Therapeutic neuroprotection by an engineered neurotrophin designed to broadly activate Trk-receptors and to circumvent p75 receptors

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Running title: In vivo efficacy of a pan-Trk agonist with low p75 affinity
Abstract

The neurotrophin growth factors bind and activate two types of cell surface receptors: the Trk family, and p75. TrkA, TrkB or TrkC are bound preferentially by NGF, BDNF, or NT3 to activate neuroprotective signals. The p75 receptors are activated by all neurotrophins, and paradoxically in neurodegenerative disease p75 is upregulated and mediates neurotoxic signals. To test neuroprotection strategies, we engineered NT3 to broadly activate Trk receptors (mutant D), or to reduce p75 binding (mutant RK). We also combined these features in a molecule that activates TrkA, TrkB and TrkC but has reduced p75 binding (mutant DRK). In neurodegenerative disease mouse models in vivo, the DRK protein is a superior therapeutic agent compared to D, to RK, or to wild-type neurotrophins, and protects a broader range of stressed neurons. This work rationalizes a therapeutic strategy based on the biology of each type of receptor, avoiding activation of p75 toxicity while broadly activating neuroprotection in stressed neuronal populations expressing different Trk receptors.

Significance Statement.

Neurotrophins NGF BDNF and NT3 growth factors, each can activate a TrkA or TrkB or TrkC receptor respectively, and all can activate a p75 receptor. Trks and p75 mediate opposite signals. We report the engineering of a protein that activates all Trks, combined with low p75 binding, as an effective therapeutic agent in vivo.
Introduction

Neurotrophins (NF) are growth factors with key roles in the embryonic and the adult nervous systems (Chao, Rajagopal et al. 2006). Mature NFs include NGF, BDNF and NT3. NFs bind to two classes of receptors, Trk and p75, which play opposite and often paradoxical roles in vivo.

Trk-receptors are bound by mature NFs with relative selectivity. NGF is the preferred ligand for TrkA, BDNF for TrkB, and NT3 for TrkC. In some cellular contexts, NT3 can activate TrkA and TrkB with lower efficiency (Ivanisevic, Banerjee et al. 2003, Ivanisevic, Zheng et al. 2007). Activation of Trk-receptors preserves the phenotype and function of neurons, and promotes neuronal survival in neurodegenerative diseases (Saragovi, Hamel et al. 2009, Josephy-Hernandez, Jmaeff et al. 2017, Saragovi, Galan et al. 2019).

Regarding p75-receptors, they are promiscuous and are bound by all NFs and the NF precursors (pro-NFs) (Hempstead 2002, Hempstead 2006, Ibanez and Simi 2012). The p75 receptors are upregulated in acute and chronic neurodegenerative diseases, and mediate multiple actions, such as elevation of pro-inflammatory TNFα (Lebrun-Julien, Duplan et al. 2009, Bai, Dergham et al. 2010, Lebrun-Julien, Bertrand et al. 2010, Barcelona, Sitaras et al. 2016, Mossa, Galan et al. 2020) resulting in neuronal dysfunction and death.

The NFs have been investigated as therapeutic agents for neurodegeneration (Josephy-Hernandez et al. 2017; Saragovi, Galan, and Levin 2019), but clinical trials with mature NF have not been successful to date. Many reasons have been postulated for the lack of clinical efficacy of NFs, such as poor pharmacokinetics due to their short half-lives, the difficulty of delivery to target neuronal compartments, and the challenges of stable expression/manufacturing, and lack of receptor selectivity leading to upregulated p75 activity prevailing over the desired neuroprotective signals of Trk-receptors.

Several strategies have been explored to develop neurotrophic-targeted therapies (Saragovi, Hamel et al. 2009, Longo and Massa 2013, Josephy-Hernandez, Jmaeff et al. 2017, Saragovi, Galan et al. 2019). Here we discuss combining two of them. Selectively activating a single type of Trk-receptor used Trk-agonistic small molecules
or mAbs that do not bind to p75 (Bruno, Clarke et al. 2004, Zaccaro, Lee et al. 2005, Bai, Dergham et al. 2010, Bai, Jing Xu et al. 2010, Aboulkassim, Tong et al. 2011, Simmons, Belichenko et al. 2013, Brahimi, Maira et al. 2016, Szobota, Mathur et al. 2019, Brahimi, Galan et al. 2020). A variation of this was to mutate NGF or NT3 at their p75-binding domain to abolish interactions between p75 and NF<sup>p75-less</sup> mutant, but leaving TrkA or TrkC activation intact (Urfer, Tsoulfas et al. 1994, Guo, Meyer et al. 1996, Mahapatra, Mehta et al. 2009, Bai, Dergham et al. 2010, Enomoto, Bunge et al. 2013). These agents have been evaluated therapeutically in vivo.

A separate approach was to activate all Trk receptors simultaneously using engineered proteins with sequences from NGF, BDNF and NT3 (pan-NF) (Urfer, Tsoulfas et al. 1994, Rydén and Ibanez 1996, Ryden and Ibanez 1997, Urfer, Tsoulfas et al. 1997). Since neuronal subpopulations express different Trk receptors (e.g. TrkA cholinergic, TrkB dopaminergic), a pan-NF would promote survival simultaneously in different injured neuronal phenotypes. However, purified proteins have not been evaluated in vivo in models of disease, and moreover in these proteins there remains the potential for unintended activation of p75 which can be toxic.

Here, we report work combining the two strategies of mutating NFs to enhance activation of all Trk-receptors and avoiding p75 activation. We generated a pan-NF protein that can activate all Trk receptors, combined with mutations that reduce binding to p75 (hereafter “DRK” based on the engineered mutations). In experimental models of acute (optic nerve axotomy) or chronic (diabetic retinopathy) retinal neurodegeneration in vivo, DRK has superior therapeutic efficacy compared to NF mutants that only reduce binding to p75, or to NF mutants that only activate all Trk receptors, or to control wild-type NFs. Efficacy was significant with DRK administered as a single dose, after injury, with ongoing degeneration, and at low concentrations. This work helps in the rationalization of therapeutic strategies by accounting for the pattern of receptor expression and the biology of each targeted receptor.

**Materials and Methods**

**Synthesis of mutants**: Mutant forms of NT3 (D, RK, DRK) were codon-optimized and expressed in E. Coli. Proteins were purified and refolded from the collected
inclusion bodies. Appropriate refolding and purity were confirmed by SDS-PAGE. Mutant D15A (D) was designed to enhance TrkB binding, and mutant R114A/K115A (RK) was designed to reduce p75 binding (Urfer, Tsoulfas et al. 1994, Rydén and Ibanez 1996, Ryden and Ibanez 1997, Urfer, Tsoulfas et al. 1997). Mutant D15A/R114A/K115A (DRK) was designed to combine these features to generate a molecule that would potentially activate TrkA, TrkB and TrkC with reduced p75 binding. DRK is a p75less–pan-NF. Wild type NFs were used as controls. As an additional control a previously published mutant of NGF, termed NGF-C, does not bind to p75 but activates TrkA fully (without activating TrkB or TrkC) (Bai, Dergham et al. 2010, Aboulkassim, Tong et al. 2011).

