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Glial cell line-derived neurotrophic factor (GDNF) induces neuritogenesis in the cochlear spiral ganglion via neural cell adhesion molecule (NCAM)

Sara Euteneuer^{a,d,e,f,*}, Kuo H. Yang^a, Eduardo Chavez^a, Anke Leichtle^{a,d}, Gabriele Loers^e, Adel Olshansky^a, Kwang Pak^a, Melitta Schachner^e, and Allen F. Ryan^{a,b,c,**}

Sara Euteneuer: sara.euteneuer@med.uni-heidelberg.de; Allen F. Ryan: afryan@ucsd.edu

^aDepartment of Surgery/Otolaryngology, University of California San Diego, School of Medicine, 9500 Gilman Drive #0666, La Jolla, CA 92093-0666, USA

^bDepartment of Neurosciences, University of California San Diego, School of Medicine, 9500 Gilman Drive #0666, La Jolla, CA 92093-0666, USA

^cVeteran's Affairs San Diego Health Care Systems, San Diego, CA 92161, USA

^dDepartment of Otorhinolaryngology — Head and Neck Surgery, University of Lübeck, Ratzeburger Allee 160, Haus 28, 23538 Lübeck, Germany

^eZentrum fuer Molekulare Neurobiologie Hamburg, University Medical Center Hamburg-Eppendorf, Falkenried 94, 20251 Hamburg, Germany

^fHeidelberg University Hospital, Department of Otorhinolaryngology, Im Neuenheimer Feld 400, 69120 Heidelberg, Germany

Abstract

Glial cell line-derived neurotrophic factor (GDNF) increases survival and neurite extension of spiral ganglion neurons (SGNs), the primary neurons of the auditory system, via yet unknown signaling mechanisms. In other cell types, signaling is achieved by the GPI-linked GDNF family receptor $\alpha 1$ (GFR $\alpha 1$) via recruitment of transmembrane receptors: Ret (re-arranged during transformation) and/or NCAM (neural cell adhesion molecule). Here we show that GDNF enhances neuritogenesis in organotypic cultures of spiral ganglia from 5-day-old rats and mice. Addition of GFR $\alpha 1$ -Fc increases this effect. GDNF/GFR $\alpha 1$ -Fc stimulation activates intracellular PI3K/Akt and MEK/Erk signaling cascades as detected by Western blot analysis of cultures prepared from rats at postnatal days 5 (P5, before the onset of hearing) and 20 (P20, after the onset of hearing). Both cascades mediate GDNF stimulation of neuritogenesis, since application of the Akt inhibitor Wortmannin or the Erk inhibitor U0126 abolished GDNF/GFR $\alpha 1$ -Fc stimulated neuritogenesis in P5 rats. Since cultures of P5 NCAM-deficient mice failed to respond by neuritogenesis to GDNF/GFR $\alpha 1$ -Fc, we conclude that NCAM serves as a receptor for GDNF signaling responsible for neuritogenesis in early postnatal spiral ganglion.

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*Correspondence to: S. Euteneuer, Heidelberg University Hospital, Department of Otorhinolaryngology, Im Neuenheimer Feld 400, 69120 Heidelberg, Germany. Fax: +49 6221 56 4641. **Correspondence to: A.F. Ryan, University of California, San Diego, Department of Surgery, Div. Otolaryngology, 9500 Gilman Drive #0666, La Jolla, CA 92093-0666, USA. Fax: +1 858 534 5319.

Contributions

S.E., K.H.Y., E.C., A.O. and K.P. performed and analyzed rat P5 tissue culture the experiments; S.E. performed mouse tissue culture experiments; S.E., E.C. and G.L. performed Western blotting experiments; S.E. performed RT-PCR, H&E staining and immunohistochemistry. S.E. and G.L. performed confocal imaging as well as image analysis. A.L. analyzed H&E images. S.E. and A.F.R. designed the experiments and obtained funding. S.E., G.L., M.S. and A.F.R. interpreted data and wrote the article.

Keywords

Glial cell line-derived neurotrophic factor; Spiral ganglion; Neural cell adhesion molecule; Rearranged during transformation; Neurite outgrowth; Hearing loss

Introduction

The major reason for severe hearing impairment is the loss of inner and outer hair cells (HCs), the sensory cells of the inner ear. This can be due to many causes, including aging, noise exposure and ototoxic drugs (Lefebvre et al., 2002). HC loss is often followed by degeneration of primary cochlear afferent neurons (Stamatakis et al., 2006), the spiral ganglion neurons (SGNs), which relay auditory information to the brain. Furthermore, SGNs may also degenerate without HC loss (Starr et al., 2003). In cases of near or complete HC loss resulting in deafness, central auditory function, i.e. hearing, can be restored via electrical stimulation of surviving SGNs with a cochlear implant in humans. However, cochlear implantation, and thus electrically evoked auditory nerve activity, did not enhance SGN survival in pharmacologically deafened or congenitally deaf animal models (e.g. Chen et al., 2010). Thus, enhancement of SGN survival, initiation of dendritic outgrowth from these neurons and maintenance of their intrinsic electrophysiological features (Davis and Liu, 2011) are desirable to further improve the performance of this prosthesis.

Glial cell line-derived neurotrophic factor (GDNF), first identified as a trophic factor for embryonic midbrain dopaminergic neurons (Lin et al., 1993), has been found to be a potent SGN survival factor *in vitro* and *in vivo* (e.g. Yagi et al., 2000; Ylikoski et al., 1998). For intracellular signaling, GDNF first binds to glycosylphosphatidylinositol (GPI) anchored GDNF-family receptor $\alpha 1$ (GFR $\alpha 1$), but, since GFR $\alpha 1$ lacks an intracellular signaling domain, it must recruit a trans-membrane receptor to induce intracellular signaling. Ret (rearranged during transformation) and the 140 kDa isoform of NCAM (neural cell adhesion molecule) have each been identified as receptors that can bind to the GDNF/GFR $\alpha 1$ complex and induce subsequent intracellular signaling, primarily via the PI3K/Akt and/or MEK/Erk MAPK pathways (Airaksinen et al., 1999; Jing et al., 1996; Paratcha et al., 2001).

GDNF, GFR $\alpha 1$, Ret and NCAM have been detected in SGNs as well as in the cochlear sensory epithelium (Ohgami et al., 2010; Stöver et al., 2000; Whitlon and Rutishauser, 1990; Ylikoski et al., 1998). However, the signaling mechanisms of the GDNF/GFR $\alpha 1$ complex in SGNs have not been investigated, and it is not known whether the reported survival effects are mediated by NCAM, Ret or both molecules, downstream of the initial binding of GDNF to GFR $\alpha 1$. We, therefore, analyzed GDNF and GDNF/GFR $\alpha 1$ effects *in vitro* using neo- and perinatal rat and mouse SGNs in organotypic tissue culture, and examined the significance of intracellular signaling cascades by both Western blot analysis of the PI3K/Akt and MEK/Erk MAPK signaling pathways in combination with specific inhibitors of these pathways. We further evaluated the downstream effects of GDNF via GFR $\alpha 1$ in SGNs derived from the NCAM knock-out (KO) mouse. Since Ret KO mice die at birth, the importance of Ret as a receptor for postnatal GDNF signaling via GFR $\alpha 1$ could not be investigated.

Results

GDNF, GFR $\alpha 1$, Ret and NCAM are expressed in the developing and adult SGNs of rats

To determine the components of the GDNF signaling complex and the downstream signal transduction pathways in the SG, we first measured the expression of components of cognate GDNF signaling complexes in SGNs. Therefore, reverse transcription PCR (RT-

PCR) and Western blot analysis were performed to examine messenger RNA (mRNA) and protein levels in this ganglion. Cell lysates were prepared from rat SGNs at different time points during development before and after the onset of hearing (about P10). mRNAs of GDNF, GFR α 1, as well as of the 140 kDa isoform of NCAM were amplified at different time points from P3 to P180. Low levels of the Ret mRNA were detected at P10 (Fig. 1A), with trace levels barely detectable at P3, P5 and P30.

