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Genetic evidence for selective neurotrophin 3 signalling through TrkC but not TrkB *in vivo*

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Neurotrophins control neuronal survival in a target-derived manner during the period of naturally occurring cell death in development. The specificity of this mechanism has been attributed to a restricted spatio-temporal expression of neurotrophin ligands in target tissues, as well as a selective expression of their cognate tyrosine kinase (Trk) receptors in different neuronal subpopulations. However, several *in vitro* and *in vivo* studies of null mutant mice have suggested that neurotrophin 3 (NT3) also signals through the non-preferred TrkB receptor. In this study, we have directly addressed the *in vivo* preference of NT3 to signal through TrkB or TrkC, by crossing the NT3 knock-in mice (*BDNF*$^{NT3/NT3}$ mice) with the TrkB- or TrkC-null mutant mice. We find that TrkB is dispensable, whereas TrkC is required for the neuronal rescue by the NT3 allele in the brain-derived neurotrophic factor- and NT3-dependent cochleovestibular system. Our results show that NT3 maintains survival of cells as well as target innervation only through interactions with TrkC *in vivo*. TrkB and TrkC receptors are thus not functionally redundant for NT3, even when coexpressed in neurons of the cochleovestibular system.

Keywords: cochlear ganglia; inner ear; promiscuity; trophic factors; vestibular ganglia

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INTRODUCTION

Analyses of null mutant mice for neurotrophins or their receptors suggest diverse requirements of different neurotrophins for selective neuronal populations (Ernfors, 2001; Huang & Reichardt, 2001). The varying requirements of neurotrophins between subpopulations of sensory neurons are set by the availability of ligands in the target tissues, as well as a selective expression of Trk receptors in different neurons. TrkB and TrkC messenger RNAs are ubiquitously expressed in vestibular ganglion neurons at embryonic and postnatal stages as well as in the adult (Ylikoski *et al*, 1993), and both brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT3) are present in the vestibular sensory epithelia (Ernfors *et al*, 1992; Pirvola *et al*, 1992). Yet, ligand- and receptor-null mutant mice show distinct and specific deficits. In the *BDNF*$^{-/-}$ mice, 80% of the vestibular ganglion neurons are lost, accompanied by a near-complete loss of innervation of the target sensory hair cells at birth, whereas only 56% of the neurons are lost in the TrkB mutant mice. In the *NT3*$^{-/-}$ and *TrkC*$^{-/-}$ mice, 34% and 16%, respectively, of the vestibular ganglion neurons are lost. In the cochlea, 87%, 51%, 7% and 15% of cochlear ganglion neurons are lost in the *NT3*$^{-/-}$, *TrkC*$^{-/-}$, *BDNF*$^{-/-}$ and *TrkB*$^{-/-}$ mice, respectively (Ernfors *et al*, 1994, 1995; Schimmang *et al*, 1995). In a study by Farinas *et al* (1998), it was claimed that TrkB can be directly activated by NT3 in primary neurons, both *in vitro* and *in vivo*. Recent results using knock-in mice of BDNF and NT3 show that both BDNF and NT3 are redundant in parts of the peripheral nervous system (Coppola *et al*, 2001; Agerman *et al*, 2003), but receptor engagement was not addressed in the latter studies. Thus, several reports have drawn attention to the issue of whether ligand redundancy is mirrored by a receptor redundancy. None of the previous studies has directly addressed whether the control of cell death and target innervation by neurotrophins is mediated by preferred or non-preferred receptor signalling *in vivo*.

In this study, we have taken a genetic approach to determine the *in vivo* preference of NT3 to signal through either TrkB or TrkC in the vestibular and auditory systems. For this purpose, we used our previously established mice, in which the coding part of the *BDNF* gene has been replaced by the *NT3* gene. In these mice, NT3 can partly and completely rescue neuronal numbers in the vestibular and cochlear systems, respectively, but only a few of the remaining neurons still innervate the sensory epithelia (Agerman *et al*, 2003). This is consistent with the fact that fibre growth (Tessarollo *et al*, 2004), short-range innervation and synaptogenesis (Agerman *et al*, 2003) are largely mediated by BDNF in the inner ear. By crossing the *BDNF*$^{NT3/NT3}$ mice with the TrkB- or TrkC-null mutant mice, we separately eliminated each of the two potential signalling pathways of NT3. We have focused our analysis on the cochleovestibular system, as both TrkB and TrkC mRNAs are expressed ubiquitously by the cochlear and vestibular

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neurons. Therefore, non-promiscuous receptor signalling can directly be identified as such, without being confounded by the restricted presence of receptors in different neuronal subpopulations. Analysis of the BDNF-dependent cochleovestibular system in the two mouse strains, $BDNF^{NT3/NT3} \times TrkB^{-/-}$ and $BDNF^{NT3/NT3} \times TrkC^{-/-}$, shows exclusive signalling of NT3 through the TrkC receptor.

