0% of explants exhibiting a preference, n=20; Fig. 4B). To further substantiate that axonal preference for the NrCAM/Laminin lanes is specifically caused by NrCAM, coverslips were pre-incubated with NrCAM F(ab) fragments, which makes the substrate-NrCAM inaccessible to its axonal binding partners (Fig. 4C). In this case, preference is almost completely abolished (preference observed in 8% of the explants, n=38). By contrast, when substrates are pre-incubated with non-specific F(ab) fragments, axonal preference for NrCAM is still clearly present (preference in 78% of the explants, n=27; Fig. 4D), indicating that pre-incubation with F(ab) fragments per se does not affect NrCAM preference.

Together, the data show that NrCAM is capable of directing RGC axon growth in vitro.
Role of NrCAM in axon growth and pathfinding

RGC axons to extend only where this CAM is present. In vivo NrCAM could thereby cause protruding RGC axons to grow along and keep to pre-existing RGC axons, guiding them towards the centre of the retina and into the optic nerve head.

**NrCAM is crucial for slim and correctly directed growth cones in the retina**

To investigate the impact of NrCAM on RGC axons and growth cones in vivo, we performed confocal microscopy studies on RGC axons in retina flat-mounts, which allow the 3D reconstruction of the growth cone morphology in the histotypic context (Fig. 5). In retinae kept in the presence of non-specific F(ab) fragments, RGC growth cones show a slim ‘streamlined’ morphology, focussed towards the optic fissure (Fig. 5A). Typically, one or very few filopodia are formed at the tip of the growth cone, which extend in contact with other axons/axon bundles and point in the growth direction (Fig. 5B). Only very rarely is a process extending away from the growth direction formed (Fig. 5B).

In the presence of NrCAM F(ab) fragments, growth cones display a complex morphology with an irregular shape: numerous lateral lamellipodia and filopodia are formed, which make the growth cones about as wide as they are long (Fig. 5C,D). The protrusions typically extend perpendicular to the growth direction, exploring the growth cone environment away from the correct pathway. Some of these protrusions, which can extend up to 10 μm, reach through the forming OFL into the GCL. Occasionally the entire growth cone was found to be bending away from the original growth direction (towards the fissure); this is never observed under control conditions (Fig. 5D). These growth cones explore the environment to the left and right of the correct pathway, yet still stay in contact with the basal membrane. Entire growth cones diving into deeper retinal layers are not observed.

Quantitative analysis of the 3D micrographs (Fig. 5E-H) reveals that the number of complex growth cones is more than doubled in retinae under NrCAM inhibition when compared with controls (45% and 20%, respectively; P<0.01; Fig. 5E). The proportion of growth cones with two or more protrusions is almost 50% higher than in control retinae (79% and 53%, respectively; P<0.01; Fig. 5F). Under NrCAM inhibition, the growth cone surface is enlarged by 19% when compared with controls (3298±896 μm² and 2770±926 μm², respectively; P<0.003; Fig. 5G) and the growth cone volume is enlarged by 32% (322±122 μm³ and 244±131 μm³, respectively; P<0.0016; Fig. 5H). The increase in surface area is proportionate to the increase in volume; the formation of lateral protrusions (increasing surface area) is counterbalanced by the shorter shape of the growth cone.

**Fig. 5. NrCAM inhibition modulates growth cone morphology.** (A-D) Two examples each of growth cones in flat-mounted retinae (E4.5) in the presence of non-specific F(ab) fragments (A,B) or NrCAM F(ab) fragments (C,D); top and side view of each growth cone is depicted; orientation of the growth cones in the retina is as indicated in D. Under control conditions, growth cones typically have a simple slim ‘torpedo-like’ shape (A,B). UnderNrCAM inhibition, growth cones acquire a complex morphology with a surplus of protrusions and a shorter, irregular form (C,D); in C, four protrusions (1-4) of a growth cone are indicated. Protrusions are directed towards deeper layers of the retina (3,4 in C; D) or to the left and right of the growth direction (1,2 in C). Occasionally the entire growth cone bends away from the correct growth direction; in contrast to the protrusions, it does not dive into the retina but stays in contact with the basal lamina (D). (E-H) Quantification of changes in growth cone morphology. (E) When NrCAM is inhibited, the proportion of complex growth cones is significantly increased compared with under control conditions. (F) The number of growth cones with two and more protrusions is significantly increased under NrCAM inhibition compared with controls. (G) The surface area of growth cones under NrCAM inhibition is significantly larger than those under control conditions. (H) NrCAM inhibition also causes a significant increase in growth cone volume P<0.01. OF, optic fissure; VIT, vitreal side of the retina; VEN, ventricular side of the retina. Scale bar: 10 μm. (See also the 3D reconstructions and rotations in Movies 1 and 2 in the supplementary material.)
Together, our 3D microscopy data show that the presence of NrCAM is crucial for the formation of slim growth cones focussed towards the optic fissure. The absence of functional NrCAM leads to large and complex growth cones extending away from the correct growth direction, indicative of aberrant explorative activities.

