The neurotrophin receptors TrkA and TrkC but not TrkB cause neuronal death

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Neurons of the peripheral nervous system have long been known to require survival factors to prevent their death during development. But why they selectively become dependent on secretory molecules has remained a mystery, as is the observation that in the central nervous system, most neurons do not show this dependency. Using engineered embryonic stem cells, we show here that the neurotrophin receptors TrkA and TrkC instruct developing neurons to die, both in vitro and in vivo. By contrast, TrkB, a closely related receptor primarily expressed in the central nervous system, does not. These results indicate that TrkA and TrkC behave as dependence receptors, explaining why developing sympathetic and sensory neurons become trophic factor-dependent for survival. The expansion of the Trk gene family that accompanied the segregation of the peripheral from the central nervous system is suggested to have generated a novel mechanism of cell number control.

Neurotrophins and their receptors are components of a signaling system known to play major and diverse roles in the nervous system of vertebrates ranging from the control of cell death to memory retention[1]. Decades of work with avian and rodent embryos have clarified some of the basic functions of the neurotrophins, such as the ability of target-derived nerve growth factor (NGF) to regulate the survival of neurons in the peripheral nervous system (PNS), including most sympathetic and sensory neurons[1]. Currently, the prevention of developmental cell death is explained by the ability of neurotrophins to activate specific tyrosine kinase receptors when axon terminals begin innervating their target tissues[2,3]. While this has been firmly established in the PNS, it turned out that most neurons in the central nervous system (CNS) do not depend on neurotrophins or other diffusible growth factors for survival, even though naturally occurring cell death at the time of target innervation is a feature of both the PNS and the CNS[4]. Brain-derived neurotrophic factor (BDNF) is the most widely expressed neurotrophin in the CNS, but

its elimination fails to affect neuronal numbers in most CNS areas, even several weeks after birth[5,6]. The neurotrophins bind and activate one of 3 tyrosine kinase receptors identified in mammals and primarily expressed by neurons[7]. They are designated TrkA, B and C, are highly related and seem to have evolved from an ancestral gene by domain and exon shuffling. A single Trk gene of hitherto unknown function has bee identified in the amphioxus *Branchiostoma floridae*[8] and the absence of any *bona fide* Trk gene in *Caenorhabditis elegans* or *Drosphila melanogaster*, and of forward genetics in general, has critically hampered progress in the understanding of neurotrophin receptor signaling. While TrkA, B and C are thought to transduce similar functions following neurotrophin binding[7], it has been essentially impossible to rigorously test this assumption as acute transfection of neurons with the corresponding cDNAs leads to expression levels that are difficult to control and typically leads to receptor auto-activation[9]. To circumvent these difficulties, we used a novel *in vitro* assay based on the neuronal differentiation of mouse embryonic stem (ES) cells[10,11] engineered to express the 3 Trk receptors. These ES cells were also used to test and compare the *in vivo* impact of the forced expression of these receptors in all developing neurons.

TrkA and TrkC trigger cell death, TrkB does not

The neuron-specific *mapt* (tau) locus was targeted in ES cells[12] with cDNAs encoding TrkA, TrkB or TrkC with or without a hemagglutinin (HA) epitope used as a tag inserted at their amino terminals to facilitate quantification of expression levels[13]. In most experiments, the second *mapt* allele was also targeted with a GFP cDNA. Detailed examination of neurons derived from ES cells with or without targeted *mapt* alleles failed to reveal any differences with regard to speed of differentiation, rate or pattern of outgrowth or cell survival. These engineered ES cells were differentiated using a protocol resulting in the generation of virtually pure progenitors expressing Pax6, a transcription factor instructing them to synchronously differentiate into glutamatergic neurons[11,14]. The *mapt* locus begins to be expressed when progenitors exit the cell cycle[12,15] and quantification of the 3 receptors using HA antibodies indicated indistinguishable levels of expression (Fig. 1A, B). TrkB under the control of its endogenous promoter begins to be detectably expressed only later in this ES cell-based system[11] (see also Fig. 1C). Unexpectedly, we found that neurons expressing TrkA and TrkC all died between 4 and 6 days in vitro (div, Fig. 1D,E). Identical results were obtained with tagged and untagged TrkA and TrkC receptors and the death of neurons could be prevented by the addition of NGF or NT3 respectively (Fig. 1D,E). By contrast, TrkB-expressing neurons did not die (Fig. 1D). This was not due to endogenous BDNF, as the addition of a BDNF blocking antibody or of the kinase inhibitor K252a did not cause any detectable effects (Suppl. Fig. 1). In line with our results with TrkC, a previous report indicated that acute expression of TrkC in 293 cells leads to increased levels of cell death, suggesting that it is a dependence receptor, a notion also tested and verified with NT3-dependent sensory neurons[16]. However, the same study failed to reach similar conclusions with TrkA[16], presumably due to uncontrolled levels of expression and receptor autoactivation[9]. Like several previous reports on the mechanism of action of dependence receptors[17], this study also focused on the caspase-dependent proteolysis