RESULTS

TrkB and TrkC expression in $BDNF^{NT3/NT3}$ mice

As any discrepancies in neuronal survival between neurotrophin- and cognate receptor-null mutant mice could be caused by changes in receptor expression as a consequence of eliminating the ligands, we first investigated whether genetically removing BDNF as well as introducing the NT3 allele in place of BDNF affected TrkB and TrkC mRNA expression. Real-time PCR showed that, in the wild-type mice, only the full-length form of TrkC (TrkC-FL) was expressed in the inner ear (Fig 1A), as there was an equal number of copies of extracellular domain (TrkC-all) and TrkC-FL mRNA transcripts (Fig 1B). TrkB-FL transcripts were present at levels similar to those of TrkC-FL in the inner ear. However, unlike TrkC, TrkB-all expression was fivefold that of TrkB-FL, suggesting a 4:1 ratio of truncated to full-length TrkB receptors. Expression of full-length and truncated TrkB and TrkC isoforms did not differ in the wild-type and $BDNF^{NT3/NT3}$ mice (Fig 1B). These results indicate that there is no misregulation of Trk receptors that could confound interpretations of receptor promiscuity in the present study.

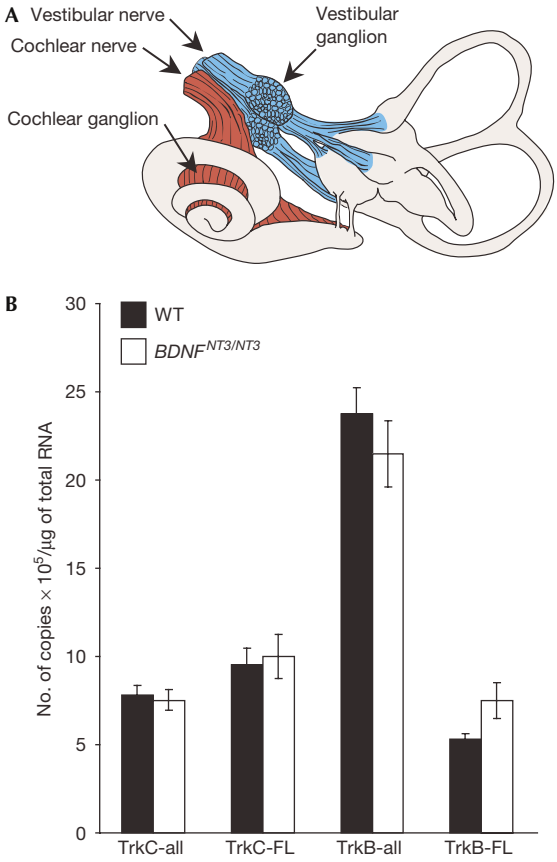

Fig 1 | TrkB and TrkC messenger RNA expression in mice of different genotypes. (**A**) Illustration of the tissue dissected for real-time PCR in (**B**). (**B**) Quantitative real-time PCR for TrkB and TrkC measuring both truncated and full-length TrkB or TrkC (TrkB-all and TrkC-all) or only full-length receptors (TrkB-FL and TrkC-FL). The data are presented as copy numbers per microgram RNA. Note that full-length TrkB and TrkC are expressed at similar levels, that there is abundant expression of truncated TrkB and that there are no changes in the expression of any isoforms in the $BDNF^{NT3/NT3}$ mice compared with the wild-type (WT) mice.

Signalling of NT3 exclusively through TrkC *in vivo*

Quantitative data based on morphological criteria identifying peripheral neurons obtained from cresyl violet stainings in the vestibular ganglion correlated well with the overt changes in ganglion size (Fig 2; Table 1).