**Cell lines.** Cells have been described (Maliartchouk and Saragovi 1997, Guilmard, Ivanisevic et al. 2010, Barcelona and Saragovi 2015, Szobota, Mathur et al. 2019, Brahimi, Galan et al. 2020). HEK293 or NIH3T3 cells were stably transfected to express either TrkC, or TrkB, or TrkA, or p75. The nnr5 cells are a variant of the rat PC12 cells (TrkA+ p75+) that lost TrkA expression. Nnr5 cells were stably transfected with human TrkC cDNA (nnr5-TrkC) and are TrkC+ p75+. Receptor expression was routinely verified by flow-cytometry, using selective monoclonal antibodies directed to the ectodomain. All cells were monitored routinely to exclude mycoplasma contamination (Cat:M208001, ZM Tech Scientific).

**Mice.** The animal protocols and endpoints were approved by the Lady Davis Institute Animal Care Committee and by McGill University IACUC. Experiments were done according to the guidelines of the Canadian Council on Animal Care (CCAC) and the 3R principle of replacement, reduction and refinement was applied. Healthy wild-type male C57BL/6N mice, 8-10 weeks of age, 18-21 grams (Charles River Laboratories) were used for the optic nerve axotomy model. C57BL/6J (Jackson Laboratories) were used for the mouse model of diabetic retinopathy. A maximum of five mice per cage were kept in a 12 hrs dark-light cycle with food and water ad libitum.

**Direct binding assay.** Wild type NT3 or mutant DRK proteins were biotinylated following manufacturer’s instructions (EZ-Link™ Sulfo-NHS-LC-biotin, cat: 21335, Thermofisher scientific). The stoichiometry of biotin:protein was verified to be ~4:1 for both wild type NT3 and DRK mutant. Not all mutant NFs could be biotinylated due to
lack of pure protein. Direct binding of the biotinylated ligands (a range of 0, 2, 10, and 50 nM concentrations) was evaluated by quantitative flow cytometry with detection by fluorescein-conjugated-avidin (avidin-FITC, cat: A 2050, SIGMA). Binding and washes were carried out in binding buffer (PBS/0.2% BSA/0.1% Na Azide pH 7.3) at 4°C, on of cells stably transfected to express TrkA, TrkB, TrkC, or p75. Receptor expression was routinely verified using specific anti-receptor mAbs.

**Binding competition to biotinylated NT3.** The binding competition assay was performed as described (Maliartchouk and Saragovi 1997, Guillemaurd, Ivanisevic et al. 2010, Barcelona and Saragovi 2015, Brahimi, Galan et al. 2020). Cells were pre-incubated for 15 min in binding buffer at 4°C with negative control vehicle (no competition), positive control competition wild-type NT3 (Cat: 450-03, Pepro Tech), or test proteins (1 µg). Then, biotinylated NT3 (NT3-bio, 40 nM) was added for 15 min. After washes in binding buffer, cells were incubated with fluorescein-conjugated-avidin (avidin-FITC, SIGMA) for 15 min at 4°C, washed at 4°C, and analyzed immediately by flow cytometry. Mean channel fluorescence (MCF) values of bell-shaped histograms were standardized to control vehicle = 100% binding, and avidin-FITC without NT3-bio = 0% binding. Shown are % MCF, mean of three independent experiments ± SD.

**Cell Metabolism/Survival Assays.** The growth/survival profile of the cells were quantified in 96-well plates using the tetrazolium salt reagent 4-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; cat: M2128, Sigma) 48 hrs after plating; by reading the optical density (OD). Cells cultured in serum-free-medium (SFM) die by apoptosis, but they can be rescued if they express TrkA or TrkC or TrkB and are supplemented with NGF (Cat: 450-01, Pepro Tech) or NT3 or BDNF (Cat: 450-02, Pepro Tech) respectively. Vehicle is the negative control and serum the positive control. Under these conditions MTT assay reflects cell survival and is comparable with live cell counting (Maliartchouk and Saragovi 1997, Guillemaurd, Ivanisevic et al. 2010, Barcelona and Saragovi 2015, Brahimi, Galan et al. 2020). All assays were in quadruplicate and were repeated n>3 independent times. MTT data are standardized to optimal concentration (2 nM) of wild-type neurophin (NF) = 100% survival, and serum-free medium (SFM) = 0% survival, using the formula 

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\frac{(OD_{test} - OD_{SFM}) \times 100}{(OD_{optimal\ NF} - OD_{SFM})}
\]
**Western Blot Analysis.** The activation of signaling proteins (Trk, Akt, and MAPK) was studied after treatment of starved cells with wild-type NT3 or mutants (2 nM) for 15 min. Detergent lysates were analyzed by Western blotting with anti-pTyr mAb 4G10 (generic anti-phosphotyrosine, cat: 05-321, Millipore), anti-phospho-MAPK (p-ERK1/2, cat:4377S, Cell Signaling), or anti-phospho-Akt (cat: 4060S, Cell Signaling). After stripping, membranes were re-probed with anti-actin (cat: A5316, Sigma) to standardize loading.

**AlphaLISA.** HEK293 or NIH3T3 cells that over-expressed either human TrkB or human TrkC were grown in 96-well plates. Cells were serum-starved for 1-4 hrs, then incubated with test agonists for up to 20 min. Cells were lysed and a portion of the cell lysate was incubated with AlphaLISA reagents (PerkinElmer) in 384 well plates. p-Erk 1/2 (MAPK) was quantified using an EnSpire plate reader (PerkinElmer) as described (Szobota, Mathur et al. 2019).

**Differentiation Assays in cells.** nnr5 cells stably transfected with human TrkC cDNA (nnr5-TrkC) were plated on cover slips with full media in 24-well plates. Twenty-four hours after plating, cells were untreated (control), or were treated for 72 hrs with wild-type NT3 or mutants at 25 pM, 0.1 nM and 2 nM. Cells on the coverslip were immunostained with rabbit-anti-MAP2 (cat: ab183830, Abcam) followed by anti-rabbit Alexa fluor 488 (cat:A11034, ThermoFisher Scientific), then washed, and further stained with DAPI. Pictures were taken by microscopy (40x). Differentiation was scored morphologically as % of cells with neurites (> 2 cell body long). Quantification was done by image analysis (Galan, Barcelona et al. 2017, Galan, Jmaeff et al. 2017), from at least three independent assays.