At the protein level, the specificity of the 1 β 2 NCAM antibody was first confirmed on crude brain lysates from an adult NCAM WT mouse and P23 rat spinal cord where bands of NCAM120, 140 and 180 kDa isoforms were detected, while they were absent in brain lysate from an adult NCAM KO littermate (Fig. 1B). In rat spinal cord lysates (P23) and in P0 brain lysates from the Ret mutant mouse line, the antibody reliably detected higher molecular weight polysialylated NCAM, a posttranslational modification of NCAM typically seen during development (Fig. 1B, for review, see Maness and Schachner, 2007). The Ret antibody specifically detected a single band of approximately 150–170 kDa in the P0 WT brain lysate corresponding to fully glycosylated Ret, which was absent in the brain lysate from P0 Ret KO mice (Fig. 1C).

In lysates from P3 to P180 SGNs, NCAM-140 and NCAM-120 were detected in a developmentally regulated manner while the levels of the NCAM-180 were below the detection limit (Fig. 1D). Remarkably higher molecular weight polysialylated NCAM, regularly seen in the neonatal central nervous system - for comparison see Fig. 1B showing P23 rat spinal cord and mouse P0 whole brain lysate -, was not observed in the SG at any age.

Despite the presence of GFR α 1- mRNA and low levels of Ret-mRNA throughout SG development and in the adult, we could not detect either GFR α 1 or Ret protein in SG lysates using various antibodies listed in Experimental methods section. The Ret antibody reliably detected a protein band of the correct size in mouse brain lysates processed alongside the rat SG lysates (Fig. 1C), while we could not detect specific GFR α 1-bands even in mouse brain lysates.

The GDNF antibody reliably detected ~36 kDa and ~50 kDa bands in lysates from adult and neonatal mouse brain (Fig. 1E), consistent with the fully glycosylated, disulfide-bonded homodimers of mature GDNF and of pro form homodimers of GDNF, respectively (Geng et al., 2011; Lonka-Nevalaita et al., 2010). The ~36 kDa band was clearly observed from P5 to P30 in SGNs as well as in brain (Fig. 1D, E). The ~50 kDa band was well detected in SGNs during early development up to the onset of hearing (P10), and then decreased to undetectable levels in the adult (Fig. 1D). Bands consistent with GDNF monomers were not detected at any age in SGNs or brain.

The inability to detect GFR α 1 and Ret protein in SG lysates after having detected the respective mRNAs prompted us to probe frozen sections from P20 cochlea for GFR α 1 and Ret protein by immunohistochemistry (Fig. 1F). Using this technique with much lower detection thresholds than Western blotting, we detected co-localization of GFR α 1 and Ret protein (as well as GDNF and NCAM) in P20 SGNs.

Ret and NCAM KO mice show normal SGN numbers but increased afferent dendrite innervation of the organ of Corti in developing NCAM KO mice

To determine the significance of the two cognate GDNF receptors Ret and NCAM for SGN survival, neurogenesis and target innervation *in vivo*, we analyzed SGN numbers and the innervation patterns of afferent dendrites in the organ of Corti in P0 Ret KO mice (Fig.

2A1–4), in P0 NCAM KO mice (Fig. 2B1–4), in P5 NCAM KO mice (Fig. 2C1–4), and in P49 (7 weeks) NCAM KO mice (Fig. 2D1–4).

The number of SGNs appeared to be normal in Ret KO mice at P0 when compared to their corresponding WT littermates (data not shown, $p=0.27$). Because Ret KO mice die soon after birth, neuron numbers as well as dendrite distribution had to be assessed at P0 in this mouse line and could not be evaluated shortly before or after the onset of hearing. There were no obvious differences in P0 Ret KO mice in the innervation patterns of afferent dendrites, including the radial dendrites of type I SGNs targeting the inner HCs (IHCs, $p>0.89$, Fig. 2A3) and the spiral dendrites of type II SGNs targeting the three rows of outer HCs (OHCs, $p>0.08$, $p>0.26$ and $p>0.51$, Fig. 2A4) when compared to their WT littermates.

In the NCAM KO mouse, SGN numbers in mid-modiolar cross-sections were comparable between WT and KO mice during development (P5, $p=0.1$, data not shown) and in adulthood (P49, $p>0.82$, data not shown). There were also no obvious differences in P0 NCAM KO mice in the innervation patterns of afferent dendrites, including the radial dendrites of type I SGNs targeting the IHCs, ($p>0.21$, Fig. 2B1–B3) and the spiral dendrites of type II SGNs targeting the three rows of OHCs ($p>0.29$, $p>0.25$ and $p>0.21$, Fig. 2B1–2 and B4) when compared visually to their corresponding WT littermates. However at P5, the time point in development at which the innervation in the organ of Corti is refined and surplus dendrites are eliminated (Huang et al., 2007), the number of radial dendrites projecting to IHCs was increased in whole mount preparations of P5 NCAM KO mice when compared to their WT littermates (68.3 ± 8.9 versus 54.6 ± 7.3 , $p<0.0001$, Fig. 2C1–3). Innervation of the three rows of OHCs by spiral dendrites was also increased in P5 NCAM KO whole mounts as compared to the WT littermates ($p<0.011$, $p<0.009$, $p<0.04$, Fig. 2C4). Interestingly, this increased innervation was not observed in the mature organ of Corti (P49) (Fig. 2D1–D4), indicating a transient difference between KO and WT innervation pattern.

GDNF stimulates neurite outgrowth in rat P5 SG explants in vitro

Having observed a significant effect of NCAM on the innervation pattern in the organ of Corti *in vivo* in P5 mice, we utilized an organotypic culture system for analyzing and manipulating the effects of GDNF stimulation in the SG *in vitro*. Given that the mRNA data indicated that all elements of GDNF signaling are present in SGs, and given that Western blot analysis revealed high GDNF and NCAM-140 protein levels in P5 SGs (Fig. 1D), this developmental time point was chosen for functional analysis of the effects of GDNF and GFR α 1 on rat SG explants *in vitro*. In culture, SG explants extended an average of 27.2 ± 9.9 neurites per explant in serum-free control cultures. To determine a dose–response for neurite extension, 12.5 to 150 ng/ml GDNF was added to the culture medium. The number of neurites extending from SG explants increased in a dose-dependent manner, reaching a significant increase in neurite number per explant at 100 ng/ml GDNF (39.8 ± 8.3) and 150 ng/ml GDNF (47.6 ± 15.0 neurites/explant, one-way ANOVA: $F=7.7$; $p<0.0001$, Tukey's post-hoc tests $p<0.001$; examples in Fig. 3A, B, E). However, GDNF did not increase the total lengths of extending neurites, which were similar in GDNF-treated and in control cultures (one-way ANOVA: $F=1.7$; $p=0.06$; Fig. 3A, B, F). GDNF also did not enhance migration of neuronal cell bodies out of the explants nor did it produce obvious alterations in neurite branching patterns. In many explants, neurites emanated in a radial pattern only from one side of the explant (Fig. 3B, D), as generally seen by us and others in organotypic cultures of these bipolar neurons.

Exogenous GFR α 1 enhances the stimulatory effect of GDNF on neurite number in rat P5 organotypic cultures

To determine whether GPI anchored GFR α 1 could serve as a functional ligand-binding receptor for GDNF in SGs, we evaluated its effect on SG explants, both alone and in combination with GDNF. When 12.5 to 150 ng/ml GFR α 1-Fc alone was added to the culture medium in a dose-response experiment, no increase in number or length of neurites per explant was observed (27.0 ± 11.4 neurites with 150 ng/ml GFR α 1-Fc alone, one-way ANOVA $p > 0.44$; examples in Fig. 3A, C, E, F) suggesting that intrinsically available GDNF levels are too low to stimulate activity even in the presence of an exogenously added receptor in the form of GFR α 1-Fc.

When 12.5 to 100 ng/ml GFR α 1-Fc were presented in combination with 100 ng/ml GDNF, neurite numbers per explant were further increased beyond that seen with GDNF alone (39.8 ± 8.3). In particular, GFR α 1-Fc levels of 50 ng/ml GFR α 1-Fc (50.5 ± 5.0) and 100 ng/ml GFR α 1-Fc (50.3 ± 12.1) significantly enhanced the effects of GDNF while lower GFR α 1-Fc levels of 12.5 and 25 ng/ml in combination with 100 ng/ml GDNF did not boost neurite numbers any further than GDNF (one-way ANOVA: $F = 7.8$; $p < 0.0001$, examples in Fig. 3A–E, Tukey's post-hoc test $p < 0.03$). Again, neurite length per explant was unaffected ($F = 1.7$; $p = 0.06$, Fig. 3A–D, F) as were branching and radial patterns of extending neurites.