of the receptor as the mechanism causing cell death[16]. But in lysates of ES cell-derived neurons, TrkA or TrkC proteolytic fragments were not detected, even in the presence of proteasome inhibitors. By contrast, proteolysis of the neurotrophin receptor p75 was observed in these same lysates, including the intracellular domain previously reported to be involved neuronal death[18]. This intracellular domain was only observed in the TrkA and TrkC in the absence of NGF or NT3, and inhibiting gamma secretase activity with LY411575 blocked both the generation of this domain and of cell death (Suppl. Fig. 2).

Embryos generated from *mapt::Trk* ES cells

To test the relevance of these *in vitro* findings in the context of the developing nervous system, we next used the so-called tetraploid complementation system to generate embryos entirely derived from ES cells[19]. These cells expressed TrkA, TrkB, or TrkC and GFP from the *mapt* locus, thus allowing a comprehensive visualization of the nervous system[20]. ES cells expressing GFP from both *mapt* alleles were used as control. At embryonic age 11.5 (E11.5), no overt differences were seen between the 4 genotypes examined, indicating that the initial steps of neurogenesis proceeded normally. In particular, peripheral ganglia could readily be observed in all 4 cases (Fig. 2). However, a widespread and massive loss of the GFP signal was observed 2 days later in TrkA- and TrkC-expressing embryos, while those expressing only GFP or TrkB appeared identical and normal (Fig. 2). Immunohistochemistry confirmed that at E13.5, most neurons were eliminated in both TrkA- and TrkC-expressing embryos (Fig. 3A). It also revealed higher than normal levels of cell death at E11.5 in the spinal cord and dorsal root ganglia (DRG), in spite of the normal appearance of the embryos (Fig. 3B). These findings indicate that the death-inducing function of TrkA and TrkC has the potential to eliminate essentially all developing neurons, regardless of their identity and developmental history. We also quantified the levels of Trk receptor expression in embryos derived from TrkA-ES cells, and found them to be higher than normal through the nervous system, including the DRG (Fig. 3C). The lack of any cell death with TrkB is of special interest as it is expressed by most neurons in the CNS, much more widely than TrkA, and at higher levels than TrkC (Suppl. Fig. 3 and Ref. [21]). Immunoprecipitation of E13 brain and spinal cord lysates with TrkB- or TrkC-specific antibodies followed by Western blot detection with pan-Trk antibodies confirmed that TrkB is expressed at considerably higher levels than TrkC in the developing CNS (data not shown). We also noted that, unlike in the CNS, rare DRG neurons escaped cell death at E13.5 (Fig. 3A, arrows), presumably corresponding to those coming into contact with sufficient quantities of the corresponding neurotrophin. These results indicate that just like neurons can escape normally occurring cell death when NGF levels are artificially increased during development[22], TrkA or TrkC expression beyond physiological levels also causes their death.