**Upregulation of TNFα by activated p75.** HEK293-p75 cells (stably transfected and expressing p75) were treated with the indicated compounds for 6 hrs. Quantification of TNFα mRNA was performed by real-time quantitative PCR and primers for TNFα and RNAs18 (Barcelona and Saragovi 2015, Brahimi, Galan et al. 2020). Data are expressed as the mean ± SD relative to the untreated (2-3 independent experiments, each in triplicate).

**Optic nerve axotomy.** The optic nerve axotomy model was described (Bai, Dergham et al. 2010, Galan, Dergham et al. 2014). The optic nerve of one eye was exposed and
was completely transected 0.75–1.0 mm posterior to the eyeball with the use of micro
tweezers, sparing vessels. The contralateral eye was mock-surgery without damaging
the optic nerve. Normal blood circulation in the retina was ascertained. This model
axotomizes the Retinal Ganglion Cells (RGC) axonal fibers, and the RGC cell body in
the retina degenerates with fast kinetics (~50% RGC loss at day 7 post-axotomy, ~90% 
RGC loss at day 14 post-axotomy). In the optic nerve axotomy model animals received
1x treatment immediately after injury (under the same anesthesia), and RGC survival
was quantified at the day-14 end-point. RGC survival was quantified as described
below.

The eyes subjected to optic nerve axotomy were injected with test agent, or control
vehicle, or control wild type NFs. The uninjured contralateral eyes (mock surgery)
acted as naive normal controls for each individual mouse, n = 8 per group (each group
is the indicated treatment of the injured eye). Surviving RGCs were quantified by
counting Brn3+ cells in flat-mounted retinas (Bai, Dergham et al. 2010, Barcelona,
counts for each individual mouse were analyzed as the ratio of the injured treated-eye
to the uninjured contralateral eye (set to 100% RGCs) to derive a % surviving RGCs for 
the injured eye of that individual mouse. The % surviving RGCs data for n = 8 mice
within a group was then averaged ± SD. This experimental design accounts for possible
mouse-to-mouse individual variations in RGC numbers.

**Diabetic Retinopathy.** Hyperglycemia was induced as described (Barcelona, Sitaras
et al. 2016). Male 10-week-old mice received an intraperitoneal injection of STZ (60
mg/kg) (Sigma-Aldrich) dissolved in sodium citrate buffer (0.01 m, pH 4.5) on five
consecutive days. Age-matched, nondiabetic C57BL/6 mice injected with sodium citrate
buffer were used as controls. Blood glucose was measured 7-days after completion of
STZ dosing (e.g. the induction of diabetes). Fasting blood glucose levels >17 mmol/L
(300 mg/dl) were considered to be diabetic. In this model there is loss of RGC nerve
fibers of ~20% at week 6 of diabetes (quantified *in vivo* by Tomography, see below)
reflecting the progressive injury of RGCs (Barcelona, Sitaras et al. 2016). In the diabetic
retinopathy model, animals received 1x treatment after 2.5 weeks of hyperglycemia, at a
time of ongoing degeneration, and the endpoint was at the 6-week time point.
For intravitreal injections mice were anesthetized in 3% isoflurane and 2 μl containing a total of 2 μg wild-type NF, mutant NFs, or vehicle (PBS) was slowly delivered into the vitreous chamber using a Hamilton syringe, and confirmed microscopically. After the injection, the syringe was left in place for 30 seconds and slowly withdrawn to prevent efflux.

**Quantification of the Nerve Fiber Layer by Tomography.** A non-invasive spectrometer-based Fourier-domain (FD)-OCT system was used to acquire retinal images. Fourier domain OCT is a non-invasive method that allows time-kinetic studies in the same animal, with consistent and reproducible axial resolution in tissue nominally better than 2.2 μm (Bai, Dergham et al. 2010, Jian, Zawadzki et al. 2013, Barcelona, Sitaras et al. 2016). In each B-scan, the thickness of the nerve fiber layer (NFL)—ganglion cell layer (GCL)—inner plexiform layer (IPL), hereafter referred to as NGI, was measured at three adjacent points, using ImageJ software (http://imagej.nih.gov/ij) as described (Barcelona, Sitaras et al. 2016, Galan, Barcelona et al. 2017, Galan, Jmaeff et al. 2017). The outer nuclear layer (ONL) is a layer not affected in the time-frames of the disease models and was also measured at the same locations as internal control for potential geographic differences in retinal thickness. Representative data are shown, and quantified as average absolute thickness in μm ± SEM in control versus diabetic injected with either vehicle (PBS) or with the indicated treatment, n = 3 independent experiments, 3–4 mice per group; and as percentage of nerve fiber loss ± SEM by setting the NGI damage in vehicle-treated diabetic eyes as maximal (100%).

**Statistical analysis.** In all experiments, one-way ANOVA with significance α = 0.05 or lower followed by Bonferroni post-hoc analysis was used for calculating significance between groups. Inter-group comparisons were specified before data were viewed. All comparisons are reported. The present study is exploratory and the p values are considered only descriptive.

**Results**

The mutant D15A (D) reportedly enhances TrkB binding, and the mutant R114A/K115A (RK) reportedly reduces p75 binding. Mutant D15A/R114A/K115A (DRK) was designed to combine these features to generate a molecule that would potentially
have the properties of NT3 plus enhanced TrkA and TrkB binding and reduced p75 binding. These agents were compared to each other, and to control wild-type NFs, in binding and in biological assays.

**Receptor binding profile of the Mutant NFs**

We evaluated ligand interactions with TrkC, TrkB, or p75 in a binding competition assay. The binding competition assay tests the ability of mutants to competitively inhibit the binding of biotinylated wild-type NT3 (NT3-biotin). Inhibition by unlabeled wild-type NT3 ("cold competition") is the positive control (Table 1).

In TrkC-expressing cells, all three mutants significantly competed the binding of NT3-biotin to a similar degree, and comparable to cold competition by wild-type NT3. In TrkB-expressing cells all three mutants significantly competed the binding of NT3-biotin, comparable to cold competition by wild-type NT3 or by wild-type BDNF. In p75-expressing cells, mutant D significantly competed the binding of NT3-biotin, comparable to cold competition by wild-type NT3. In contrast, mutants RK and DRK did not compete NT3-biotin binding to p75 significantly (Table 1). The mutant proteins were not evaluated for competition of NT3-biotin binding to TrkA, because detectable NT3-biotin binding to TrkA requires very high concentrations >75 nM (Ivanisevic, Banerjee et al. 2003, Ivanisevic, Zheng et al. 2007).