GDNF/GFR α 1-Fc stimulation of rat SG explants induces Akt and Erk phosphorylation and inhibition of these signaling pathways reduces GDNF/GFR α 1 induced neurite outgrowth

Having established the stimulatory effect of 100 ng/ml GDNF and 100 ng/ml GFR α 1-Fc on neurite outgrowth in SGN explants *in vitro*, we evaluated the downstream signaling pathways mediating this effect. The transduction of signals from GPI-anchored GFR α 1 after GDNF binding requires the recruitment of a transmembrane signaling receptor, either Ret or NCAM-140. After GDNF/GFR α 1 binding, Ret can either signal via the PI3K/Akt- or MEK/Erk-pathways depending upon its localization in lipid raft or non lipid raft compartments at the cell membrane, while NCAM is well recognized to signal via the MEK/Erk-pathway after activation of the Src-family kinase Fyn (Beggs et al., 1997; Paratcha and Ledda, 2008). Stimulation of PI3K and Akt via NCAM has also been reported (Ditlevsen et al., 2008a, 2008b).

To investigate a possible activation of the MEK/Erk- or PI3K/Akt-pathways downstream of GDNF in SGs, we first probed lysates of freshly dissected P5 rat SG explants, stimulated with 100 ng/ml GDNF/GFR α 1-Fc for up to 120 min, and P5 rat SG explants cultured for 24 h, for the phosphorylation status of Erk-1/-2 and Akt. Western blot analysis revealed strong, sustained phosphorylation of Akt for 120 min (Fig. 4A). After 24 h, Akt signals were still enhanced when compared to signals obtained from unstimulated cells (Fig. 4C). Erk phosphorylation peaked at 5 to 15 min with GDNF/GFR α 1 stimulation, then decreased below baseline at 120 min of stimulation and returned to basal levels after 24 h (Fig. 4A, C). In fresh lysates from P20 SGs, however, Erk phosphorylation briefly peaked at 5 min and then showed a sustained increase above baseline levels from 30 to 120 min after stimulation. Fresh SG lysates from P20 rats showed a barely detectable baseline Akt phosphorylation, which increased noticeably for up to 120 min after GDNF and GFR α 1 stimulation (Fig. 4B).

We then evaluated the effects of the irreversible PI3K inhibitor Wortmannin (Ui et al., 1995) and the MEK-1/-2 noncompetitive inhibitor U0126 (Duncia et al., 1998) on GDNF/GFR α 1 stimulated SGN explant cultures (Fig. 5). Since both inhibitors were dissolved in DMSO, a solvent potentially harmful to developing neurons (Hanslick et al., 2009), we first investigated the effects of DMSO on our GDNF/GFR α 1-stimulation assay of P5 SGN explants. DMSO even at 0.05%, a higher volume percentage than the 0.01% applied during

the inhibitor experiments, slightly decreased the number of neurites per explant in the GDNF/GFR α 1 stimulated P5 SGN explants (Fig. 5C, J, K, L), an effect which was not statistically significant. In contrast, we found that Wortmannin eliminated the stimulatory effects of GDNF/GFR α 1 on SGN explant neurite numbers at concentrations of 10 and 100 nM ($F=5.3$; $p<0.0004$; Fig. 5C, D, E), while there was no effect of 100 nM Wortmannin alone (data not shown), indicating a role for PI3K signaling downstream of GDNF/GFR α 1 in SGNs. There was also no effect of Wortmannin on neurite length, either in control or in GDNF/GFR α 1 treated cultures ($F=1.2$; $p>0.31$; Fig. 5F). Similarly, the MEK-1/-2 inhibitor U0126 had no effect on SGN explant cultures by itself up to 100 nM (data not shown), while it abolished the stimulatory effects of GDNF/GFR α 1 on SGN neurite number at 10 and 100 nM ($F=8.6$; $p<0.0001$; Fig. 5C, G, H), indicating a role for MEK/Erk signaling downstream of GDNF/GFR α 1. Again, neurite length remained unaffected ($F=1.3$; $p<0.26$; Fig. 5I).

SGN explants from NCAM KO mice fail to respond to GDNF and GFR α 1

Having observed activation of both the MEK/Erk as well as the PI3K/Akt pathways in SGNs with strong effects of inhibition of either pathway on SGNs *in vitro*, and since both Ret and NCAM are competent to activate either pathway upon GDNF/GFR α 1 stimulation, we attempted to investigate the effects of GDNF/GFR α 1 stimulation on SGN explants from both KO mice lines to identify the receptor that mediates the GDNF/GFR α 1 signaling effects in SGNs *in vitro*. Because of early neonatal lethality, Ret KO mice had to be evaluated at P0. However, in WT mice neither GDNF nor GDNF/GFR α 1 stimulated neurite numbers at this early developmental stage (data not shown). We therefore could not evaluate GDNF/GFR α 1 stimulation utilizing this KO model. We and our collaborators (personal communication) were also unable to effectively block Ret-function in tissue culture by commercial or laboratory-made c-Ret antibodies or peptides. Therefore we could not further elucidate the role of this receptor in mediating GDNF/GFR α 1 function in SGNs.

As described for P5 rats, explants of SG from P5 NCAM KO and WT mice were exposed to 100 ng/ml GDNF or 100 ng/ml GDNF/GFR α 1-Fc (Fig. 6A, B). WT SGN explants were observed to respond to GDNF and GDNF/GFR α 1-Fc with a strong increase in neurite number (Fig. 6C, 2-way-ANOVA, treatment effect $p<0.001$, Tukey's post hoc tests $p<0.036$ and $p<0.006$). In agreement with the observations on explant cultures from rat SGNs, neither GDNF nor GDNF/GFR α 1 increased the neurite length of SGNs in P5 WT mice (Fig. 6D, 2-way-ANOVA $p>0.67$). In contrast to explants from WT mice, GDNF/GFR α 1 did not produce a significant increase in neurite number when applied to SG explants from NCAM KO mice. These results indicate that NCAM functions as the receptor in SGNs to mediate the GDNF/GFR α 1 signaling in the neonatal SG.

To determine whether additional mechanisms for activation of PI3K or MEK-1/-2 are detectable in the SGNs of NCAM KO mice, the PI3K inhibitor Wortmannin and the MEK-1/-2 inhibitor U0126 were added to SGN explant cultures from P5 NCAM KO and WT mice (Fig. 7). As observed in the P5 rat SGN cultures, neither 10 nM Wortmannin nor 10 nM U0126 affected the number of neurites in WT cultures without GDNF/GFR α 1-Fc stimulation (Fig. 7B1–3, D, one-way ANOVA $p>0.66$). Moreover, neither 10 nM Wortmannin nor 10 nM U0126 decreased the number of neurites in cultures from NCAM KO mice (Fig. 7A1–3, C, one-way ANOVA $p>0.77$), indicating that the role of these signaling pathways in neurite extension is specific to NCAM activation.

Discussion

In the present study, we have investigated the substrates of GDNF signaling in SGNs. We present evidence that GDNF utilizes the 140 kDa isoform of NCAM as a transmembrane

receptor in SGNs after GFR α 1 binding for mediating its neuritogenic effect in the neonatal cochlea. However, NCAM does not appear to influence SGN survival.

SGN numbers in the developing organ of Corti are normal in Ret and NCAM KO mice

SGN counts revealed no significant differences in the numbers of SGNs in the P0 Ret KO mouse nor in the NCAM KO mouse from P0 to P49. Similarly, Ohgami et al. (2010) observed comparable neuron densities in the SG of homozygous c-Ret Y1062F phosphorylation deficient mice at P2.5. However, later during development (P8 and P14), they observed lower SGN densities in these mutant mice, implicating neuroprotective c-Ret signaling via this phosphorylation site, known to be activated by GDNF/GFR α 1 (Tsui-Pierchala et al., 2002; Wells and Santoro, 2009), in more mature SGNs. Due to early postnatal lethality of the Ret KO mouse, we could not investigate these later developmental stages in our *in vitro* GDNF stimulation assay. In the NCAM KO mouse line, however, SGN counts *in vivo* were still comparable, with no obvious signs of neuronal pathology in the ganglion, in mice aged up to 7 weeks (P49).

