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Pan-neurotrophin 1: A genetically engineered neurotrophic factor displaying multiple specificities in peripheral neurons *in vitro* and *in vivo*

(nerve growth factor/brain-derived neurotrophic factor/neurotrophin 3/site-directed mutagenesis)

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ABSTRACT **Pan-neurotrophin 1 (PNT-1) is a synthetic trophic factor engineered by combining active domains of the neurotrophins nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin 3 (NT-3) into an NT-3 backbone. This molecule was produced in transiently transfected COS cells or in baculovirus-infected insect cells and subsequently purified to homogeneity. Saturation binding in embryonic spinal sensory neurons demonstrated a greater number of high-affinity binding sites for PNT-1 than for its parental molecule NT-3. PNT-1 was shown to efficiently block the chemical crosslinking of NGF, BDNF, and NT-3 to their cognate Trk receptors and to the low-affinity NGF receptor expressed on neuronal and nonneuronal cells. PNT-1 stimulated survival and proliferation of MG87 fibroblasts expressing either TrkA, TrkB, or TrkC. PNT-1 also promoted survival of a greater number of embryonic dorsal root ganglion neurons than any of the other neurotrophins alone, and its effects were equivalent to a combination of NGF, BDNF, and NT-3. Analysis of receptor-specific neurotrophic activities demonstrated that PNT-1 efficiently rescued TrkA mRNA-containing sympathetic neurons and TrkB and TrkC mRNA-containing sensory neurons from the dorsal root and nodose ganglia. Finally, PNT-1 showed robust retrograde transport to DRG neurons *in vivo* after injection into the sciatic nerve. Radiolabeled PNT-1 accumulated in small-, medium-, and large-sized neurons. Coinjection with different unlabeled neurotrophins inhibited PNT-1 transport in distinct subpopulations of neurons of different sizes, suggesting that this molecule affects sensory neurons of different modalities. These results indicate that PNT-1 is a potent and multispecific neurotrophic factor that may be useful in the treatment of peripheral neuropathies and nerve damage.**

The neurotrophins are a family of structurally and functionally related polypeptides that control the development, survival, and maintenance of peripheral and central neurons (1). This family includes four members, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4 (NT-4), which share ≈50% amino acid sequence identity (2, 3). The specificity of neurotrophin action is believed to be achieved by the selective interaction between the different neurotrophins and members of the Trk family of tyrosine kinase receptors. Thus, NGF binds to $p140^{trk}$ (TrkA), BDNF and NT-4 interact with $p145^{trkB}$ (TrkB), and NT-3 interacts with $p145^{trkC}$ (TrkC) and, to a lesser extent, also with TrkA and TrkB (for review, see ref. 4). The ability of the neurotrophins to promote survival of peripheral and central neurons during development and after neuronal damage suggests that these molecules may constitute therapeutic agents for the treatment of brain injury and disease.

The neurotrophins affect both overlapping and distinct subpopulations of neurons in the peripheral and central nervous systems, including sensory and sympathetic neurons (1, 5–10), motor neurons (11, 12), and several other subpopulations of central neurons (13–17). Within the dorsal root ganglion (DRG), the neurotrophins support distinct functional groups of sensory neurons. Smaller-sized neurons, conveying nociceptive sensory information (heat, pain), are supported by NGF (10, 18). NT-3, however, has been shown to affect large proprioceptive sensory neurons carrying information on the position and movement of skeletal muscles (9, 19). BDNF appears to have effects on medium-sized neurons of different modalities (8). Although the different subpopulations of sensory neurons in the DRG, as well as spinal cord motor neurons, respond to different trophic factors, their axons extend through the same peripheral nerves. Hence, functional regeneration of damaged peripheral nerves is likely to require concomitant action of different neurotrophins. Using mutant and chimeric neurotrophins, structural determinants of binding to and activation of Trk receptors have recently begun to be delineated (20, 21), providing a structural basis for the specificity of neurotrophin action. On the basis of this information, a chimeric neurotrophin, designated pan-neurotrophin 1 (PNT-1), was constructed by recruiting residues from the active sites of NGF, BDNF, and NT-3 into an NT-3 backbone (20). In this paper, we characterize the biochemical and biological properties of purified PNT-1 in both neuronal and nonneuronal systems.