The binding competition data indicate that: (i) all mutants D, RK and DRK bind TrkC; (ii) all mutants bind TrkB; and (iii) mutant D binds to p75 like wild-type NT3, but mutants RK and DRK have reduced affinity for p75.

This conclusion about the binding profile of mutant DRK was confirmed in direct binding assays. Quantitative flowcytometry assays compared the direct binding of NT3-biotin and DRK-biotin to cells expressing TrkA, TrkC, TrkB, or p75 (Figure 1). Ligand binding is concentration-dependent. Compared to wild type NT3, the DRK mutant maintains equal or enhanced binding to TrkC, TrkA, and TrkB; but has significantly reduced binding to p75.

**Mutants promote survival in cells expressing TrkC or TrkB**

Survival assays evaluated whether the mutants have agonistic activity at Trk receptors. In a serum-deprivation cell culture model, the NFs promote survival. The bioactivity of the mutants was compared versus the corresponding wild-type NFs as
positive controls (NT3 for TrkC-expressing cells; BDNF for TrkB-expressing cells, and NGF for TrkA-expressing cells). Concentrations of the NFs at low (L: 0.2 nM, suboptimal) or high (H: 2 nM, optimal) molarity, were tested versus untreated control. The 0.2 nM and 2.0 nM ligand concentrations were pre-defined respectively as suboptimal and optimal for these cells in these assays (Ivanisevic, Banerjee et al. 2003, Zaccaro, Lee et al. 2005, Ivanisevic, Zheng et al. 2007, Guillemard, Ivanisevic et al. 2010, Brahimi, Ko et al. 2014).

In TrkC-expressing cells, mutant RK promoted survival significantly better than wild-type NT3 (p < 0.01) (Figure 2A). The D and DRK mutants promoted survival better than NT3, but without reaching statistical differences. These data indicate that mutations expected to enhance TrkB interactions could also have a positive impact on TrkC-mediated trophic survival, because these cells do not express TrkB or p75.

In TrkB-expressing cells the mutants D and DRK promoted survival above untreated control cells. Mutant DRK was significantly better than mutant D (p < 0.05) indicating enhanced TrkB activation. The mutant RK and control wild-type NT3 did not induce significant survival at 0.2 nM or 2 nM (Figure 2B), or even at the higher 10 nM concentration (10 nM not shown).

In TrkA-expressing cells, neither the mutants nor wild-type NT3 promote survival at the concentrations tested (Figure 2B). This is consistent with previous reports that wild-type NT3 promotes significant survival via TrkA only at >75 nM concentrations (Ivanisevic, Banerjee et al. 2003, Ivanisevic, Zheng et al. 2007).

The survival data (Figure 2) are generally consistent with the binding data (Table 1 and Figure 1) and validate the concept that mutants D and DRK retain full or enhanced TrkC activation, and also have enhanced TrkB activation.

However, it is noteworthy that binding and bioactivity assays are not always aligned, this is a recurrent theme in neurotrophin biology (Ivanisevic, Banerjee et al. 2003, Ivanisevic, Zheng et al. 2007). For example, at the concentrations tested, both wild type NT3 and DRK bind to TrkA but do not promote survival in biological assays. Also, compared to wild type NT3, mutant RK promotes enhanced TrkC survival without enhanced binding to TrkC (nor enhanced biochemical signals, see below). Perhaps this
is due to the RK mutant being more stable at 37°C, a feature that could be evident in
the long-term biological assays (48 hrs) at cell culture temperatures.

**Mutants induce differentiation via TrkC**

In addition to promoting survival signals, NT3 promotes neurite growth and synapse
formation. Hence, we tested TrkC-mediated induction of neurite outgrowth in nnr5-TrkC
cells, which differentiate in response to NT3.

Given that nnr5-TrkC cells express TrkC and p75, and that p75 can impact negatively
or positively on Trk agonists (Ivanisevic, Banerjee et al. 2003, Zaccaro, Lee et al. 2005,
Ivanisevic, Zheng et al. 2007, Guillemand, Ivanisevic et al. 2010, Brahimi, Ko et al.
2014) we first evaluated function on these cells using survival assays.

The nnr5-TrkC cells survive equally well in response to wild-type-NT3 and to the
mutants D, RK and DRK (Figure 3A). However, we note that in cells lacking p75 the
mutant RK promoted TrkC–mediated survival significantly better than wild-type NT3
(Figure 2A), and this observation remains intriguing.

In differentiation assays the mutants and wild-type NT3 all increased differentiation,
defined as the percent of cells bearing >2 axons with axonal length >2 cell bodies
(Figure 3B). In negative controls, treatment with mouse IgG, or 10 nM NGF did not
induce differentiation. Quantification demonstrates that the mutants and wild-type NT3
have similar neuritogenic activity (Figure 3C). These data indicate that expression of
p75 does not impact on the TrkC-mediated neurogenic differentiation induced by RK
and DRK although these mutants have reduced affinity for p75. The lack of impact by
p75 on the ability of mutants to induce differentiation (Figure 3B, 3C) is consistent with
lack of impact by p75 on the ability of mutants to promote equivalent survival (Figure
3A).

**Mutants induce signal transduction via TrkC and TrkB**

As a correlate of the biological endpoints of cell survival and cell differentiation, we
evaluated biochemical endpoints of signal transduction in Western blot studies using
specific antibodies to quantify signaling pathways.

The phosphorylation of TrkC (pTrkC) or TrkB (pTrkB), and the phosphorylation of
downstream effectors AKT (pAKT) and pERK1/2 were quantified (Figure 4). For
quantification, densitometry data were standardized relative to actin and to total AKT;
and the bands corresponding to p-ERK1/2, p-AKT, and p-Trks were quantified as a function of wild-type NT3 positive control. As a positive internal control for each cell type, the corresponding wild-type NFs was used (NT3 for TrkC; BDNF for TrkB, and NGF for TrkA).

In TrkC-expressing cells (Figure 4A), wild-type NT3 and all the mutants afforded significant pTrkC, and activated the downstream pERK1/2 and pAKT. In TrkB-expressing cells (Figure 4B), D and DRK were the most potent, followed by wild-type NT3 and to a lesser degree RK. These assays are consistent with the survival data for TrkC or TrkB expressing cells.

In TrkA expressing cells (Figure 4C), wild-type NT3 and mutants D and DRK activated biochemical signals that, while statistically significant, represent a small fraction of the positive control NGF-activated signals. This may explain why the agents did not promote detectable survival of TrkA-expressing cells in biological assays (see Figure 2B).