Transient hyperinnervation in the organ of Corti of P5 NCAM KO mice

Having seen comparable numbers of SGNs in NCAM and P0 Ret KO mice when compared to their WT littermates, we went on to determine the relevance of the two possible GDNF receptors for innervation of the organ of Corti *in vivo*. We found no differences in afferent dendritic innervation of IHCs and OHCs in P0 NCAM KO and P0 Ret KO mice compared to WT mice. In contrast, we found increased afferent innervation of IHCs by radial dendrites of type I SGNs as well as increased innervation of the three rows of OHCs by spiral dendrites of type II SGNs in NCAM KO mice compared to the WT at P5, the developmental stage used in our *in vitro* experiments. This developmental stage is the critical period of dendritic refinement and retraction during development of the organ of Corti in C57Bl6/J mice (Huang et al., 2007). Huang et al. (2007) described an initial mismatch with both type I and type II SGNs extensively innervating both IHCs and OHCs at P0. By P6, the innervation pattern had reached the mature pattern with type I neurites innervating IHCs and type II neurites innervating OHCs, confirming the transient hyperinnervation and consecutive withdrawal of neurites. Since the neurofilament immunostaining used in our study does not differentiate between type I and type II neurites, we can only speculate about the cause for the transiently observed hyperinnervation in the NCAM KO at P5 *in vivo*.

It should be noted that Ret KO mice could not be investigated at P5 due to neonatal lethality. It is thus possible that changes would have been observed in Ret KOs at this later age which would be interesting to investigate, since Ohgami et al. (2010, 2012) found that homozygous c-Ret Y1062F phosphorylation deficient mice displayed loss of SGNs in the adult cochlea, indicating that Ret becomes more important as a signaling molecule in SGNs at some point after P0. Unfortunately, these mice were not available for our study.

Both GDNF and GDNF/GFR α 1 stimulate SGN neurite number in vitro

Application of GDNF in organotypic SGN explant cultures of developing rats (P5) increased the number of neurites extending from the SGN explants in a dose-dependent manner, suggesting an important role for GDNF in SGN survival and/or neuritogenesis. These observations are akin to those of others in SGNs (Stöver et al., 2007; Wefstaedt et al., 2005; Wei et al., 2007) and other neuronal cell types (Ledda et al., 2002; Paratcha et al., 2001); e.g. Wefstaedt et al. (2005) found that 100 ng/ml of GDNF increased the number of neurite-bearing SGNs in dissociated neuron cultures. The fact that GDNF application enhanced SGN neurite numbers in P5 rat and mouse SG explants indicates the presence and activation of an endogenous GDNF receptor in this ganglion. Furthermore, endogenous levels of GDNF in SGN cultures are apparently low, since application of GFR α 1 alone did not

enhance SGN neurite numbers. It can be speculated that SGN explants *in vitro* might lack a GDNF source that is present *in vivo*, such as represented by the organ of Corti or neurons in the cochlear nucleus complex (Del Fiacco et al., 2002; Stöver et al., 2000). Alternatively, GDNF production by neurons or glia in the SG may be down-regulated in culture.

In this study, co-application of GDNF and exogenous GFR α 1-Fc further increased the effect of GDNF on neurite number in developing rat SGN explants in a dose-dependent manner. This suggests that soluble GFR α 1-Fc, not inserted into the SGN plasma membrane, retains the ability to bind GDNF and signaling receptors, as has been shown in nodose and sympathetic neurons (Ledda et al., 2002), in murine Neuro2a and cerebellar granule cells transfected with Ret (Tansey et al., 2000), as well as in an immortalized neural precursor cell line from embryonic rat Raphe nucleus and motoneurons (Paratcha et al., 2001). Furthermore, our data suggest that rat SGN neurons contain sufficient transmembrane receptors to complex with the additional GFR α 1. Interestingly, in P5 mice the addition of exogenous GFR α 1-Fc did not increase neurite number beyond that observed with GDNF alone, suggesting that in mice, unlike to the rat, endogenous GFR α 1 receptors may fully recruit all of the available transmembrane signaling receptors in SGNs.

GDNF/GFR α 1 stimulation activates Akt and MEK/Erk signaling and inhibition of both signaling cascades inhibits GDNF effects in vitro

GDNF stimulation alone produced little or no Erk or Akt phosphorylation in prehearing (P5) SGN explants, whereas GDNF/GFR α 1-Fc co-stimulation induced strong Akt stimulation and moderate Erk phosphorylation in SGNs from both pre-hearing (P5) and hearing (P20) SGNs. The activation of both pathways in SGs, containing SGNs as well as Schwann cells, is in agreement with the activation of both the PI3-K/Akt and MEK/Erk signaling cascades in other systems (Hauck et al., 2006; Paratcha et al., 2001, 2003; Tansey et al., 2000; Zhang et al., 2006). For instance, Hauck et al. (2006) reported simultaneous phosphorylation of Erk and Akt in rat retinal Mueller cells after GDNF stimulation and Zhang et al. (2006) detected changes in both Erk and Akt phosphorylation in Ret-transfected PC12 cells after GDNF stimulation. Since both pathways were activated with GDNF alone, this suggests ample GFR α 1 expression in these glial cells as well as the presence of either one or both transmembrane signaling receptors. Also in agreement with our observation in SGNs, Tansey et al. (2000) noted enhanced Erk and Akt phosphorylation in murine Neuro2a cells co-transfected with Ret and GFR α 1, and Paratcha et al. (2001) described a more pronounced phosphorylation of Akt and Erk downstream of Ret in an immortalized motoneuron-derived cell line as well as in sensory and sympathetic neurons, when co-stimulated with GDNF/soluble GFR α 1 than with GDNF stimulation alone.

Differences in downstream signaling from GDNF interactions with GFR α 1 appear to be widespread. In our study, the PI3-kinase inhibitor Wortmannin as well as the MEK/Erk inhibitor U0126 completely abolished GDNF- and GDNF/GFR α 1-stimulated increases in neurite number on rat P5 SGN, strongly implicating both pathways. Similarly, Paratcha et al. (2001) observed the abolishment of GDNF/GFR α 1-mediated neurite initiation in an immortalized motoneuron-derived cell line treated with the PI3K inhibitor LY294002. As in rat SGNs, they observed comparable effects when replacing LY294002 with the MEK/Erk inhibitor PD98059, indicating that both signaling pathways are required for neurite initiation in these cells. In contrast, Villegas et al. (2006) observed a reduction of GDNF pro-survival effects in a rat glial-like cell line only after concurrent treatment with both MEK/Erk- and PI3K-inhibitors, but not with either inhibitor alone, suggesting multiple signaling via the two downstream pathways.

Transmembrane GDNF receptors Ret and NCAM are differentially expressed in SGNs during development

As noted above, responsiveness of SGNs to GDNF and GDNF/GFR α 1 suggests the presence of endogenous signaling receptors that mediate intracellular signaling after GDNF binding to GFR α 1. Previous work in other systems has indicated that both Ret and NCAM 140 can function as transmembrane signaling receptors with GFR α 1 (Jing et al., 1996; Paratcha et al., 2003). Indeed, mRNAs for GDNF, GFR α 1 and NCAM 140 were observed at all stages from birth through adulthood, consistent with Stöver et al.'s (2000) identification of GDNF and GFR α 1 mRNAs in adult rat SGNs as well as Simonneau et al.'s (2003) immunohistochemical detection of NCAM protein in developing and adult rat SGNs.