The neuronal loss in the vestibular ganglion of $BDNF^{-/-}$ mice was markedly rescued by the *NT3/NT3* alleles in the BDNF locus (22% versus 56% neurons remaining). The rescue of neuronal numbers in the $BDNF^{NT3/NT3}$ mice was not affected by eliminating signalling through the TrkB receptor, as was seen after introducing a null mutation of the *TrkB* allele (56% remaining). In contrast, the $BDNF^{NT3/NT3} \times TrkC^{-/-}$ mice showed a complete loss of mature vestibular ganglion neurons (Table 1). In the cochlear ganglion, 75% of the neurons remained in the $BDNF^{-/-}$ mice and 99% in the $BDNF^{NT3/NT3}$ mice (Agerman *et al*, 2003). Similar to vestibular neurons, the rescue of cochlear neurons by the NT3/NT3 alleles was not affected in the $BDNF^{NT3/NT3} \times TrkB^{-/-}$ compound mutant mice, whereas no remaining neurons could be found in the $BDNF^{NT3/NT3} \times TrkC^{-/-}$ mice (Table 2).

We next used immunohistochemistry for βIII tubulin (Tuj1) to address more directly whether there is a complete absence of neurons in the $BDNF^{NT3/NT3} \times TrkC^{-/-}$ mice (Fig 2F–J). The results were similar to those obtained in the histological analysis, and the marked loss of vestibular neurons in the $BDNF^{NT3/NT3} \times TrkC^{-/-}$ mice (Fig 2J) was confirmed.

Early rescue in $BDNF^{NT3/NT3}$ mice

Most of the naturally occurring cell death in the vestibular ganglion takes place between embryonic day 13 (E13) and E16, and it is also during this period in development that most of the excessive cell death in the $BDNF^{-/-}$ mice occurs (Ernfors *et al*, 1995). We examined whether the neuronal rescue in $BDNF^{NT3/NT3}$ mice coincided with the period of naturally occurring cell death by counting the number of vestibular neurons at E16 and E18. A statistical difference was found in cell numbers between these stages in the wild-type mice, which showed $4{,}615 \pm 234$ neurons at E16 ($n=6$) and $3{,}823 \pm 182$ neurons at E18 ($n=8$; $P<0.05$). This result shows that close to 20% of the neurons present at E16 have died at E18 in the wild-type mice, indicating that the period of normal cell death continues until E18. Much of the neuronal rescue in the $BDNF^{NT3/NT3}$ mice had already taken place at E16 ($2{,}019 \pm 64$ neurons, $n=6$) and the number of neurons remained unchanged between E16 and E18 in the $BDNF^{NT3/NT3}$ mice (E18, $2{,}143 \pm 248$ neurons, $n=4$). These data show that there is a rescue of neurons in the $BDNF^{NT3/NT3}$ mice throughout the period of naturally occurring cell death. We conclude that the neuronal rescue in the $BDNF^{NT3/NT3}$ mice coincides with the period of excessive neuronal loss in the $BDNF^{-/-}$ mice.