As independent confirmation of the biochemical endpoints, Trk receptor activity was assessed by AlphaLISA, a commercial assay that quantifies Trk-dependent MAPK (pErk1/2) activation after stimulation with the various ligands or controls for 15 min.

In cells expressing human TrkB, the mutants showed a trend towards lower EC50 values when compared with NT3, with similar % maximal stimulation values (Figure 5A, Table 2). In cells expressing human TrkC, all three mutants produced concentration-response curves for ERK1/2 activation that were similar to NT3, with equivalent EC50 and % maximal stimulation values (Figure 5B, Table 2), and a trend towards lower EC50 values with respect to BDNF.

p75 activation by RK and DRK mutants

In cells expressing p75 in the absence of Trks, ligand-dependent p75 activation leads to an increase in TNFα mRNA and protein. We studied the mutants for their ability to promote TNFα production in HEK293 cells expressing p75. At 10 nM all mutants induced significant TNFα expression compared to untreated control, and not different from the positive control LPS (1 µg/ml). The TNFα expression induced by mutants RK and DRK was lower, but not significantly different from wild-type NT3 (p = 0.09, one-way
ANOVA) (Figure 6). This is somewhat surprising considering that RK and DRK mutants have lower p75 binding than wild-type NT3.

Unfortunately, this in vitro assay requires high ligand concentrations (10 nM NF), and is not robust to evaluate lower agonist concentrations (e.g. at 1 nM ligands do not induce TNFα expression significantly over background control). At high ligand concentrations the differences in p75-activation may not be evident (Barcelona and Saragovi 2015). Nonetheless, the relatively lower induction of TNFα expression, and the enhanced Trk-activation profiles were encouraging for evaluating the agents in vivo.

**Neuroprotective efficacy of mutants in in vivo models of retinal neurodegeneration**

We evaluated the therapeutic action of the mutant NFs in two in vivo mouse models of retinal neurodegeneration; the optic nerve axotomy causing rapid and acute neuronal death (Figure 7), and the diabetic retinopathy model causing slower and chronic neuronal death (Figure 8). In these in vivo models, activation of TrkA or TrkB (expressed in neurons) is neuroprotective; whereas activation of p75 (upregulated in glia) causes neuronal death through elevation of pro-inflammatory TNFα (Bai, Dergham et al. 2010, Barcelona and Saragovi 2015).

**DRK mutant prevents retinal ganglion cell death in optic nerve axotomy**

Optic nerve axotomy is a model of acute injury in which the optic nerve is completely severed, causing the RGCs to die and their cell bodies in the retina to degenerate rapidly (Figure 7). Equal protein concentrations of test agents were injected intravitreally (1.6 μg/2 μL), and RGC survival was quantified at the 14-day post-axotomy end-point.

In these experiments the right eye of each mouse was injured, while the uninjured left eye represents 100% RGCs in that individual mouse. Randomized groups of mice received injections of treatments or controls (n = 8 per group). The % RGC survival data (right eye/left eye ratio) of each single mouse was averaged by group.

In the PBS-treated (control) axotomy group, only ~10% of the RGC cell bodies remained detectable. Injection of PBS control had no effect (9.2 ± 1%). Mutant RK (12.9 ± 1.0 %) and control wild-type NT3 (11.6 ± 1.0 %) did not preserve RGCs compared to controls, likely due to the poor TrkC expression in RGCs.
Significant preservation of RGCs was promoted by mutant D (19 ± 1.3 \%) \( (p \leq 0.0001 \) vs. PBS control group); and even more significantly by mutant DRK (27.7 ± 1.8 \%) \( (p \leq 0.0000061 \) vs. PBS control group). Mutant DRK was significantly better than D, likely due to lower p75 binding by DRK, since is the only difference between these mutants. Indeed, mutant DRK is significantly better than wild type BDNF \( (p \leq 0.001) \) (Figure 7), which is the gold standard growth factor for neuroprotection in this model.

Ligands lacking p75 binding are more efficacious at preserving retinal ganglion cells after optic nerve axotomy

To further evaluate the consequence of ligands binding to p75, and as a benchmark comparison, we used mutant NGF-C. NGF-C is a previously reported mutant of NGF that activates TrkA but does not bind to p75. NGF-C is reported to be significantly protective of RGCs in the optic nerve axotomy model, while wild-type NGF is not protective (Bai, Dergham et al. 2010).

The DRK mutant and NGF-C are significantly protective when compared to untreated or to vehicle controls. Together these data support the concept that after injury in vivo activation of a Trk receptor while circumventing p75-binding is beneficial, as demonstrated when comparing mutant DRK with mutant D, and comparing wild-type NGF versus NGF-C.

Mutants prevent loss of RGC Nerve Fibers and synapses in Diabetic Retinopathy.

We then compared the neuroprotective effect of NFs or mutants in vivo in a chronic degenerative mouse model of diabetic neuropathy (Figure 8). Retinopathy affects both eyes. Hence, mice received an intravitreal injection of the indicated agent in one eye; and PBS vehicle (control) in the contralateral eye to represent maximal degeneration in that individual mouse. This design provides with the ability to internally standardize the data to the untreated diabetic eye (100% damage) in each animal. Then, the groups are averaged ± SD. Drug delivery was at week 2.5 of diabetes, at a time of ongoing degeneration.

To evaluate long-term effects in this chronic disease model, endpoint and quantification was at the 6-week time point. This is a time of significant retinal degeneration but the animals remain relatively healthy in spite of persistent
Hyperglycemia. Quantification of retinal structures was done by OCT, a technique that quantifies the thickness of the Nerve fiber layer (axons), the Ganglion cell layer (RGC cell bodies) and the Inner Plexiform layer (synapses), which we termed NGI. Quantification of the NGI thickness is validated as a marker of RGC and synaptic health (Bai, Dergham et al. 2010, Barcelona, Sitaras et al. 2016, Galan, Jmaeff et al. 2017, Barcelona, Galan et al. 2018). In all eyes, the OCT measurements were taken at an equivalent geographic topology, guided by fundoscopy markers and confirmed by measuring the thickness of the Outer Nuclear Layer (ONL) at the same locations. The ONL is not affected at 6 weeks of diabetes, and its thickness serves as internal control (Platon-Corchado, Barcelona et al. 2017).

Quantification of the neuronal structures of the retina at 6 weeks after onset of diabetes showed that mutant DRK achieved a significant ~37% preservation (p < 0.001 vs. untreated) (Figure 8A, B). Mutant D achieved a significant ~17% protection (p <0.05 vs. untreated) (Figure 8C). In the diabetes model the efficacy of the DRK and D mutants is not significantly different when compared to each other, but DRK is significantly better than D when they are compared to the untreated group. Moreover, in the diabetes model mutant DRK performs significantly better than BDNF control (p <0.05).