While we did observe Ret m-RNA during early postnatal development consistent with Stöver et al. (2000), we did not detect Ret protein in SGNs by Western blot analysis, hinting at the possibility that immunohistochemical methods could be more sensitive. This is confirmed by our immunohistochemical detection of Ret in SGNs of P20 rats, and Ohgami et al.'s (2010) immunohistochemical observation of phosphorylated Ret protein increasing in mouse SGNs throughout postnatal development, with the highest levels in young adults. The same argument could apply to our inability to detect GFR α 1 protein by Western blot analysis, since GFR α 1 mRNA were detected in early postnatal to adult SGNs and GFR α 1 protein immunohistochemically in P20 SGNs.

Regarding GDNF in rat SG, we only observed bands at ~36 kDa consistent with fully glycosylated mature GDNF homodimers, and at ~50 kDa consistent with pro-form homodimers of GDNF, respectively (Geng et al., 2011; Lonka-Nevalaita et al., 2010), but not the 16 kDa or 25 kDa bands consistent with either monomers. The functional significance of pro-GDNF isoform is presently unresolved. It has been suggested that it may have functions distinct from those of mature GDNF (Geng et al., 2011), as has been observed for the pro-forms of other neurotrophins. With this in mind, it is noteworthy that pro-GDNF expression predominates in the early postnatal SG, but that mature GDNF predominates at mature ages. Clarification of the function of pro-GDNF may thus be of importance for SGN development.

SG explants from P5 NCAM KO mice lack the ability to respond to exogenous GDNF/GFR α 1, implicating NCAM as a receptor for GDNF/GFR α 1

It is noteworthy in this context that SGNs from WT mice failed to respond to exogenous GDNF/GFR α 1 stimulation *in vitro* at P0, an age where only the pro-form but not the mature GDNF homodimer could be detected *in vivo*. Due to the impossibility to investigate the role of Ret in mediating GDNF signaling utilizing our explant culture assay, we tried to functionally block Ret signaling in tissue culture utilizing anti-Ret antibodies. However, testing of a number of commercial as well as laboratory-made anti-Ret antibodies all resulted in partial activation, but not blocking, properties. Moreover, silencing RNAs are not effective in SG explants in general, perhaps due to the myelin sheath that encases even the cell soma of SGNs. It thus remains to be investigated which functions would be affected by blockade of Ret.

Regarding the functions of NCAM, P5 NCAM KO mice failed to respond to GDNF/GFR α 1 stimulation while SGNs from WT littermates showed the robust increase in neurite number that we observed in rat P5 SGN. So far, only in one study (Wei et al., 2007) the effects of GDNF on mouse SGNs were investigated in SGNs from adult C57BL/6J mice, the background strain of the NCAM KO mouse line used in the current experiments, failing to detect a response to GDNF after culture for 7 days. However, early postnatal SGNs were not used in this study and a dose of GDNF (100 pg/ml) was used, being substantially lower than

doses that were effective in our hands as well as those of several investigators using other systems (e.g. Hauck et al., 2006; Uchida et al., 2006; Villegas et al., 2006), including SGs of different species (Maruyama et al., 2007; Stöver et al., 2007). Numbers of cell bodies per explant could not be discerned in our explants, being too bulky for visualization of individual structures within. However, *in vivo* we observed normal SGN numbers in P5 and in mature (P49) NCAM KO mice rendering the failure to detect an increase in neurite numbers upon GDNF/GFR α 1 stimulation in NCAM KO mice *in vitro* unlikely to be due to a loss in numbers of neurons.

In fact, the current finding that GDNF does not stimulate neurite extension in cultured SGNs from NCAM KO mice implicates NCAM as a functional receptor for GDNF/GFR α 1, at least in early postnatal ages. NCAM dependency of GDNF induced neurite outgrowth has been confirmed in cultured embryonic hippocampal and cortical neurons (Nielsen et al., 2009; Paratcha et al., 2003) which do not express Ret. Paratcha et al. (2003) observed severely compromised neuritogenesis upon GDNF stimulation in NCAM KO neurons when compared to WT neurons. This NCAM dependency of neuritogenesis upon GDNF stimulation in cultures of hippocampal neurons was confirmed by Nielsen et al. (2009). In both studies an increase in length of neurites was reported rather than an increase in neurite number as observed in the present study, pointing towards a unique response of SGNs to GDNF/GFR α 1 stimulation. Furthermore, it is likely that cochlear neurons behave differently from nodose neurons, in which Ret but not NCAM activates PI3K/Akt signaling upon GDNF/GFR α 1 stimulation (Paratcha et al., 2001). It would have been desirable to validate the role of NCAM using a function-blocking antibody as reported by Paratcha et al. (2003) in rat SGNs. However, when we applied antibodies against the extracellular domain of NCAM to SGN explants, they stimulated neuritogenesis, making them unsuitable for potentially inhibiting the response of GDNF signaling in our model system. Application of these antibodies in culture can indeed result in activation of NCAM functions (Kleene et al., 2010; Schuch et al., 1989).

It is noteworthy that our *in vivo* observations of increased organ of Corti innervation in NCAM KO mice at P5 conflict with our *in vitro* observation that lack of NCAM abolished the stimulatory effect of GDNF/GFR α 1 on P5 NCAM KO SGN neurite number. One explanation for the observed discrepancy might be the substantial difference in the cell types present *in vivo* when compared to our *in vitro* experiments: As described in Experimental methods section, SGN explants for culture were dissected at the level of Rosenthals's canal, by cutting off the distal part of the dendrites and the organ of Corti containing their target cells. Yet, these IHCs and OHCs produce neurotrophic factor receptors, such as the FGF receptor and TrkB, both known to interact with NCAM and to modulate NCAM-mediated neurite outgrowth (Cassens et al., 2010; Ponimaskin et al., 2008). Another potential explanation for this observation might be increased branching of terminal neurites, innervating multiple HCs. However, we did not see obvious increases in branching in P5 NCAM KO explant cultures. Another possible explanation is failure to retract surplus neurites from the IHCs and OHCs in the NCAM KOs during maturation of the organ of Corti (Huang et al., 2007). Interestingly, Rafuse et al. (2000) observed transient hyperinnervation of the musculus senitendinosus neuromuscular junction of NCAM KO mice at P11, at a stage when WT littermates already showed singly innervated muscle fibers of the mature innervation type.

Conclusions

The present study demonstrates that developing SGNs *in vitro* respond to GDNF with an increase in the number of neurites, with addition of exogenous GFR α 1 increasing this effect. Both PI3K/Akt and MEK/Erk signaling pathways mediate these *in vitro* effects. Moreover,

our results suggest that considerable differences exist in GDNF-mediated activation patterns in SGNs before and after the onset of hearing. Thus, at early postnatal ages NCAM appears to be the dominant signaling receptor that partners with GFR α 1 in mediating the neurotogenic effects of GDNF. At later stages, when neuritogenesis is complete, Ret is an important mediator of SGN survival, as demonstrated by Ohgami et al. (2010, 2012).

Experimental methods

Animals

The local animal subject committees of the Veterans Affairs San Diego Health Care Systems and the University of Lübeck approved all procedures in accordance with the guidelines laid down by the National Institute of Health regarding the care and use of animals for experimental procedures (NIH Publication No. 85-23, revised 1996). Animals were deeply anesthetized with an intraperitoneal injection of rodent cocktail (ketamine 60 mg/kg, xylazine 6.4 mg/kg, acepromazine 1.2 mg/kg) before decapitation for SGN removal.

Spiral ganglion (SG) explants were obtained from postnatal day 5 and 20 (P5, P20) Sprague–Dawley rats (Harlan, Indianapolis, IN, USA), as well as P0, P5 and P49 NCAM KO mice (strain B6.129P2-*Ncam*^{tm1Cgn/J}, Jackson Laboratories, Bar Harbor, ME, USA and a kind gift from Dr. Harold Cremer (Cremer et al., 1994)). Explants were obtained only from P0 Ret KO mice (a gift from Dr. Costantini, Columbia University, NY, USA; Schuchardt et al., 1994) due to postnatal lethality of the *Ret* gene deletion. Corresponding wild-type (WT) littermates, generated from the heterozygous breeding pairs, yielded control tissue.