**NrCAM is crucial for efficient advance and direct routing of RGC axons towards the optic fissure**

To analyse the role of NrCAM for growth cone dynamics and axonal orientation in vivo, time-lapse studies of RGC axons navigating in retina flat-mounts were performed. In the presence of non-specific F(ab) fragments, RGC axons \( n=25 \) grow towards the optic fissure in a straight fashion, no axon was observed to deviate from the correct growth direction (see Movie 3 in the supplementary material). Moreover, the axons strictly extend on RGC axons/axon bundles and do not stray away. By contrast, under NrCAM inhibition 20% of the axons \( n=30 \) detach from the axons/axon bundles and turn away at various angles from the direct route to the optic fissure (Fig. 6A-F; see Movie 4 in the supplementary material); some even turn towards the periphery. Half of these axons did not correct their deviations, the other half re-orientated towards the OF. Such misrouting or wandering of RGC axons was never observed in controls. Under NrCAM inhibition axons proceed slower and show a less steady forward movement with long pauses (Fig. 6E,F). Short stops, however, are also observed under control conditions. In the pause phases, growth cones show a strong explorative behaviour, with numerous filopodia being rapidly formed and retracted, both in controls and under NrCAM inhibition.

Phases of axon growth and pausing alternate (Fig. 7A,B). The overall advance speed in control retinae is \( 81\pm19 \mu m/hour \); the elongation rate measured selectively during the advance phase is \( 117\pm32 \mu m/hour \) \( n=25 \). When NrCAM is inhibited, the overall advance speed of RGC axons is reduced by 17% \( (67\pm25 \mu m/hour, P<0.025, n=30; \text{Fig. 7C}) \). The elongation rate in the growth phases \( (125\pm48 \mu m/hour) \), however, is not significantly changed (Fig. 7C). Inhibition of NrCAM does not make axons slower but increases the length of pauses by 34% when compared with controls \( (6.0\pm2.5 \text{ minutes and } 4.4\pm1.6 \text{ minutes, respectively; } P<0.013; \text{Fig. 7D}) \). Concomitantly, advance phases are 25% shorter than in controls \( (6.2\pm2.2 \text{ and } 8.3\pm3.9 \text{ minutes, respectively; } P<0.017; \text{Fig. 7D}) \). As a result, a 23% shorter distance is covered per advance phase under NrCAM inhibition. The frequency of pauses \( (4.9\pm1.6/hour) \) and advance phases \( (5.6\pm1.4/hour) \) under control conditions is not altered when NrCAM is inhibited \( (5.2\pm1.6/hour \text{ and } 5.6\pm1.6/hour, \text{respectively}) \). Retractions are rare events under control conditions; only one of the observed axons \( n=25 \) showed such a behaviour (Fig. 7A). By contrast, when NrCAM is inhibited, 50% of the axons \( n=30 \) exhibited one or more retractions. The average frequency of retraction events is only 0.7/hour and therefore the average loss of axon length is merely 5 \( \mu m \) (Fig. 7A,B). Thus, the less efficient advance of axons under NrCAM inhibition is mainly due to longer pauses and shorter advance phases, only to a minor degree is it due to the more frequent retractions.

Taken together, the data show that, in the retina, NrCAM keeps advance phases long, pauses short, and the frequency of RGC axon retractions low. Presence of NrCAM also limits