Together, the data for the optic nerve injury and the diabetic retinopathy models indicate that DRK has overall better efficacy than the other mutants or than wild type NFs, and induces a prolonged physiological response leading to the preservation of retinal neurons and retinal structures in acute and in chronic models of disease.

**Discussion**

Strategies to achieve neuroprotection in neurodegenerative states have evolved over the last 4 decades. Approaches based on treatments with NFs have to account for the biology of these systems. In the present studies, we explored the idea that it is possible to engineer desirable features into a neurotrophin molecule that would improve its ability to protect neuronal function *in vivo*.

This was based on the idea that a molecule possessing agonist activity at more than one Trk receptor would have the benefit of enhancing the survival and function of a
A wider range of neurons, or potential synergistic effects within a neuron expresses more than one Trk receptor subtype. These benefits would be further enhanced by lowering affinity for p75 and thereby reducing the deleterious consequences of activating this receptor, particularly under pathological conditions when p75 is upregulated (Josephy-Hernandez, Jmaeff et al. 2017, Saragovi, Galan et al. 2019).

The present work shows in models of retinal degeneration the improved therapeutic efficacy of DRK, a pan-NF that can activate multiple Trks while bypassing p75. In terms of mechanisms that explain a benefit, signal transduction data indicated that both the D and DRK mutants have a greater ability to activate TrkB in terms of autophosphorylation and stimulation of the downstream ERK and AKT pathways. This was evident for pERK1,2 in cells expressing human TrkB where in dose-response analyses DRK showed a trend toward a lower EC$_{50}$ (i.e. greater potency) than NT3. Signal transduction was similar between the mutant NFs and NT3 in TrkC assays, however in assays of TrkA signal transduction, again, D and DRK showed increased activity relative to NT3. The overall picture emerging from these studies is that the D and DRK mutants retain the ability to activate TrkC, and have improved functional activity for TrkB and TrkA relative to the parent NT3 and are thus pan-Trk agonists. This is consistent with the original report that the D15A mutant of NT3 increases the affinity of NT3 for TrkB (Urfer et al. 1994).

With regards to p75, the RK and DRK mutants showed significantly less affinity than NT3 in ligand displacement experiments. However, all three mutant NFs stimulated TNFα production in p75-expressing cells. Although the NFs with the RK mutation did so to a lesser extent, the difference was not significant in this assay that requires high concentration of test agent (preventing detailed dose range studies). Consequently, the data from the binding and signal transduction assays are consistent with the D mutation providing improved agonist activity at TrkB, and the RK mutation potentially providing for reduced p75 binding and activation.

In cell survival assays, the consequence of the improved TrkB agonist activity of D and DRK translated into improved activity relative to NT3 in TrkB-expressing cells, and surprisingly all three mutants had superior survival activity relative to NT3 in TrkC-expressing cells. Although the signal transduction assays indicated improved activity in
TrkA-expressing cells for D and DRK, this did not translate into improved survival of TrkA-expressing cells likely because the level of activity remained below a critical threshold for survival under these conditions.

All three mutant NFs promoted survival and differentiation in nnr5-TrkC cells that express both TrkC and p75, indicating that this activity was retained. Indeed, neurite outgrowth and complexity were also shown to be improved with D, RK and DRK in rat spiral ganglion neurons that express both TrkB and TrkC (Szobota et al, 2019 ARO abstract; manuscript in preparation), with mutant DRK showing robust activity.

**In vivo therapeutic paradigms.**

Taken together, these data predict that the mutant NFs and in particular DRK which combines pan-Trk activity with reduced p75 affinity have activity profiles that bode well for improved efficacy *in vivo*. The two *in vivo* models of retinal neurodegenerative disease (and the *ex vivo* model of hearing loss) suggest that these disorders may be indications suitable for treatment with mutant DRK.

In the *in vivo* experimental paradigms we apply the therapeutic agents *once only*, after injury. In the optic nerve axotomy model the agents are administered shortly after injury because this is an acute model with a short therapeutic window. In the DR model the agents are administered after 2.5 weeks of continuous injury, as this is a chronic model. Drug dosing and timing was selected to better represent a real-life scenario of intervention in trauma versus a chronic disease that requires a diagnosis.

In optic nerve axotomy DRK is better than D. In the DR, where injury is chronic but modest and subtle, it would be difficult to see differences between D and DRK. However, a comparison of DRK or D versus controls (BDNF positive control or untreated negative control) shows that DRK provides a more significant benefit than D.

In addition, we aimed to avoid administration of a Trk-agonist able to fully activate p75, especially in chronic diseases that require chronic drug administration. For example, evaluation of wild type NFs in optic nerve injury shows that higher frequency of administration results in higher toxicity *unless p75 action if blocked*. For example, wild type NGF given 2x (day 1 and 7 post-optic nerve injury) is ineffective, even though more drug is administered. In contrast, wild type NGF 1x (immediately following injury) or 2x (immediately following injury and at day 7 post-injury) given *together* with p75
antagonist (or in p75 knockdown or knockout mice) is effective and provides a more than additive benefit than when using each agent alone (Shi, Birman et al. 2007, Lebrun-Julien, Morquette et al. 2009, Bai, Dergham et al. 2010).

In a different approach (Enomoto, Bunge et al. 2013) Schwann cells transfected with the cDNA of a mutant NFs similar to DRK were implanted in spinal cord at the time of spinal cord damage, aiming to achieve high local expression of the mutant NF concomitant with injury. That work reported more surviving Schwann cells and sensory fibers, but did not result in significant functional locomotive improvement or recovery. In our work soluble proteins are used as the therapeutic agents, with the DRK mutant protein being efficacious at a relatively low dose, and given at low frequency (single administration), even when the treatment was administered after neuronal injury and with ongoing degeneration. Moreover, in vivo the DRK mutant protein was more effective than wild type NT3 or than the related mutants D and RK.

In summary, we present data supporting the concept that the administration of NF molecules that are capable of broad Trk activation while at least partially avoiding p75 activation is beneficial for neuronal survival, regeneration and delay of disease progression. These concepts may help to rationalize therapeutic strategies for neurodegeneration.
Disclosures

Patent applications have been filed on behalf of authors (HUS, ACF). HUS is a paid consultant to Otonomy Inc.