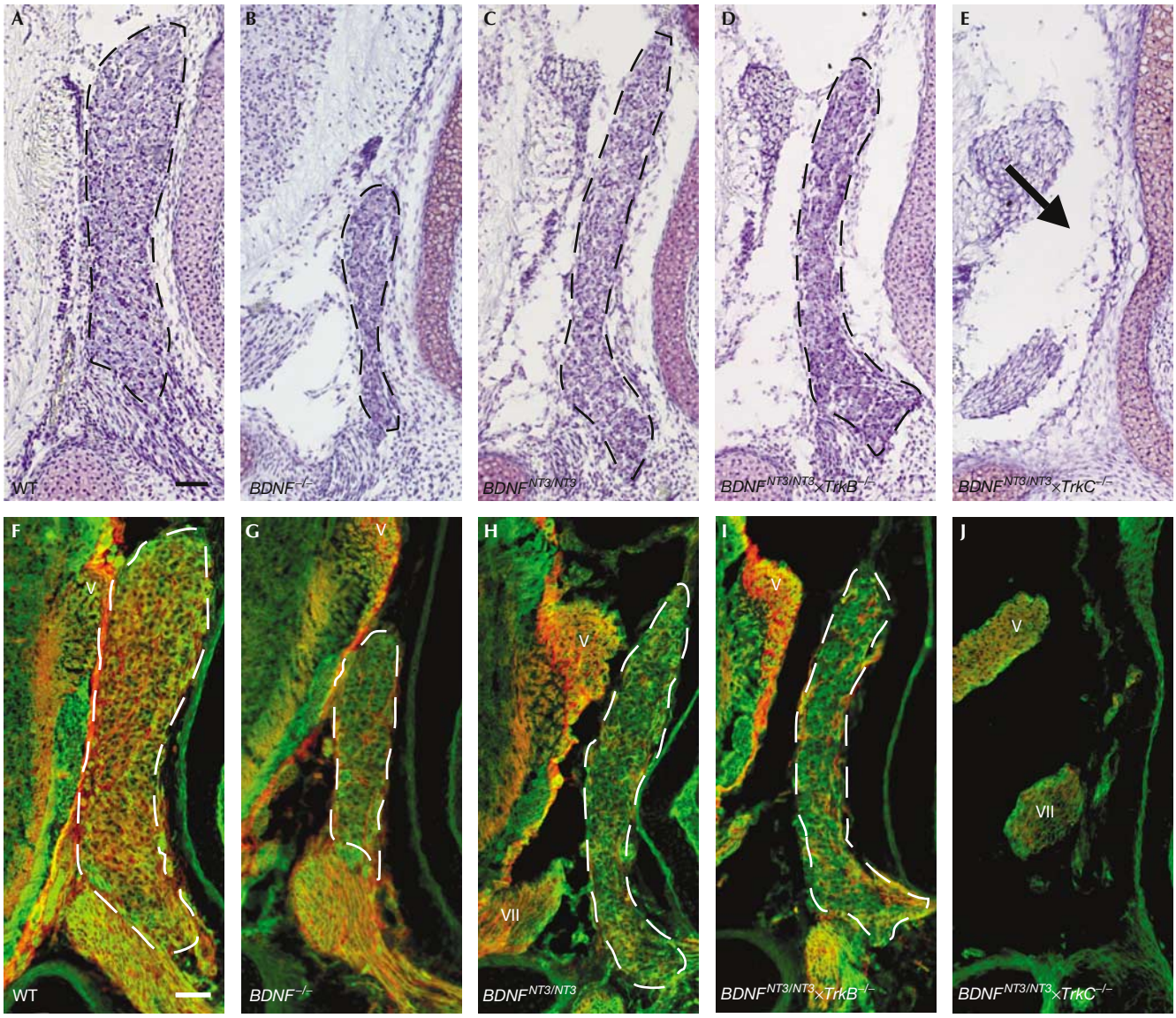

Fig 2 | Histological and immunohistochemical analyses of the vestibular ganglion. (**A–E**) Cresyl violet staining of embryonic day 18 (E18) vestibular ganglion in mice of indicated genotypes. The arrow in (**E**) indicates the location at which the ganglion is missing in the $BDNF^{NT3/NT3} \times TrkC^{-/-}$ mice. (**F–J**) Immunohistochemical staining for $p75^{NTR}$ (red) and βIII tubulin (green) of E18 vestibular ganglion in mice of indicated genotypes. WT, wild type; V, fifth cranial nerve; VII, seventh cranial nerve. Scale bars, 50 μm.

Rescue of innervation is dependent on TrkC signalling

Previous results illustrate that BDNF is the most important trophic factor for a proper terminal innervation and maturation of functional sensory nerve endings in the vestibular organ, but that NT3 expressed from the BDNF locus in $BDNF^{NT3/NT3}$ mice is sufficient to rescue a minor afferent innervation (Agerman *et al*, 2003). We studied whether the remaining nerve fibre innervation of the $BDNF^{NT3/NT3}$ mice was dependent on TrkB or TrkC signalling. We stained for βIII tubulin, which is present in both afferents and efferents of the inner ear (Fig 3A–E; Kim *et al*, 2001). Semiquantification of the staining (Fig 3F) showed a greater loss in the epithelial layer than in the subepithelial layer of the $BDNF^{-/-}$ mice (Fig 3B). A distinct increase in number and thickness of nerve fibres in the sensory epithelium was found in the $BDNF^{NT3/NT3}$ mice (Fig 3C) as compared with the $BDNF^{-/-}$ mice, and this effect remained in the $BDNF^{NT3/NT3} \times TrkB^{-/-}$ mice (Fig 3D), showing that the effect of NT3 is independent of TrkB. In agreement with this, nerve fibres were nearly completely absent in both layers in the $BDNF^{NT3/NT3} \times TrkC^{-/-}$ mice (Fig 3E).

A greater rescue of efferent than of afferent innervation

We next examined βIII tubulin and $p75^{NTR}$ double immunohistochemistry at high power to compare possible differences between efferent and afferent innervation. Afferents are known to express both $p75^{NTR}$ and βIII tubulin, whereas efferents express only βIII tubulin. Consistently, $p75^{NTR}$-positive fibres in the wild-type mice also expressed βIII tubulin, whereas there were many βIII tubulin-expressing fibres that did not stain for $p75^{NTR}$ (supplementary Fig 1A online).