**Fig. 6.** Axon navigation in the retina under NrCAM inhibition. (A-D) Axons growing towards the optic fissure in a retina flat-mount under NrCAM inhibition (top view). (A) Four axons (marked 1-4) grow on pre-existing axons and have large, complex growth cones. Axon 1 forms a growth cone with wide, laterally exploring protrusions (B), detaches from the other axons and turns away at an almost rectangular angle (C). This aberrant growth direction, perpendicular to the pathway to the optic fissure, is maintained for more than 20 minutes (D). Axon 2 turns away from the other axons at a smoother angle (B,C); later it performs a compensatory turn, returning to the correct direction (D). At 54 minutes, axon 3 also starts to deflect (D). Axon 4 grows towards the optic fissure during the observation period, displaying a growth behaviour typically found under control conditions. (E,F) Growth cone kinetic plots of two axons each, growing in retina flat-mounts in control conditions (E) and with NrCAM inhibition (F); each dot represents the position of the growth cone neck localised every two minutes (observation time in brackets). The lower plot in F corresponds to axon 2 shown in A-D. Under control conditions, axons grow rather straight and steadily towards the optic fissure (E). Under NrCAM inhibition the pathway is more crooked and deviating from the correct direction; in addition long pauses and retractions are observed (F). The overall distance covered is considerably shorter under NrCAM inhibition; note that the observation period is about 20% longer in F than in E. Scale bar: 20 \( \mu m \).
Role of NrCAM in axon growth and pathfinding

We analysed the impact of NrCAM on RGC axonal pathfinding at the optic fissure; here, the axons have to perform rectangular turns to dive into the optic nerve head and leave the retina. E4.5 chick eyes were kept in culture as intact organs for 1 div in the presence of NrCAM F(ab) fragments or non-specific F(ab) fragments. Retinae were then spread out as flat-mounts and groups of RGC axons labelled for the detection of their routing (Fig. 8). In eyes organ-cultured under control conditions (n=13), RGC axons turn at the optic fissure to dive into the optic nerve head and into the optic nerve; none of the control retinae axons pass across the optic fissure (Fig. 8A,B). By contrast, aberrant axons extending across the optic fissure were observed in 50% of the NrCAM-inhibited retinae (n=16). Most of these misrouting axons covered a substantial distance on the opposite side of the retina. These axons followed the RGC axon bundles in an anti-parallel fashion, not leaving the OFL, but aberrantly heading towards the periphery (Fig. 8C,D). Taken together, the data show that NrCAM plays a crucial role in RGC axon pathfinding at the optic fissure, and in their turning into the optic nerve head to leave the retina.

Discussion

Besides NrCAM, three other IgSF-CAMs have been shown to be functionally involved in the routing of RGC axons to the centre of the retina/optic nerve. Inhibition of N-CAM causes misrouting in the vicinity of the OF (Pollerberg and Beck-Sickinger, 1993). L1 inhibition results in misrouting in the rat midretina, with RGC axons heading back to the periphery (Brittis et al., 1995), a phenomenon observed only once in our time-lapse studies. DM-GRASP was demonstrated to play a role for RGC axon navigation to/into the optic nerve head in chick and mice (Avci et al., 2004; Weiner et al., 2004). IgSF-CAMs are present on probing growth cones as well as on pre-existing axons, therefore they could function both as sensory (receptor) and/or substrate (ligand) molecules.

‘Classical’ receptor/ligand systems also contribute to RGC axon guidance. In retinae deficient for the Ephrin receptors EphB2/EphB3, axons split away from the direct trajectory to the optic nerve head and fail to reach it (Birgbauer et al., 2000). Deficiency for Netrin or its receptor DCC results in a failure of RGC axons to exit into the optic nerve (Deiner et al., 1997). Netrin lines the optic nerve head, forming a cuff around the RGC axons, probably acting in a repulsive manner (together with Laminin) (Hopker et al., 1999). Suppression of Slit1, a ligand that acts positively on RGC axons, causes abnormal axon trajectories towards the optic nerve head (Jin et al., 2003). Overexpression/inhibition of Sonic hedgehog (Shh), a released protein that promotes and guides RGC axons in vitro, leads to a loss of centrally directed projections of RGCs (Kolpak et al., 2005). Chondroitin sulphate proteoglycan, an inhibitory/repellent ECM component located in the peripheral retina, is necessary to prevent RGC axons from growing into the periphery (Brittis et al., 1992). The proteins mentioned above do not form gradients across the developing retina and therefore cannot serve as peripheral-central cues for RGC axon navigation. The chemokine SDF1
is expressed by optic stalk cells (of zebrafish embryos), and conceivably might spread over the retina as an attractive central-peripheral gradient (Li et al., 2005). SDF1 does not attract (chick) RGC axons; however, it does reduce their repulsion by Slit2 (Chalasani et al., 2003). The transcription factor Zic3 is expressed in a gradient (high in periphery) and might induce the expression of inhibitory factors for RGC axons; its overexpression induces abnormalities in RGC projections towards the optic nerve head (Zhang et al., 2004).