Authorship Contributions

Participated in research design: FB, AG, HUS, ACF
Conducted experiments: FB, AG, SS, SSz
Performed data analysis: FB, AG, MVS, HUS, ACF
Wrote the manuscript: HUS
Edited or contributed to the writing of the manuscript: FB, AG, SS, SSz, ACF
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Footnote

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Figure 1. Direct binding biotinylated NT3 or biotinylated DRK mutant to Trk or p75 receptors. Cells transfected with the indicated receptor were bound with the indicated concentrations of biotinylated-NT3 or biotinylated-DRK mutant, followed by labeled fluorescein-avidin as secondary. Cells were analyzed by flow cytometry. Shown is the ratio of the mean channel fluorescence of bell-shaped histograms, with each cell type having 50 nM biotinylated-NT3 (highest concentration) normalized to 1. Average ± SD (n=3 biological replicates, 10,000 cells analyzed in each assay). Statistical analysis was done by two-way ANOVA with significance α < 0.05, followed by Bonferroni's correction for multi-comparisons. In p75-expressing cells, mutant DRK (50 nM) has significantly reduced binding compared to NT3 (*p< 0.05). In TrkB-expressing cells, mutant DRK (2 and 10 nM) has significantly enhanced binding compared to NT3 (*p< 0.05, ****p< 0.0001, respectively). In TrkA-expressing cells, mutant DRK (2, 10 and 50 nM) has significantly enhanced binding compared to NT3 (*p< 0.05, ****p< 0.0001, (*p< 0.05, respectively).
Figure 2. Cell survival induced by mutants. MTT assays after culture in SFM ± the indicated compounds, for 48 hrs. Data are standardized to the corresponding wild-type neurotrophin (NT3 for TrkC, BDNF for TrkB, NGF for TrkA = 100% survival). Agents tested at sub-optimal (0.2 nM) or optimal (2 nM) concentrations. The values from untreated controls (SFM without supplementation) are set to 0% survival. Survival was calculated using the formula [(OD test – OD SFM) x 100 / (OD optimal NF – OD SFM)]. Data shown are the mean of n= 3 independent biological replicate experiments ± SD, each independent experiment had n=4 or n=6 technical replicate wells per condition. Statistical analyses are one-way ANOVA with significance α < 0.05, followed by Bonferroni's correction for multi-comparisons. (A) NIH-TrkC cells, the asterisk (*) indicates differences between RK and NT3 (**p<0.05) at high concentrations. There are no significant differences between treatments at lower concentrations. (B) NIH-TrkB and control NIH-TrkA cells, only the 10 nM NF concentration is shown. Asterisks (*) indicate differences between the cognate wild-type NF for each receptor (BDNF for TrkB and NGF for TrkA) and the test agents (*** p< 0.001, ** p< 0.01). The number sign (#) indicates differences between DRK and NT3 in the survival of NIH-TrkB cells (### p< 0.001). The difference between D vs. DRK is significant (*p< 0.05). However, in NIH-TrkB cells D vs. RK is not significant, and RK vs. DRK is significant (**** p< 0.0001) (for clarity, this is not shown in the graph).
Figure 3. Mutants induce cell survival and differentiation in cells expressing TrkC+ and p75+.

(A) Survival of nnr5-TrkC cells (a PC12 cell variant expressing TrkC+ p75+). Data are the mean of n= 3 independent biological replicate experiments ± SD, each independent experiment had 4-6 technical replicate wells per condition. No significant differences are seen between wild-type NT3 and the mutants when compared at their equivalent concentrations. (B) Representative assay of cell differentiation. Nnr5-TrkC cells were untreated (control), or were treated with wild-type NT3 or mutants at 0.1 nM, and after 72 hrs they were immunostained with anti-MAP2. Nuclei were counterstained with DAPI. Magnification 40x. (C) Quantification of neurite outgrowth (differentiation). Data are shown as % of differentiated cells ± SD, relative to NT3 (n=3 independent experiments). Statistics were calculated by one-way ANOVA with significance α < 0.05, followed by Bonferroni’s correction for multi-comparisons, *** p< 0.001 with respect to untreated control. Treatment groups show no significant differences.
Figure 4. Mutants activate TrkC and TrkB signal transduction
Fibroblast cells transfected to express TrkC (A), TrkB (B) or TrkA (C) were serum-starved for 2 hrs and then were treated with the indicated NF or mutants (2 nM) for 15 min. Positive controls are NT3 for TrkC-expressing cells, BDNF for TrkB-expressing cells, and NGF for TrkA-expressing cells. Cell lysates were analyzed by Western blots for pTrk, or pAkt, or pErk1,2 by exposing membrane strips cut based on a molecular weight marker to the corresponding primary antibody. After stripping, membranes were re-probed with anti-actin, and the actin band shown is the standard loading control for that panel. The blots shown for a protein are assembled form the same exposure, and data are representative. Densitometry was done from proper linear exposures of the blots to quantify p-TrkC, or p-TrkB, or p-TrkA, the phosphotyrosine-containing bands at ~140 kDa in each experiment. Assays were repeated at least three independent times with unique biological samples. Quantification reports the average ± SD of all independent biological experiments (n=3), relative to wild-type NT3 (set to 1). Statistical analysis was done by one-way ANOVA with significance α < 0.05, followed by Bonferroni's correction for multi-comparisons. * p< 0.05, ** p< 0.01, *** p< 0.001.
Figure 5. Trk-mediated pErk1,2 activation by Mutants. Concentration-dependent pERK phosphorylation (AlphaLISA tests) in cells expressing (A) human TrkB, or (B) human TrkC. Concentration-response curves were generated after normalization of responses to 10 nM BDNF or 10 nM NT3 as respective controls. Curve fitting was performed using the "log(agonist) vs. response (three parameters)" function in GraphPad Prism 8. EC$_{50}$ and maximal inhibition values calculated from these data are shown in Table 2. Data are mean ± SD values from the number of biological replicates indicated in Table 2.

Figure 6. Effects of mutants on p75-dependent TNFα expression. The levels of TNFα transcript were quantified by quantitative real-time PCR after 6 hrs of the indicated treatment in HEK293-p75 cells (stably expressing p75). Each sample was assayed in triplicate technical replicate wells, in at least 3 independent biological replicate experiments. Data shown are the mean of the independent experiments ± SD, with values standardized to the untreated (Unt) control. Statistical analysis was done by one-way ANOVA with significance α < 0.05, followed by Bonferroni's correction for multi-comparisons. * p< 0.05, ** p< 0.01, *** p< 0.001.
Figure 7. Mutants delay loss of RGC somata after optic nerve axotomy.
For each treatment, the surviving RGCs were quantified. Data for each mouse is the ratio of the axotomized eye ± treatments and the contralateral control healthy eye (100% RGC cell bodies). Percent data are averaged per group. Shown is the average (n=8 mice/group) of the measurements at the 14-day end point. Mutant DRK affords significant RGC neuroprotection compared to untreated or PBS-treated control, and compared to wild-type NGF, BDNF or NT3. NGF-C is mutant that does not bind to p75 but activates TrkA selectively. One-way ANOVA was used with significance α < 0.05, followed by Bonferroni’s correction for multi-comparisons * p< 0.05, ** p< 0.01, *** p< 0.001, **** p< 0.0001. The differences between D and RK are not significant, RK vs. DRK are significant (p< 0.0001), and D vs. DRK are significant (p< 0.001).