Table 1 | Neuronal cell counts in vestibular ganglion

	Mean number of neurons ± s.e.m.	Percentage of control ± s.e.m.
Wild type	3,823 ± 182 ($n = 8$)	100 ± 5
BDNF$^{-/-}$	854 ± 64 ($n = 4$)	22 ± 2*
BDNF$^{NT3/NT3}$	2,143 ± 248 ($n = 4$)	56 ± 6* °
BDNF$^{NT3/NT3}$ × *TrkB*$^{-/-}$	2,136 ± 105 ($n = 8$)	56 ± 3* °
BDNF$^{NT3/NT3}$ × *TrkC*$^{-/-}$	0 ± 0 ($n = 6$)	0 ± 0* °‡§

Cryostat sections from embryonic day 18 mice of the indicated genotypes were prepared and stained with cresyl violet. Vestibular ganglion neurons with a clear nucleus and nucleoli were counted in every third section. ANOVA Bonferroni's *$P < 0.001$ between wild-type and mutant mice, °$P < 0.001$ between *BDNF*$^{-/-}$ and other mutant mice, ‡$P < 0.001$ between *BDNF*$^{NT3/NT3}$ and *BDNF*$^{NT3/NT3}$ × *TrkC*$^{-/-}$ and §$P < 0.001$ between *BDNF*$^{NT3/NT3}$ × *TrkB*$^{-/-}$ and *BDNF*$^{NT3/NT3}$ × *TrkC*$^{-/-}$.

Table 2 | Neuronal cell counts in cochlear ganglion

	Mean number of neurons ± s.e.m.	Percentage of control ± s.e.m.
Wild type	6,499 ± 198 ($n = 3$)	100 ± 3
BDNF$^{-/-}$	4,872 ± 270 ($n = 3$)[a]	75 ± 4[a]
BDNF$^{NT3/NT3}$	6,455 ± 351 ($n = 3$)[a]	99 ± 5[a]
BDNF$^{NT3/NT3}$ × *TrkB*$^{-/-}$	6,434 ± 244 ($n = 7$)	99 ± 4
BDNF$^{NT3/NT3}$ × *TrkC*$^{-/-}$	0 ± 0 ($n = 6$)	0 ± 0* °

Cryostat sections from embryonic day 18 mice of the indicated genotypes were prepared and stained with cresyl violet. Cochlear ganglion neurons with a clear nucleus and nucleoli were counted in every third section.
[a]Data from Agerman *et al* (2003), reproduced with permission.
ANOVA Bonferroni's *$P < 0.001$ between wild-type and *BDNF*$^{NT3/NT3}$ × *TrkC*$^{-/-}$, °$P < 0.001$ between *BDNF*$^{NT3/NT3}$ × *TrkB*$^{-/-}$ and *BDNF*$^{NT3/NT3}$ × *TrkC*$^{-/-}$.

In the subepithelial layer, efferents were abundantly present in all genotypes except the *BDNF*$^{NT3/NT3}$ × *TrkC*$^{-/-}$ mice, which displayed a marked paucity. In contrast, the presence of afferents reflected the number of vestibular ganglion neurons remaining in each genotype (supplementary Fig 1A–E online). In the sensory epithelia, the efferents were very sparse in the *BDNF*$^{-/-}$ and *BDNF*$^{NT3/NT3}$ × *TrkC*$^{-/-}$ mice, whereas many fibres were present in the *BDNF*$^{NT3/NT3}$ and *BDNF*$^{NT3/NT3}$ × *TrkB*$^{-/-}$ mice. However, afferent innervation of the sensory epithelial layer did not correlate with the number of fibres present in the subepithelial layer, as there was an absence in the *BDNF*$^{-/-}$ and *BDNF*$^{NT3/NT3}$ × *TrkC*$^{-/-}$ mice, and only a minor presence in the *BDNF*$^{NT3/NT3}$ and *BDNF*$^{NT3/NT3}$ × *TrkB*$^{-/-}$ mice (supplementary Fig 1A–E online, and summarized in Fig 4). From this, it is clear that NT3 expressed from the BDNF locus is sufficient for afferent axonal growth and projection to the subepithelial layer but not for significant target innervation of hair cells in the sensory epithelia (which depends on BDNF; Agerman *et al*, 2003).