Histology

Whole cochleas were isolated from P0, P5 and P49 (7 weeks) NCAM KO mice, as well as P0 Ret KO mice. WT tissue was from littermate controls. Cochleas were immediately immersed in 4% PFA in 0.1 M phosphate buffer. Cochleas were manually perfused with this buffer through the oval and round windows, post-fixed over night at room temperature (RT) and washed with phosphate buffered saline, pH 7.4 (PBS). P49 cochleas from the NCAM KO mouse line were then decalcified in 10% EDTA (pH=8) for 1 week at RT with slight agitation. For analysis of frozen sections, cochleas were cryopreserved with 30% sucrose overnight, followed by mounting in Shandon Cryomatrix™ (Thermo Electron Corp., Milford, MA, USA) and 30% sucrose (1:1) and frozen in liquid nitrogen. Seven and 12 μ m sections were cut on a CM1900 cryostat microtome (Leica Microsystems, Wetzlar, Germany) and gathered on Superfrost Ultra Plus glass slides (Menzel Glaeser, Braunschweig, Germany).

Haematoxylin and eosin (H&E) staining was performed according to standard protocols (Mayer's acid Haematoxylin, Hollborn & Sons, Leipzig, Germany; Eosin G 0.5% aqueous solution, Carl Roth®, Karlsruhe, Germany).

For immunohistochemistry, cell membranes were permeabilized with 5% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA) for 10 min, followed by blocking with 5% donkey serum in PBS (Sigma-Aldrich) for 20 min. Sections were then stained with mouse monoclonal anti-GFR α 1 (1:40, MAB560, R&D Systems, Minneapolis, MN, USA) and polyclonal goat anti-c-Ret (1:50, cat.# sc-167-G, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS containing 5% donkey serum overnight at 4 °C, followed by appropriate Texas Red (TR) or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (both 1:1000, Jackson ImmunoResearch, West Grove, PA, USA) for 1 h in PBS at RT. Sections were then mounted in ProLong® Gold Antifade (Invitrogen, Carlsbad, CA, USA).

For whole mount preparations, fixed cochleas were transferred to PBS and the membranous otic capsule, followed by the spiral ligament, Reissner's membrane and the tectorial membrane were removed under a dissection microscope. Half-turn segments of the organ of Corti and SG were dissected and transferred to 4-well culture slides (BD Falcon, BD Biosciences, Bedford, MA, USA) for staining as floating preparations. Cell membranes were permeabilized with 5% Triton X-100 (Sigma-Aldrich) for 10 min, followed by blocking with 15% donkey serum in PBS (Sigma-Aldrich) for 20 min. Explants were then stained for neurofilament using a rabbit polyclonal 200 kDa anti-neurofilament primary antibody in PBS containing 5% donkey serum overnight at 4 °C (N4142, 1:500; Sigma-Aldrich) followed by a FITC-conjugated secondary donkey anti-rabbit antibody (1:200, Jackson ImmunoResearch) for 1.5 h in PBS with 2% donkey serum at RT. Whole mounts were then carefully removed, placed on individual Superfrost Ultra plus glass slides (Menzel Glaeser) and mounted in Aqua Polymount (Polysciences Inc., Eppelheim, Germany).

SG explant preparation

The SG was dissected from the inner ear as described by Van de Water and Ruben (1971). Briefly, temporal bones were removed after a mid-sagittal opening of the skull and transferred into sterile PBS for further dissection under a microscope (Leica MZ6, Leica Microsystems). The cartilaginous otic capsule was opened and the exposed membranous labyrinth with the sensory epithelium, the organ of Corti, was removed from the modiolus and the SG was carefully separated from the modiolus.

Tissue culture

For tissue culture, the freshly dissected SG were cut into 200–300 µm lengths to form explants, which were immediately transferred into culture medium. Forty-eight well tissue culture plates (Costar, Acton, MA, USA) were coated with 10 µg/ml fibronectin from human plasma (Sigma-Aldrich) and 10 µg/ml laminin from Engelberth-Holms sarcoma (Sigma-Aldrich) in PBS (Gibco by Invitrogen Corporation, Grand Island, NY, USA) overnight at 4 °C. After two washes with PBS, culture plates were incubated with poly-L-lysine (5 µg/ml, Sigma-Aldrich) for one hour at 37 °C. After rinsing three times with PBS, the wells were filled with 75 µl primary attachment medium, consisting of Dulbecco's modified Eagle medium (DMEM; Gibco), 10% fetal bovine serum (Sigma-Aldrich), 25 mM HEPES buffer (Gibco) and 300 U/ml penicillin (Sigma-Aldrich). Individual explants were transferred into the wells of the plate and incubated for 24 h in the attachment medium at 37 °C, 5% CO₂ and 95% humidity. Then the culture medium was changed to serum-free maintenance medium consisting of DMEM, 30 µl/ml N2 supplement (Gibco), 6 mg/ml glucose (Sigma-Aldrich), 25 mM HEPES (Gibco) and 300 U/ml penicillin (Sigma-Aldrich). SG explants were incubated for 72 h either in maintenance medium (control cultures) or in maintenance medium supplemented with recombinant GDNF and/or GFRα1-Fc (both from R&D Systems), Wortmannin and U0126 (both from Calbiochem, EMD Chemicals, Gibbstown, NY, USA). The latter two inhibitors were dissolved in dimethylsulfoxid (DMSO; Calbiochem, EMD Chemicals) as recommended by the manufacturer, yielding stock solutions of 1 mM each.

After 72 h of culture in maintenance medium, explants were fixed in 4% paraformaldehyde (PFA) in PBS for 20 min at RT. Cell membranes were permeabilized with 5% Triton (Sigma-Aldrich) for 10 min, followed by blocking with 5% donkey serum (Sigma-Aldrich), all at RT. Explants were then probed for neurofilament (200 kDa form) immunostaining using a rabbit polyclonal 200 kDa anti-neurofilament primary antibody with an overnight incubation at 4 °C (N4142, 1:500; Sigma-Aldrich) followed by a FITC-conjugated secondary donkey anti-rabbit antibody (1:100, Jackson ImmunoResearch) for 2.5 h at RT.

RT-PCR

Freshly dissected rat SGs were rapidly transferred into RNAlater (Qiagen, Valencia, CA, USA), incubated overnight at 4 °C and then stored at –80 °C until further processing. RNA was isolated using TRIzol® Reagent (Invitrogen) according to the manufacturer's protocol and ultimately dissolved in DEPC treated water (Invitrogen). cDNA was synthesized via reverse transcription using the SuperScript™ First-Strand Synthesis System (Invitrogen) according to the manufacturer's protocol. Subsequently, 35 cycles of polymerase chain reaction (PCR) were performed using Taq DNA Polymerase (Invitrogen) with optimized annealing temperatures and magnesium (Mg, 50 mM) using the following primers and PCR conditions: NCAM-140: sense GAA CCC ATT GTA GAG GTC CGA AC, antisense GTG TGT GTG TGT GTG TGT GTG TGA G, 54 °C, Mg 1.5 µl; Ret: sense TGA AGA AGC AGG GCA TTT GG, antisense CTC AGT CGT TGT CAG GGA AAC C, 57 °C, Mg 3.5 µl; GFRα1: sense CAA AAG GAA CGG CAG TTT ATG G, antisense ACT TCA GAC ACC AAA GCG TCC C, 57 °C, Mg 1.5 µl; GDNF sense: ACC AGG CTG TCG TCT AAA AAC G, antisense AGA TAA ACA AGC GGC GGC AC, 54 °C, Mg 1.5 µl; actin: sense GCT CGT CGT CGA CAA CGG CTC, antisense CAA ACA TGA TCT GGG TCA TCT TCT, 56 °C, Mg 1.5 µl.

PCR products were analyzed by agarose gel electrophoresis in TAE-buffer. For sequence verification of the PCR products, gel bands were excised and PCR products were purified according to the QIAquick gel extraction kit protocol (Qiagen), cloned into *Escherichia coli* using the OneShot® Topo10 Kit (Invitrogen), and then sequenced by UCSD's Bio Core facility.