**NrCAM distribution in the developing retina**

In the E6 retina, NrCAM is selectively present on the axons of RGCs and is absent from their somata. A few other CAMs are also restricted to RGC axons in this phase: L1/NgCAM (Rathjen et al., 1987b), DM-GRASP (Avci et al., 2004; Pollerberg and Mack, 1994), Neurofascin (Rathjen et al., 1987a), TAG1/Axonin 1 (Ruegg et al., 1989), Thy 1 (Sheppard et al., 1988), and F3/F11/Contactin (Rathjen and Rutishauser, 1984). The absence of these CAMs from the RGC somata could make the GCL a zone that is avoided by RGC axons, and thus limit their growth to the OFL.

The enrichment of NrCAM in the distal axon portion and growth cone suggests that NrCAM acts as a membrane receptor probing the environment. NrCAM could, however, play a dual role, as RGC axons extend on older RGC axons, which present NrCAM as a growth substrate, i.e. as a ligand. In vitro, in alternating stripe assays or homogeneous substrate assays for example, the selective role of substrate-NrCAM for the RGC axons can be studied. Inhibition of NrCAM in vivo affects both the NrCAM on growth cones (receptor-NrCAM) and on axons (substrate-NrCAM); the two functions cannot be separated. NrCAM could interact homophilically (Mauro et al., 1992) and/or heterophilically with TAG1/Axonin 1 (Suter et al., 1995), F3/F11/Contactin (Morales et al., 1993), and Neurofascin (Volkmer et al., 1996), which are known interaction partners present in the OFL at this stage.

**Impact of NrCAM on axons and growth cones**

NrCAM offered as a substrate is by itself not able to promote axon formation in single RGCs. Initial axon outgrowth therefore seems to be independent of NrCAM. This is in concordance with the observation that, in the retina, initial outgrowth takes place in contact with the basal lamina in an environment devoid of NrCAM. By contrast, once the RGC axon is extending, NrCAM by itself is capable of increasing axon advance as a substrate. This could reflect the phase of axonal growth when the RGC axon has contacted the first NrCAM-displaying (older) RGC axon, conceivably increasing axon elongation. This is probably enhanced by other IgSF-CAMs. L1 is capable of promoting RGC axon growth by itself (Morales et al., 1996). DM-GRASP and F3/F11/Contactin promote axon growth of other neuron types, but not of RGCs (DeBernardo and Chang, 1995; Treubert and Brummendorf, 1998), indicating a differential use of receptors or intracellular signalling pathways in various neuron types.

RGC axons extend in the retina with their growth cones in contact with both the basal lamina, which is strongly positive for Laminin, and, as soon as they have reached a RGC axon, with older RGC axons carrying NrCAM. We therefore tested the impact of NrCAM as co-substrate of Laminin on RGC growth cones. In vitro, NrCAM substrate induces RGC growth cones to form more protrusions; however, growth cone size is not influenced. By contrast, L1 and DM-GRASP lead to enlarged growth cones as co-substrates (Avci et al., 2004; Burden-Gulley et al., 1995). The specific effect of NrCAM on growth cone shape but not on size is suggestive of an instructive role of this CAM rather than a merely adhesive function.

In contrast to the in vitro assays, in the developing retina NrCAM is only presented as a growth substrate by RGC axons/axon bundles. If NrCAM is undisturbed, the pre-existing axons focus the growth cones to extend on them and thereby guide them towards the OF. Under inhibition of NrCAM, more growth cone protrusions are formed, extending in all directions, including away from the substrate axons. This is the first study revealing such details of growth cone morphology in the histotypic context in 3D. These findings do not contradict the observation of increased protrusion formation caused by substrate-NrCAM in vitro. In both cases, substrate-NrCAM induces the formation of protrusions; in vitro, NrCAM is ubiquitously present in the growth cone environment and therefore causes a unidirectional formation of protrusions all around the growth cone. In vivo, the focussing impact of the selective occurrence of NrCAM on RGC axons/axon bundles...
is lost when NrCAM is inhibited: growth cones and substrate-axons are not able to interact effectively, thus, growth cone protrusions extend away from the substrate axons. This could be the first step initiating the growth cones to splay off the axon bundles and, ultimately, leading to axonal misrouting.