DISCUSSION

By establishing the *BDNF*$^{NT3/NT3}$×*TrkB*$^{-/-}$ and *BDNF*$^{NT3/NT3}$×*TrkC*$^{-/-}$ mice and comparing neuronal survival and target innervation in these mice with those in the *BDNF*$^{NT3/NT3}$ and wild-type mice, we have characterized the selectivity of NT3 to its cognate receptor, TrkC, *in vivo*. Earlier studies (Agerman *et al*, 2003) had shown a partial rescue of vestibular neuronal numbers in the *BDNF*$^{NT3/NT3}$ mice but a failure to rescue the balance defect, presumably a result of the failure of rescued neurons to innervate the hair cells. The present results indicate that NT3 activation of TrkC is not equivalent to BDNF activation of TrkB in supporting hair-cell innervation and recovery of balance. Although trkB and trkC

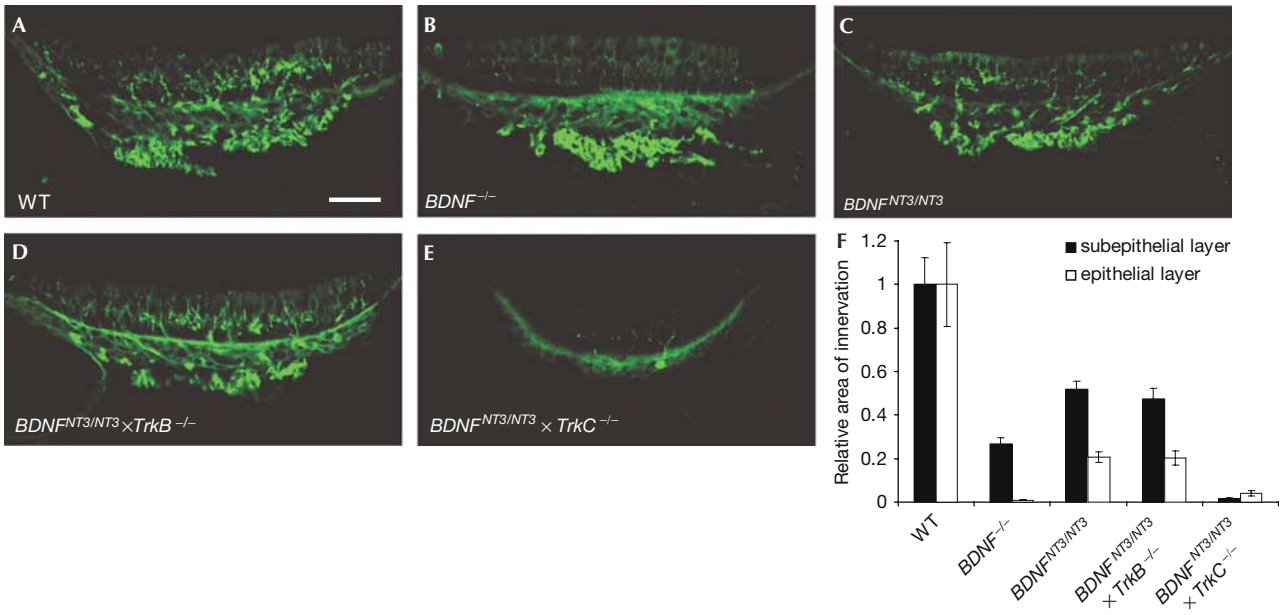

Fig 3 | Rescue of innervation in *BDNF*$^{NT3/NT3}$ mice occurs independent of TrkB. (**A–E**) Immunohistochemistry for βIII tubulin, which stains both efferent and afferent innervation in the utricular maculae at embryonic day 18 (E18) of mice of indicated genotypes. (**F**) Semiquantification of nerve fibres in the epithelial and subepithelial layers of the different genotypes. Note the greater loss of innervation in the epithelial layer in all knockout animals. Data are ± s.e.m. WT, wild type. Scale bars, 50 μm.