Survival of neurons lacking TrkA

To test the prediction that Trk expression is involved in the elimination of neural crest-derived neurons during development, we focused our analyses on TrkA neurons as TrkC is expressed by many progenitors already during their migration[3,23]. This complicates the analysis as NT3 affects early aspects of gangliogenesis, before target-derived NT3-

dependency for survival[24]. Sympathetic neurons were isolated from the superior cervical ganglion (SCG) of animals lacking TrkA[25] at E17 when many neurons begin to depend on target-derived NGF for survival[26]. While wild-type neurons were rapidly eliminated in the absence of NGF, those lacking TrkA survived in large numbers (Fig. 4A,B). However, their processes were not maintained and the addition of NGF caused their rapid death (Fig. 4A,C). Previous work by others has indicated that the survival of sympathetic neurons and their axons is regulated by neurotrophin activation of p75, such that the activity-dependent-release of BDNF causes axon pruning during development[27]. In line with this, exuberant innervation of some target tissues innervated by sympathetic axons has been reported in animals lacking p75[28], as well as higher numbers of neurons in their SCG at birth, but not at E16.5[29]. To test if p75 expression accounts for the elimination of neurons lacking TrkA upon NGF addition, we isolated SCG neurons lacking both TrkA and p75[30] and found that they survived and elongated processes both in the presence of NGF, as well as in its absence (Fig. 4A-C). Neurons lacking only p75 did not survive in large numbers in the absence of NGF (20.3% ± 2.5 SEM, $p<0.05$ compared with wild-type), suggesting that at E17, molecules others than p75 may also be involved in TrkA-mediated killing. We next quantified cell death in DRG, another neural crest-derived structure containing a high proportion of TrkA-expressing neurons[31]. At E11.5, in DRG lacking TrkA, the levels of cell death were not different from control, in line with a previous report[32]. However, cell death was significantly increased at E11.5 in *ngf -/-* animals, confirming NGF-dependency for survival at this early age[33] (Fig. 5A,B). As DRG neurons lacking TrkA were previously reported to die at later time points[32], we examined sections of mutants lacking both TrkA and p75 as there is a marked developmental increase of p75 expression in developing DRG[34]. We found the levels of cell death to be significantly reduced at E14.5 in animals lacking both neurotrophin receptors (Fig. 5). As a control, *trkA-/-* animals were also analyzed at E10.5 and as expected, no differences were found between control and mutant ganglia (Suppl. Fig. 4). At this developmental age, TrkA levels are still very low and NGF-dependency for survival insignificant[34].

Cell death without Trk kinase activation

We next sought to determine whether death mediated by TrkA and TrkC requires activation of their kinase domain. Western blot experiments with antibodies against phosphotyrosine failed to reveal autoactivation, likewise with TrkB. Phosphorylation was only observed after addition of NGF, NT3 or BDNF respectively (data not shown). While the addition of K252a completely prevented Trk phosphorylation and the survival of TrkA- or TrkC-expressing neurons promoted by NGF or NT3, it did not prevent neuronal death in the absence of neurotrophin (Fig. 6A,B), nor did it recognizably affect the TrkB-expressing neurons (Suppl. Fig. 1). In line with the notion that the death caused by TrkA and TrkC does not involve activation of their kinase domain, neurons generated from ES cells expressing a kinase-inactive mutant of TrkA (K538A[35]) died just like those expressing wild-type TrkA (Fig. 6C-E). As expected, these neurons could not be rescued by the addition of NGF (Fig. 6C,D). In view of the results obtained with sympathetic and sensory neurons lacking p75, we examined its possible involvement in the death of ES

cell-derived neurons expressing TrkA. ES cell-derived progenitors express p75 at relatively high levels[11] and we previously showed that its moderate overexpression is sufficient to cause the death of neurons and of their processes[12]. TrkA ES cells were selected to also express one of 3 different shRNA efficiently targeting p75 (Fig. 6F-H) which resulted in significantly reduced levels of cell death (Fig. 6F-H). However, cell death was not completely abolished, again suggesting that p75 may not be the only mediator of cell death induced by TrkA or TrkC. Also, the results obtained with embryos generated from the corresponding ES cells indicate that TrkA and TrkC can dominantly cause the death of all developing neurons though p75 is not expressed at high levels in most CNS areas. Alternative candidates include the p75-related molecules NRH2, Troy, DR6, all members of the large tumor necrosis factor receptor family[36]. Additional work will also be required to explain why TrkB does not initiate the death process. We observed that in membrane fractions prepared from the corresponding ES cell-derived neurons, TrkB partitions differently from TrkA and TrkC (VN and YAB, manuscript in preparation).