Western blot

For protein-level analysis of NCAM, Ret, GFRα1 and GDNF, the SGs of eight to twelve rats (i.e. sixteen to twenty-four cochleas) at ages ranging from P5 to P180 were isolated as described above and directly transferred into sterile 1.5 ml Eppendorf tubes (Fisher Scientific by Thermo, Waltham, MA, USA) on dry ice. Brain tissue lysates from WT mice were used as a positive control, while brain tissue from KO mice was used as a negative control for NCAM and c-Ret. SGs were then lysed in 200 µl ice cold T-PER lysis buffer (Pierce, Rockford, IL, USA) supplemented with phosphatase and protease inhibitors (both from Roche, Basel, Switzerland) by manual grinding on ice followed by sonication for 10 min at 4 °C. For GDNF signaling analysis, SGs from eight to ten P5 and P20 rats were isolated as described above and directly transferred into 1.5 ml Eppendorf tubes containing 1 ml of culture maintenance medium supplemented with 100 ng/ml GDNF and 100 ng/ml GFRα1-Fc for 0 to 120 min at 37 °C. SG explants from another eight to ten P5 rats were treated with or without GDNF/GFRα1-Fc in tissue culture maintenance medium under the culture conditions described above. The medium was then carefully removed and SGs were lysed in 200 µl ice-cold supplemented T-PER lysis buffer. Samples were then centrifuged for 5 min at 10,000 g and 4 °C, and the protein concentration of the supernatant was determined using the BCA method (reducing agent compatible BCA Protein Assay Kit, Pierce). For further analysis, 25 µg of total protein was used.

Reduced protein samples along with molecular mass standards were loaded in NuPage® LDS sample buffer to a 1 mm 4% to 12% NuPage® Novex Bis-Tris gel (Invitrogen), separated by electrophoresis using the XCell SureLock™ Mini Cell electrophoresis system (Invitrogen), and then electroblotted to PVDF membranes (Bio-Rad, Hercules, CA, USA) for 1.5 h at 45 V using the XCell II™ Blot Module Kit (Invitrogen). Electrophoresis gels and membranes were checked for transfer quality by Coomassie blue staining and reversible Ponceau labeling, respectively.

Immunoblots were blocked in either TBS, pH 7.6 containing 0.05% Tween 20 (Fisher Scientific) and 5.5% fat free milk or 5.5% bovine serum albumin, according to the recommendations of the manufacturers of the primary antibodies for 1.5 h at RT followed by incubation with the following primary antibodies in blocking solution at 4 °C overnight: polyclonal rabbit β 2 anti pan-NCAM (Niethammer et al., 2002; 1:1,000), rabbit anti Ret (Cell Signaling Technology, Inc., Danvers, MA, USA, cat.# 3220, 1:1,000, and cat.# 3223, 1:1,000; Santa Cruz Bio-technology, Santa Cruz, CA, USA, cat.# sc-13104, 1:1,000), rabbit anti GFR α 1 (Santa Cruz Biotechnology, cat.# sc-10716, 1:100 and Sigma-Aldrich, cat.# G9290, 1:500), goat anti GFR α 1 (R&D Systems, cat.# AF560, 1:500), rabbit anti GDNF (Santa Cruz Biotechnology, cat.# sc-328, 1:500), rabbit anti phospho (Thr202/Tyr204)-Erk 1/2 (Santa Cruz Biotechnology, cat.# sc-16982-R, 1:200), rabbit anti Erk 2 protein (Santa Cruz Biotechnology, cat.# sc-154, 1:500), rabbit anti phospho (Ser473)-Akt (Cell Signaling, cat.# 9271, 1:500), rabbit anti total Akt (Cell Signaling, cat.# 9272, 1:1,000) and, as loading controls, mouse anti actin (BD Biosciences, cat.# 612656, 1:10,000) and monoclonal mouse anti GAPDH (Santa Cruz Biotechnology, cat.# sc-32233, 1:1,000). After washing, blots were incubated with appropriate HRP-labeled secondary antibodies (Jackson ImmunoResearch, 1:5,000 to 1:10,000) for 1.5 h at RT and visualized using Amersham ECL Plus reagents (Amersham, GE Healthcare, Piscataway, NJ, USA) on Kodak® BioMax™ film (Sigma-Aldrich). If needed, blots were stripped of phospho-antibody complexes using Restore Western Stripping buffer (Pierce) for 20 min at 37 °C with gentle agitation, re-blocked and reprobed with primary antibodies recognizing total proteins according to the manufacturer's instructions.

Digital imaging

For tissue culture evaluation, digital images of individual explants were obtained on an inverted fluorescence microscope (Olympus IX70, Olympus America, Inc., Melville, NY, USA) equipped with appropriate excitation and emission filters for FITC using a CCD camera and Spot advanced software (both from Spot RT KE/SE, Diagnostic Instruments Inc., Sterling Heights, MI, USA). Images were optimized to achieve uniform brightness and contrast using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA, USA).

For densitometry, Western blots were digitally imaged on the Geldoc™ XR System (Bio-Rad) with fixed settings of iris, zoom and exposure (0.169 s). For figures, the blots were imaged on a Canon San LIDE25 flatbed scanner (Canon USA Inc., Lake Success, NY, USA) and adjusted for background uniformity using Graphic Converter 6.3 for Mac OS X (Lemkesoft GmbH, Peine, Germany).

For evaluation of the dendritic innervation patterns of the organ of Corti, whole mount preparations of the basal cochlear turn were imaged in 0.25 to 0.5 μ m interval optical sections acquired by confocal laser scanning on an Olympus FV1000 microscope (Olympus America, Inc.) and processed using Olympus FluoView FV10-ASW 1.3 software (Olympus America, Inc.). Maximum intensity projections of stacks of optical sections, used to resolve labeled dendrites, were constructed by the FluoView FV10-ASW 1.3 software package and Image Pro-Plus Software (MediaCybernetics, Bethesda, MD, USA).

For evaluation of H&E stained sections, digital images were obtained on a Zeiss Axiovert 200M inverted microscope equipped with a CCD camera and AxioVision 4.3 software (all from Carl Zeiss Micro-imaging, Goettingen, Germany).

Image analysis and statistics

Tissue culture images were analyzed using the NeuronJ plug-in of NIH's ImageJ-software. Neurite number and mean length of all neurites were assessed for each explant. Data of

individual explants in every experimental condition showed normal distributions in the Kolmogorow–Smirnow test and homogeneous variances by Levene’s test. Therefore, individual explant data, grouped by experimental condition, were compared by one-way ANOVA followed by Tukey’s post-hoc test using Statistica 5.1 (StatSoft Inc., Tulsa, OK, USA).

Densities of blot analysis were determined using the ImageJ software package. Band densities, as measured by optical density, were normalized to the optical density measured at time point zero ($\text{INT}(\text{time})/\text{INT}(0)$). Relative phosphorylation status was calculated as the ratio of normalized density of phosphorylated protein to normalized density of total protein.

SGN cell bodies in Rosenthal’s canal were counted in the middle turn in P0 and P5 mice and in the apical, middle and basal turn in P49 mice. Neurons were counted only when a clear nucleus was visible in the section. Two to 3 cross-sections from 3 to 7 individual cochleas were analyzed in WT, Ret KO and NCAM KO mice respectively. Neuron cell body counts from NCAM and Ret WT and KO mice showed normal distribution but inhomogeneous variances and were therefore compared by 2-tailed Students t-test for inhomogeneous variances.

For quantification of the innervation pattern of the organ of Corti, the number of neurites in a 3-dimensional volume with the width of 10 inner HC bodies were counted as they crossed through the x–z planes at the level of the habenula perforata (for inner HC innervation) as well as the x–z planes between the inner HCs and the first row of outer HCs, the first and second row of outer HCs as well as the second and the third row of outer HCs (for outer HC innervation).

Results were considered to be significant when the likelihood for a type 1 error was less than 5% ($p < 0.05$). The data presented in the text and figures are the means and standard deviations (SD) for each experimental condition.