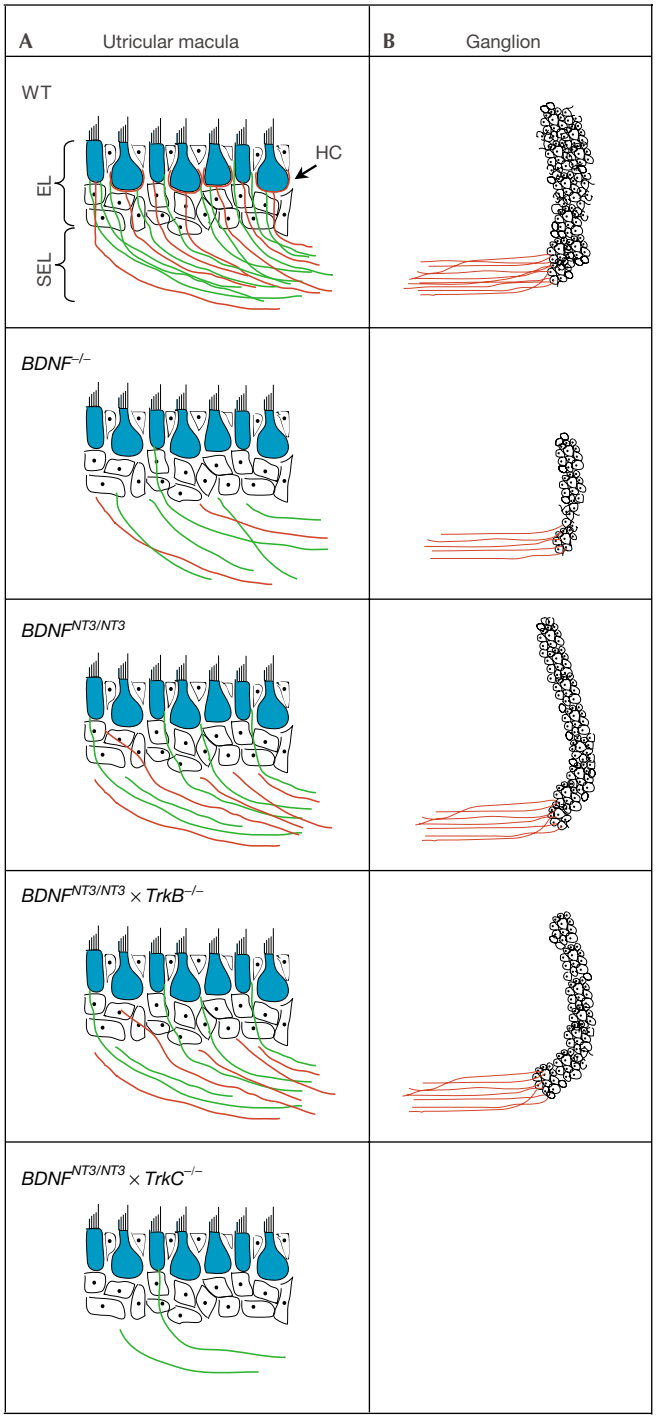

Fig 4 | Schematic drawing summarizing target innervation and neuronal survival in all studied genotypes. The illustration of the vestibular sensory epithelia (**A**) and vestibular ganglia (**B**), summarizing afferent (red) and efferent (green) innervation of the epithelial layer (EL) and the subepithelial layer (SEL) of the different genotypes. Density of afferent innervation in the subepithelial layer had a direct correlation with ganglion size. Although neurotrophin 3 (NT3) through TrkC activation rescued neurons and nerve fibres in the sensory epithelia, hair-cell (HC) innervation and functional recovery was minor in the $BDNF^{NT3/NT3}$ mice. The efferents were reduced when the number of afferents decreased. WT, wild type.

mRNAs are ubiquitous in all neurons of the vestibular ganglion, their presence at the protein level has not been examined. It is possible that the partial neuronal rescue in the $BDNF^{NT3/NT3}$ mice (56% remaining), as compared with that in the $BDNF^{-/-}$ mice, is the result of neurons expressing both TrkB and TrkC, but responding primarily to BDNF; hence, introducing the NT3/NT3 allele effectively rescues them. Others may express only TrkB protein and therefore are not rescued by NT3. A final population could contain only TrkC, and all neurons are therefore lost in the $BDNF^{NT3/NT3} \times TrkC^{-/-}$ mice.

The issue of whether neurotrophins signal through non-preferred receptors *in vivo* was raised by the findings that the NT3-null mutant mice in several peripheral ganglia show a more severe neuronal loss than the TrkC receptor-null mutant mice (Klein *et al*, 1994; Tessarollo *et al*, 1997; Ernfors, 2001; Huang & Reichardt, 2001). For example, the NT3-null mutant mice lose 85% of the cochlear sensory neurons (Farinas *et al*, 1994; Ernfors *et al*, 1995), whereas only a 51% loss has been reported in the TrkC mutant mice (Schimmang *et al*, 1995). These initial findings were followed up by studies addressing a putative non-preferred receptor signalling by NT3, both *in vivo* (Farinas *et al*, 1998; Huang *et al*, 1999, Coppola *et al*, 2001) and *in vitro* (Davies *et al*, 1995; Kuruvilla *et al*, 2004). The results from these studies are consistent with biochemical studies indicating that, in addition to TrkC, NT3 can, with lesser affinity, bind to and activate its non-preferred receptors TrkA and TrkB in non-neuronal cell lines (Barbacid, 1994). Although these results only indirectly address the issue of ligand interactions with non-preferred receptors *in vivo*, they are in agreement with a role for NT3 signalling through its non-preferred receptors, which has become generally accepted as a physiological process participating in the development of the peripheral nervous system.