To analyse the impact of NrCAM on axon extension, we offered this CAM as a co-substrate of Laminin and observed a significant increase in RGC axon length. An increase in axon length has been reported for the two axonal CAMs, F3/F11/Contactin and DM-GRASP, offered as co-substrates of ECM molecules (Avci et al., 2004; Treubert and Brummendorf, 1998); NrCAM was shown to increase axon growth when offered together with NgCAM (Morales et al., 1996). Our data indicate that substrate-NrCAM probably triggers intracellular signalling pathways independently of integrin signalling, which can be assumed to be well stimulated by the Laminin concentrations offered in vitro. The increase in axon length by NrCAM in addition to the one caused by Laminin is in concordance with our findings that the advance of RGC axons in their histotypic environment, which provides both Laminin and NrCAM, is significantly reduced by NrCAM inhibition. In particular, in vivo axon advance does not only depend on elongation rates, but also on the frequency and duration of the pauses (filled with exploration) and retractions. The less efficient advance of RGC axons in the retina in absence of functional NrCAM is due to longer pauses and shorter advance phases, and not to a decreased elongation rate. The strong increase of retractions under NrCAM inhibition does not severely affect axon advance. Inhibition of other CAMs (L1 in rat or Neurolin in fish retina) does not seem to increase retractions (Britts et al., 1995; Leppert et al., 1999). This is the first study providing insight in the differential impact of a CAM on axonal advance, pause, exploration and retraction in a histotypic context.

Role of NrCAM in axonal pathfinding

The role of NrCAM in axonal pathfinding has been studied in a histotypic context for two other neuron types: proprioceptive and commissural axons in the spinal cord are no longer able to grow under NrCAM inhibition (Perrin et al., 2001; Stoeckl and Landmesser, 1995). Here, the IgSF-CAMs TAG1/Axonin 1 and F3/F11/Contactin in the axonal membrane interact with NrCAM, which is present on cells in the environment. Inhibition of NrCAM turns the growth cone environment and prospective pathway (spinal cord grey matter, floor plate) from permissive into repulsive. In NrCAM-deficient mice, however, no defects in axon orientation at the floor plate have been observed (More et al., 2001). In the developing retina, NrCAM acts differently, as inhibition of NrCAM does not make the OF repulsive for RGC axons. In the environment of the RGC axons, NrCAM (on pre-existing RGC axons heading into the optic fissure) serves as a positive guidance cue defining the correct pathway; this is the first study demonstrating such a role for NrCAM. NrCAM lanes can indeed guide RGC axons, as seen in our substrate stripe assays. Under inhibition of NrCAM, RGC axons in the stripe assays leave the NrCAM lanes but, in the retina, they do not stray away from the OF. This is probably because of the selective presence of other axon-specific proteins in the OF, and the GCL acting as a ‘no entry zone’.

At the OF, where RGC axons dive into the optic nerve head to leave the retina, inhibition of NrCAM results in axons overshooting onto the opposite side of the retina. At the OF, RGC axons have to perform rectangular turns to dive into the optic nerve head, which conceivably requires a stronger association of their growth cones to the pre-existing axons than just tracking on a straight route does. In addition, a minor detachment of an axon from its bundle caused by NrCAM inhibition results in a substantial misrouting at the OF, as the axon is irreversibly splayed off and is not ‘picked up’ by other bundles running in parallel, as is the case on its way towards the OF. The observed effects could be due to a reduction in adhesion, as homo- and heterophilic trans-interactions between the extending axon and the pre-existing axon bundle might be weakened by NrCAM inhibition. However, NrCAM – which is not an abundant CAM – might rather have an instructive effect on navigating RGCs, e.g. by interactions with membrane proteins (TAG1/Axonin 1 or F3/F11/Contactin), triggering signal cascades, and/or by acting on cytoskeletal components in the growth cone (Davis and Bennett, 1994; Faivre-Sarrailh et al., 1999). Effects caused by NrCAM inhibition cannot be (sufficiently) counterbalanced by other mechanisms despite the presence of five other axonal IgSF-CAMs, indicating an irreplaceable, pivotal role of NrCAM in axonal pathfinding.

It is tempting to speculate that the restricted presence of proteins selectively on RGC axons – such as NrCAM – might be sufficient for RGC pathfinding into the optic nerve. Axonogenesis advances strictly regulated from the central to the peripheral retina: the first emerging RGC axons in the immediate vicinity of the optic fissure could find the exit by random probing and/or local cues, the growth cone itself already covering this short distance (about 20 μm). Later emerging RGC axons, further away from the optic fissure, also only have to span a very short distance to find the next RGCaxon (less than 15 μm) (McCabe et al., 1999) (P.Z. and G.E.P., unpublished), which could be based on random search, too, and from then on could travel on the pre-existing axons interacting with axon-specific proteins. In this scenario, the crucial gradient in the retina is not a guidance molecule gradient (which has not been found until now) but the differentiation gradient, which spreads over the retina and spatio-temporally controls RGC axon formation.

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Supplementary material

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