Discussion

The finding that TrkB, unlike TrkA and TrkC, does not trigger neuronal death suggests that in the developing CNS, mechanisms based on cell-cell contact and synaptic transmission predominate over those involving diffusible ligands in controlling the survival of neurons. During the course of the evolution of the nervous system, the diversification of the Trk family seems to have accompanied the generation of epidermal placodes and the neural crest as transient structures at the origin of the PNS. This view is consistent with the observation that the cephalochordate Amphioxus, which lacks a neural crest[37], has only one Trk receptor[8]. It will be interesting to examine the role of this ancestral gene as it is primarily expressed by neurons, both during development and in the adult. In vertebrates, the progenitors of the PNS migrate and divide extensively as single cells outside their epithelia of origin. TrkA and TrkC are both expressed at high levels by many PNS neurons at the time of target innervation, and cell numbers can then be adjusted by creating a dependency for survival on local sources of neurotrophins restricted to the newly colonized territories.

The lack of death-inducing activity of TrkB does not imply that its activation by BDNF is not required to prevent neuronal death during the development of the PNS. For example, BDNF deprivation causes substantial losses of cranial sensory neurons and these neurons are known to require TrkB activation to survive[38]. However, mechanisms not based on TrkB expression are likely to predispose these neurons to die, including high levels of death-inducing receptors such as p75, NRH2 or TrkC, the latter being expressed at levels similar to TrkB in vestibular neurons, without any detectable survival role for its ligand NT3[39]. Likewise after axotomy or in culture, CNS neurons including motoneurons or retinal ganglion cells are rescued by the addition of BDNF[40], but these neurons up-regulate death-inducing molecules such as p75 following section of their axons[41]. Mice lacking BDNF fail to show significant losses of motoneurons or of retinal ganglion cells[6,40,42].

Beyond new insights in vertebrate nervous system development, our findings also suggest an explanation for the different prognoses associated with tumors derived from the neural crest, including neuroblastomas in particular. It has long been recognized that tumors arising from progenitors of the sympatho-adrenal lineage often spontaneously regress when expressing TrkA or TrkC (for review, see Ref. [43]). By contrast, TrkB-expressing neuroblastomas have a significantly poorer prognosis.

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Author contributions

Tetraploid embryos were generated by P.G. and H.L.; J.M.F. initiated the comparison of the *ngf* and *trkA* mutant embryos; C.R performed the cloning of the HA-tagged Trk cDNAs; ES lines expressing non-tagged Trk cDNAs from the *mapt* locus were generated by M.B.; L.Z. made the first observations with the neuronal differentiation of Trk-expressing ES cells. V.N. and Y.-A.B designed and carried out all other experiments. This work was supported by the consortium EuTRACC and the Swiss National Foundation.

Summary of methods

Differentiation of ES cells and generation of embryos by tetraploid complementation
ES cells were cultured and differentiated as previously described[10] and tetraploid ES cells aggregated with Trk and/or GFP ES cells as in Ref. 19.

Culture of superior cervical ganglia
Superior cervical ganglia isolated from E17 embryos were dissociated and cultured as described[44].

Figure Legends

Figure 1. TrkA and TrkC, but not TrkB, cause the death of ES cell-derived neurons
A. Trk detection in neuronal lysates (3 div) with anti-HA. B. Quantification of HA levels (N=3, mean ± SEM, t-test). C. Detection of TrkB driven by its own (right) or the *mapt* promoter (left) with anti-panTrk. D. β-III tubulin staining of control and Trk-expressing neurons cultured without or with 50 ng/ml neurotrophins. Control, *mapt::TrkB* and *mapt::TrkC* cultures: 6 div, *mapt::TrkA*: 4 div. E. Percentage of active caspase-3 positive neurons (N=7, mean ± SEM, t-test. P values relative to wild-type neurons. t-test between NGF or NT3 treatments and the corresponding non-treated cells, P<0.0001). Scale bars: 100 μm.