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Abbreviations

DMEM	Dulbecco’s modified Eagle medium
DMSO	dimethylsulfoxid
Fc	fragment crystallizable region
FITC	fluorescein isothiocyanate
GDNF	glial cell line-derived neurotrophic factor

GFRα1	glial cell line-derived neurotrophic factor receptor alpha 1
GPI	glycosylphosphatidyl inositol
HC	hair cell
H&E	haematoxylin and eosin
IHC	inner hair cell
KO	knock-out
NCAM	neural cell adhesion molecule
mRNA	messenger ribonucleic acid
OHC	outer hair cell
P	postnatal day
PFA	paraformaldehyde
PCR	polymerase chain reaction
PBS	phosphate buffered saline, pH 7.3
Ret	rearranged during transformation
RT	room temperature
SD	standard deviation
SG	spiral ganglion
SGN	spiral ganglion neuron
TAE	Tris-hydroxymethyl-aminomethane-acetate-ethylenediamine tetraacetic acid
TBS	Tris buffered saline, pH 7.6
TR	Texas Red
WT	wild-type

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Fig. 1.

Expression of members of the GDNF signaling complex during SG development. A. RT PCR showing amplified products from P3 (neonatal) to P180 (adult) rat SG lysates and whole brain lysate (control) with primer pairs for GDNF, GFR α 1, Ret, NCAM-140 and actin. PCR product sizes are given in base pairs (bp). SG lysates at each time point originated from pools of 16–24 cochleas each. Bands shown are representative PCR results from 2 to 3 independent SG lysate preparations. B, C, E. Specificity testing of antibodies: immuno-blots of crude brain lysates from Ret- and NCAM-null-mutant mice (P0 and adult respectively) and corresponding littermates as well as P23 Sprague–Dawley rats probed with antibodies for pan NCAM (antibody 1 β 2), Ret (antibody #3223) and GDNF. Twenty-five micrograms of total lysate is loaded per lane. For molecular weight approximation of proteins, the molecular marker weights are provided next to each blot. Blots show results representative of 3 independent lysates. D. Immunoblots of P3 to P180 rat SG lysates probed with the indicated antibodies for pan-NCAM, Ret, GDNF and GAPDH as a loading control. SG lysates at each time point originated from pools of 16 to 24 cochleas. Twenty-five micrograms of total lysate is loaded per lane. Bands shown are representative of results from 2 independent SG lysate series. F. Immunohistochemistry of P20 SGs probed with antibodies for GFR α 1 (Texas red) and Ret (FITC). 20 $\times$, frozen sections, thickness 12 μ m.

Fig. 2. Ret KO and NCAM KO mice show normal patterns of afferent dendrite projection in the organ of Corti at P0, but NCAM KO mice transiently show higher innervation densities during development. A1–2, B1–2, C1–2, D1–2: Organ of Corti whole mount preparations: representative maximum intensity projections of neurofilament signal in SGN neurites from 28 consecutive 0.5 μ m optical planes. Stars mark position of IHCs and filled circles mark the three rows of OHCs. A3–D3 and A4–D4: Quantitative analysis of afferent innervation patterns from organ of Corti whole mount preparations. Means ($\pm$ SD) of afferent dendrites projecting to 10 consecutive IHCs (A3–D3) and OHCs (A4–D4). Data shown represent 6 whole mount preparations each from 3 to 4 individual knockout mice and their respective littermates. * $p < 0.05$, ** $p < 0.001$, Student's t -test.

Fig. 3. GDNF stimulation of neurite outgrowth in rat P5 spiral ganglion (SG) neurons is enhanced by exogenous GFRα1. Representative images of rat P5 SG explants after 72 h in culture: A. control explant in tissue culture medium alone; B. medium supplemented with 100 ng/ml GDNF; C. medium supplemented with 100 ng/ml GFRα1; D. medium supplemented with 100 ng/ml GDNF and 100 ng/ml GFRα1. E. Average number of neurites per rat P5 SG explant. F. Length of neurites extending from individual explants, in μm. Results in E. and F. represent means±SD of 2–3 different experiments, including counts from 6 to 25 individual explants. One-way ANOVA followed by Tukey's post-hoc test (Bonferroni corrected) * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

Fig. 4.

GDNF/GFR α 1 stimulation of rat P5 and P20 SGNs induces both Akt and Erk phosphorylation. Immunoblots of P5 before the onset of hearing (A) and P20 after the onset of hearing (B) rat total SG lysates after treatment with 100 ng/ml GDNF and 100 ng/ml GFR α 1 for 0 to 120 min. (C) For better comparison to functional tissue culture experiments, lysates from P5 SGNs after 24 h in culture were investigated as well. SG lysates originated from stimulated SGs of 16–24 cochleas each. Twenty-five micrograms of total protein lysate is loaded per lane. Blots are representative of 3 independent stimulation series. Blots were probed with the indicated antibodies for phospho-Erk1/2 (42 and 44 kDa), total Erk1/2 (42 and 44 kDa), phospho-Akt (60 kDa), total Akt (60 kDa), GAPDH (37 kDa) and actin (42 kDa). Numbers below the bands represent the fold increase of phosphorylated protein

relative to total protein, normalized to the ratio at time point zero from 2 blots each of the 3 stimulation series.

Fig. 5. Inhibition of Akt- and Erk-signaling pathways reduces GDNF/GFRα1-induced neurite outgrowth in P5 SGNs. Representative images of rat P5 SG explants after 72 h in culture, treated with inhibitors of Akt or Erk, or the solvent DMSO alone. Quantification of numbers of outgrowing neurites and mean length of neurites in μm. A. Control explants in culture medium alone, B. medium supplemented with 100 ng/ml GDNF or C. medium supplemented with 100 ng/ml each of GDNF and GFRα1 (right); D. Explant treated with GDNF, GFRα1 (100 ng/ml each) and the phosphatidylinositol 3-kinase (PI3K) inhibitor Wortmannin (100 nM). G. Explant treated with GDNF, GFRα1 (100 ng/ml each) and the MEK-1/-2 inhibitor U0126 (100 nM). J. Explant treated with GDNF, GFRα1 (100 ng/ml each) and DMSO (0.01% VoL). E, H, K. Group comparison of the numbers of neurites

per explant (means $\pm$ SD) in GDNF/GFR α 1 double stimulated cultures (100 ng/ml each) treated with Wortmannin (E), U0126 (H) and DMSO as control (K). F, I, L. Group comparison of mean length of neurites extending from the explants for the GDNF/GFR α 1 (100 ng/ml each) and Wortmannin (F), U0126 (I) and DMSO as control (L). Two to three different experiments including counts from 11 to 24 individual explants per group were analyzed by one-way ANOVA followed by Tukey's post-hoc test * p <0.05, ** p <0.001, *** p <0.0001.

Fig. 6.

SGN explants from NCAM KO mice fail to respond to GDNF and GFR α 1. Representative images of SG explants from P5 NCAM KO (A1–A3) and NCAM WT (B1–B3) null mutant mice after 72 h in culture and quantification of the mean number and length of neurites extending from each explant. A1 and B1 show explants grown in culture medium alone, A2 and B2 explants grown in medium supplemented with 100 ng/ml GDNF. A3 and B3 show explants maintained in medium supplemented with GDNF and GFR α 1 (100 ng/ml each). C. Quantitative evaluation of the average number of neurites per rat SG explant. D. Mean length of neurites extending from an individual explant (in μ m). Results in C. and D. represent means $\pm$ SD of 4 different experiments, including counts from 6 to 11 individual explants per condition. One-way ANOVA followed by Tukey's post-hoc test * p <0.05, ** p <0.001.

Fig. 7.

Neuritogenesis in SGN explants from NCAM KO mice is not further impaired by PI3K and MEK-1/-2 inhibitors. A and B. Representative images of SG explants from P5 NCAM KO (A1–A3) and NCAM WT (B1–B3) null mutant mice after 72 h in culture and quantification of the mean number of neurites extending from each explant. A1 and B1 show explants grown in culture medium alone, A2 and B2 explants grown in medium supplemented with 10 nM of the MEK-1/-2 inhibitor U0126. A3 and B3 explants were maintained in medium supplemented with 10 nM of the PI3K-inhibitor Wortmannin. C and D. Quantitative evaluation of the average number of neurites per SG explant. Results in C. (KO) and D. (WT) represent means $\pm$ SD of 3 different experiments, including counts from 6 to 16 individual explants per condition. Means did not differ significantly (n.s.) from each other by one-way ANOVA and Tukey's post-hoc test, indicating that no additional MEK-1/-2 and PI3K signaling pathways are active in NCAM KO mice.