Figure 8. Mutants prevent loss of RGC Nerve Fibers in Diabetic Retinopathy
Mice were made diabetic (STZ model, with verified hyperglycemia), causing loss of retinal structure and thinning of the Nerve Fiber Layer, Ganglion Cell Layer, and Inner nuclear layer (NGI, a structure formed by RGCs and their axons and fibers). At week 2.5 of diabetes (with ongoing disease) mice were administered an intravitreal injection of the indicated agent or vehicle (Unt.). Controls are healthy non-diabetic retinas of sibling mice, of normal thickness (n=3-4 animals/group). (A) Representative sections of B-scan OCT images at 6 weeks of diabetes; treated with NT3 or DRK at week 2.5 of diabetes, compared to age-matched control-healthy mice. (B) NGI thickness in µm. All diabetic mice are significantly worse than control non-diabetic (p< 0.001), and DRK treated eyes are significantly better than untreated (p< 0.001) or than BDNF (p< 0.05) groups. (C) Nerve fiber loss (%) in all STZ-treated groups, reflective of the quality of the RGC fibers. Compared to untreated control (100% damage), the mutants D (p< 0.05) and DRK (p< 0.001) groups experience significantly reduced damage. Compared to BDNF, DRK is also significantly better (p< 0.01). One-way ANOVA test was used with significance α < 0.05, followed by Bonferroni’s correction for multi-comparisons. * p< 0.05, ** p< 0.01, *** p< 0.001.
### Table 1. Summary of binding competition of mutants versus biotinylated-NT3.

<table>
<thead>
<tr>
<th>Biot-NT3 ± competitors</th>
<th>Biotin-NT3 binding (% of untreated control)</th>
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<tbody>
<tr>
<td></td>
<td>TrkC</td>
</tr>
<tr>
<td>No competitor</td>
<td>100</td>
</tr>
<tr>
<td>NT3 wild-type</td>
<td>60 ± 3.3</td>
</tr>
<tr>
<td>D</td>
<td>52 ± 3.8</td>
</tr>
<tr>
<td>RK</td>
<td>51 ± 5.8</td>
</tr>
<tr>
<td>DRK</td>
<td>56 ± 6.6</td>
</tr>
<tr>
<td>BDNF wild-type</td>
<td>Not done</td>
</tr>
</tbody>
</table>

Table 1. Cells transfected with the indicated receptor were pre-incubated for 15 min in binding buffer on ice without (untreated) or with competitors wild-type NF (control), or test mutants D, RK or DRK (each at 380 nM). Then, saturating biotinylated-NT3 was added for 15 min. The labeled secondary was fluorescein-avidin, and cells were analyzed by flowcytometry. The mean channel fluorescence (MCF) of bell-shaped histograms were standardized, with no competition = 100% binding. Values shown are the average ± SD (n=3 biological replicates, in each assay 5,000 cells analyzed). Statistical analysis was done by one-way ANOVA with significance α < 0.05, followed by Bonferroni's correction for multi-comparisons. In Trk-expressing cells, the three mutants, wild-type NT3 and wild-type BDNF significantly inhibited NT3-biotin binding (p< 0.001 in TrkC and TrkB). There are no significant differences in competition between NT3 wild-type versus the mutants or BDNF. In p75-expressing cells, wild-type NT3 and mutant D significantly inhibited NT3-biotin binding (p< 0.05). In p75 expressing cells, mutants RK and DRK did not significantly compete binding compared to untreated control, and binding remained significantly higher compared to control wild type NT3 competition (p< 0.05).
Table 2. Summary of Trk-mediated pErk1,2 activation by mutants

<table>
<thead>
<tr>
<th></th>
<th>BDNF</th>
<th>NT-3</th>
<th>D</th>
<th>RK</th>
<th>DRK</th>
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<tr>
<td>hTrkB</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt; nM</td>
<td>0.59 (0.43,0.80)</td>
<td>0.87 (0.57,1.32)</td>
<td>0.18 (0.05,0.70)</td>
<td>0.22 (0.08,0.61)</td>
<td>0.14 (0.03,0.62)</td>
</tr>
<tr>
<td>% Max</td>
<td>96.7 ± 9.53</td>
<td>120 ± 7.30</td>
<td>91.2 ± 14.2</td>
<td>109 ± 16.9</td>
<td>98.5 ± 26.9</td>
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<tr>
<td>n</td>
<td>7</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
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<tr>
<td>hTrkC</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt; nM</td>
<td>10.0 (2.64,37.9)</td>
<td>0.58 (0.29,1.16)</td>
<td>0.36 (0.16,0.57)</td>
<td>0.11 (0.02,0.61)</td>
<td>0.27 (0.10,0.69)</td>
</tr>
<tr>
<td>% Max</td>
<td>80.1 ± 21.7</td>
<td>101 ± 6.16</td>
<td>109 ± 61.9</td>
<td>84.9 ± 37.7</td>
<td>111 ± 32.6</td>
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<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>3</td>
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Table 2. EC<sub>50</sub> values and maximal inhibition values (% Max) were derived from the concentration-response curves in Fig 5. The EC<sub>50</sub> values are the geometric means derived from pEC<sub>50</sub> values with 95% confidence limits and the % Max values are means ± SD for n biological replicates.
Figure 2

A. NIH-TrkC cells

<table>
<thead>
<tr>
<th></th>
<th>0.2 nM</th>
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<tr>
<td>NT3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
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<tr>
<td>RK</td>
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<tr>
<td>DRK</td>
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% survival (relative to optimal NT3)

B. NIH-TrkA cells

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<thead>
<tr>
<th></th>
<th>NIH-TrkA cells</th>
<th>NIH-TrkB cells</th>
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<tbody>
<tr>
<td>NF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT3</td>
<td></td>
<td></td>
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<tr>
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<tr>
<td>RK</td>
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<tr>
<td>DRK</td>
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</table>

% survival (relative to optimal NF)
Figure 3

A. % Survival (relative to optimal NT3)

B. Anti-MAP2

C. % Differentiated cells relative to NT3

Untreated NT3 D RK DRK NT3 D RK DRK

Unt.
Figure 7
Figure 8

A. Control  STZ (Untreated)  STZ + NT3  STZ + DRK

B. NGI thickness (μm)

C. % Nerve fiber loss

Nh

Ctl  Unt.  DRK  D  BDNF  NT3

STZ  STZ