Figure 2. Visualization of the nervous system by GFP expressed from the *mapt* locus
Embryos were generated by aggregation of tetraploid blastomeres with ES cells expressing *TrkA*, *TrkB* or *TrkC* from one *mapt* allele and GFP from the second allele. Control embryos express GFP from both *mapt* alleles.

Figure 3. Sections of *mapt:.Trk* and *mapt::GFP* embryos: neuronal death and Trk expression
A. β-III tubulin staining in sections at E11.5 and E13.5. Note the loss of GFP signal in TrkA and TrkC embryos between E11.5 and E13.5. B. Active caspase-3 positive neurons in the spinal cord (SC) and dorsal root ganglia (DRG) at E11.5 (N=3, mean ± SEM, t-test, P values relative to *mapt::GFP*). C. Relative Trk expression in the DRG of embryos with the indicated genotypes at E12.5 with anti-panTrk (N=5 embryos per genotype). Scale bars: 100 μm.

Figure 4. Loss of TrkA protects sympathetic neurons from cell death *in vitro*.
A. Neurofilament staining of E17 SCG neurons of the indicated genotypes, 2 div with or without NGF. Inserts are magnification of boxed areas. Note that *TrkA-/-* neurons have healthy cell bodies in the absence of NGF, but not in its presence (inserts). Percentage of healthy neurons in the absence of NGF (B), or in its presence (C) (N=5, ± SEM, t-test, P value relative to wild-type). Scale bars: 50 μm

Figure 5. Loss of TrkA protects sensory neurons from cell death *in vivo*.
Active caspase-3 staining of E11.5 (A) and E14.5 (C) spinal cord and lumbar dorsal root ganglia (DRG) sections of the indicated genotypes. White lines encircle the ganglia. B,D. Active caspase-3 positive DRG neurons. (N=4 embryos per genotype, mean ± SEM, t-test). Scale bars: 100 μm.

Figure 6. Trk-induced death without kinase activity
A. β-III tubulin staining of *mapt::TrkA* and *TrkC* neurons cultured with or without neurotrophin, K252a (100 nM) or both. B. Percentage of active caspase-3 positive

neurons (N=9, mean ± SEM, t-test. P values relative to untreated samples). Ç. β-III tubulin staining of *mapt::TrkA* and *mapt::TrkA/KA* (kinase inactive) neurons cultured with or without NGF. D. Percentage of apoptotic neurons (N=7, mean ± SEM, t-test). E. Trk levels in 3 div neurons determined by anti-HA. F. β-III tubulin staining of *mapt::TrkA* neurons stably expressing scrambled, or p75 shRNA. G. Percentage of apoptotic neurons (N=7, mean ± SEM, t-test). H. p75 levels in *mapt::TrkA* neurons expressing scrambled shRNA, or p75 shRNAs. Scale bars: A, F: 100 μm; C: 50μm.

Supplementary figure 1. The survival of *mapt::TrkB* neurons is not caused by receptor activation

β-III tubulin staining of *mapt::TrkB* neurons (8 div) treated at 2 div with either K252a or with a monoclonal antibody blocking BDNF activity. Scale bars: 100 μm.

Supplementary figure 2. Proteolysis of p75 and effects of gamma secretase inhibition

A. The intracellular domain of p75 is generated by proteolysis of p75 and is detectable in TrkA and TrkC cells incubated with the proteasome inhibitor lactacystin. Note that its generation is blocked by NGF or NT3 and by LY 411575. B LY411575 (50 nM) completely blocks cell death caused by TrkA abd TrkC.

Supplementary figure 3. Trk receptor expression in the developing nervous system. *In situ* hybridizations with probes against TrkA, TrkB or TrkC (Allen Brain Atlas).

Supplementary figure 4. Analysis of cell death in E10.5 sensory neurons

Active caspase-3 staining of spinal cord and lumbar dorsal root ganglia (DRG) sections of wildtype and TrkA mutant E10.5 embryos. White lines encircle the ganglia. Quantification of TUNEL-positive DRG neurons (N=4 embryos per genotype, mean ± SEM). Scale bars: 100 μm.