Other work has, however, pointed to a high degree of receptor specificity *in vivo*, suggesting little, if any, interaction of neurotrophins with non-preferred Trk receptors (ElShamy & Ernfors, 1996; White *et al*, 1996; Ernfors, 2001). Consistently, in more recent studies of the $TrkC^{-/-}$ mice, the reported loss of cochlear neurons is 70% (Tessarollo *et al*, 1997), thus approaching the 85% loss in NT3-deficient mice. The high specificity of NT3 signalling through TrkC in the inner ear suggests a similar high specificity and the lack of TrkB interactions also in other neuronal populations in which NT3 is active. The evidence of NT3 acting through TrkA in sympathetic neurons (Kuruvilla *et al*, 2004) suggests that the remaining discrepancies in neuronal numbers of, for instance, the sensory trigeminal and dorsal root ganglion could be accounted for by its action through TrkA. Unlike the auditory and vestibular systems, TrkA is also expressed in many neurons of these ganglia. Because several of the analyses that suggested NT3 signalling through TrkB and TrkC were based on the detection of protein or presence of mRNA at cellular resolution and could be confounded by rapidly changing patterns of expression during development, we have taken a genetic approach to determine engagement of preferred versus non-preferred Trk receptors. Thus, in this study, we have, for the first time, *in vivo* addressed the promiscuity of receptor activation maintaining neuronal survival by NT3. Our new results are in agreement with the latter studies and show that, in the inner ear, NT3 signals exclusively through TrkC *in vivo*. Our results strongly argue that there is a high degree of specificity for NT3 receptor engagement *in vivo* and show that TrkB

and TrkC receptors are not functionally redundant, even when coexpressed in individual neurons of the cochleovestibular system.

METHODS

Animals. In this study, we used offspring from the *BDNF*$^{+/-}$ (Ernfors *et al*, 1994), *BDNF*$^{+/NT3}$ (Agerman *et al*, 2003), *TrkB*$^{+/-}$ (Klein *et al*, 1993) and *TrkC*$^{+/-}$ (Klein *et al*, 1994) mice. The offspring were genotyped with PCR.

Tissue preparation. For immunohistochemical analysis and neuronal quantification, embryos were obtained from overnight mating and the morning of the vaginal plug was considered as E0. Tissues from E18 mice were immersionfixed in 4% paraformaldehyde overnight, equilibrated in 10% sucrose followed by 30% sucrose, and frozen. The tissue was sectioned in a cryostat at a thickness of 14 μm.

Quantification of neuronal numbers. Sections were stained with cresyl violet. Neuronal numbers were established by counting neurons with a clear nucleus and nucleoli in every third section in the ganglia. The total number of cells in a ganglion was calculated as described before (Coggeshall, 1992). One-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test was used for statistical analyses. A *P*-value of <0.05 was considered to be significant.

Immunohistochemistry. Sections were stained with mouse anti-βIII tubulin (1:2,000; Promega, Southampton, UK), and rabbit anti-p75^{NTR} (1:200; Promega). Photomicrographs (Fig 3A–E; supplementary Fig 1A–E online) were obtained on a confocal microscope (Zeiss LSM510).

Semiquantification of innervation. Frames were captured from anti-βIII-tubulin-immunostained sections at $\times 20$. The total area of the utricular sensory epithelium and that of the subepithelial layer were quantified separately, and the area covered with signal above threshold was computed using ImageJ. Wild type was set to 1 and the other genotypes were normalized against this value.

Quantitative real-time PCR. Total RNA from the inner ears of E18 mice was extracted using the Absolutely RNA^{TM} Nanoprep kit (Stratagene, La Jolla, CA, USA). Primers amplifying the extracellular domain (all) or the intracellular tyrosine kinase domain (FL) of either TrkB or TrkC and primers for the HPRT (positive control) were designed in different exons to avoid amplification of eventual DNA contamination. PCR products were verified by sequencing and used for standard curves in the real-time PCR. The samples were run on a Perkin-Elmer ABI Prism 5700.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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