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Supplementary Materials and Methods

Targeting of the mapt locus in ES cells

Targeting of the endogenous *mapt* locus was performed as previously described[12]. A cassette containing a EGFP (Clontech, Palo Alto, California), TrkA, TrkB or TrkC cDNA, the *Pgk-1* polyadenylation signal and the G418-selectable marker *Pgk-Neo*^{r} was inserted between the PmeI and NotI sites of a plasmid containing exon 1 of the tau locus and 8.0 kb flanking genomic sequence. The linearized targeting vector was electroporated into J1 embryonic stem (ES) cells as described[20] http://www.nature.com/neuro/journal/v4/n1/full/nn0101_29.html - B43. 200 G418-resistant colonies were picked for each line and analyzed by Southern blot. The 5' external probe consisted of a 500-base pair (bp) *Sma*I−*Eco*RI genomic fragment whose 5' end was located 2.8 kb upstream of exon 1, and the 3' external probe consisted of a 600-bp *Bam*HI−*Eco*RI genomic fragment whose 5' end was located 6.0 kb downstream of exon 1.

Western Blot

After 2 washes with ice-cold PBS, cells were lysed in buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100 and supplemented with protease inhibitor cocktail (Roche). After centrifugation for 30 min at 4,200 r.p.m. in an Eppendorf 5415R with F45-24-11 rotor, the supernatant was collected. Samples containing 1mM (dithiothreitol) DTT, were heated at 70^{o}C for 10min in sample buffer (Nupage, Invitrogen), loaded onto gradient polyacrylamide gels (Nupage, Invitrogen) and transferred to nitrocellulose membranes using semi-dry transfer cell (Biorad). Membranes were blocked with a 5% milk solution prepared in PBS containing 0.1% Tween (TPBS) overnight, and then incubated overnight with primary antibody. Subsequently, membranes were washed several times in TPBS and incubated 1 hour with the corresponding secondary antibody in 2% milk solution in TPBS. Following several washes in TPBS, detection of signals was performed with Immobilon western ECL substrate (Millipore) or with Chemi-glow (Alpha innotech corp.)

In vivo tetraploid complementation assay

ES cell clones expressing TrkA, TrkB or TkC from one *mapt* allele and GFP from the second allele, or a control clone expressing GFP from both *mapt* alleles were aggregated with CD1 tetraploid embryos to generate ES cell-derived embryos as described[19]. Embryos were dissected at E11.5 and E13.5 and assessed for EGFP expression under fluorescence optics.

Fixation and immunofluorescence

Mouse embryos were collected at E11.5 or E13.5 in cold PBS and fixed overnight at 4^{o}C in 4% PFA (paraformaldehyde) prepared in PBS. Subsequently embryos were cryo-protected by overnight incubation in 30% sucrose solution prepared in PBS at 4^{o}C and were sectioned in a Leica cryostat. TUNEL staining was performed using the *in situ* cell death detection kit (Roche).

Antibodies and inhibitors

The following antibodies were used: mouse anti-BDNF Mab#9[45], mouse anti-HA (1:1000, as described[13]), rabbit anti-p75 (1:1000, Promega), rabbit anti-p75 (1:1000, Freund I[46], raised against a bacterially expressed intracellular domain sequence), rabbit anti-Trk (C-14) (1:1000, Santa Cruz Biotechnology), mouse anti-actin (1:5000, Sigma), mouse anti-ß tubulin (1:5000, Covance), rabbit anti-activated caspase-3 (1:1500, Chemicon). All HRP secondary antibodies were obtained from Dianova. Alexa fluorophore-conjugated secondary antibodies for immunohisto- and immunocyto-chemistry were obtained from Millipore. K252a was from Calbiochem, lactacystin (5 µM, proteasome inhibitor, Cayman). Recombinant NGF and NT3 produced in Chinese hamster ovary cells (CHO) were a gift from Genentech, Inc. All neurotrophins were used at a final concentration of 50 ng/ml.