MATERIALS AND METHODS

Recombinant Protein Production and Purification. Recombinant PNT-1 was produced in transiently transfected COS cells as described (20) or in baculovirus-infected Sf21 insect cells. For expression of PNT-1 in insect cells, the PTN-1 gene was cloned into the baculovirus transfer vector pVL1393, and recombinant virus clones were isolated by plaque purification. PNT-1 was purified by ion-exchange, size-exclusion, and reverse-phase chromatography. SDS/PAGE and silver staining showed the preparation had a purity >90%. N-terminal amino acid sequencing for 24 cycles confirmed the predicted sequence of mature PNT-1. Both COS and insect cell-derived

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Abbreviations: PNT-1, pan-neurotrophic factor 1; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin 3; NGF, nerve growth factor; DRG, dorsal root ganglion; L4 and L5, lumbar fourth and fifth, respectively; E*X*, embryonic day *X*.

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PNT-1 preparations showed identical specific biological activities on embryonic DRG neurons.

Binding Assays and Chemical Crosslinking. Purified PNT-1 and neurotrophins were labeled with ^{125}I by the lactoperoxidase method and bindings were done as described (20). For chemical crosslinking, 1×10^6 cells were incubated with 1 ng of iodinated factor followed by 30 min at 37°C in the presence of 2 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochoride/0.5 mM bis(sulfosuccinimidyl)suberate (Pierce). Immunoprecipitations using anti-panTrk antiserum 203, anti-NGF antiserum (Collaborative Research), or anti-$p75^{LNGFR}$ monoclonal antibody MC192 (Boehringer Mannheim) were performed as described (20).

Bioassays. Trk-specific biological activities of PNT-1 and native neurotrophins were assayed by using MG87 NIH 3T3 fibroblasts as described (22). Survival of specific subpopulations of developing peripheral neurons expressing different variants of Trk mRNAs was assayed by RNase protection assay using RNA probes including the tyrosine kinase domains of Trk receptors as described (21). Before RNA extraction, a small amount of *in vitro* synthesized RNA complementary to each RNA probe was added to the lysis buffer and subsequently used as recovery standard.

Retrograde Transport. Retrograde transport was done on adult Sprague–Dawley male rats (220–240 g) as described (23). Fifteen to thirty nanograms of iodinated PNT-1 was injected into the sciatic nerve alone or with 0.5 μg of NGF, BDNF, NT-3, NT-4, or with combinations of neurotrophins in a total volume of 2 μl of phosphate-buffered saline (PBS). In other animals, equivalent amounts of iodinated neurotrophins were injected. After 18 hr, radioactivity in the lumbar fourth (L4) and fifth (L5) DRG was determined, and relative transport was calculated as fmol transported per pmol injected. Sucrose-equilibrated tissues were sectioned and processed for emulsion autoradiography (24). Size-frequency analysis of labeled DRG neurons was done as described (23).

RESULTS

Receptor-Binding Specificities of PNT-1. PNT-1 was engineered by using an NT-3 backbone in which residues 94–98 and 1–9 were replaced by corresponding amino acid residues from BDNF and NGF, respectively (20). The replacements, which account for only 10% of the residues of the molecule, are located in solvent-accessible regions, not involved in the tertiary or quaternary structures of the neurotrophins (25). Recombinant PNT-1 was produced in transiently transfected COS cells or in baculovirus-infected Sf21 insect cells and subsequently purified to homogeneity. Steady-state binding of PNT-1 and NT-3 to embryonic day 8 (E8) chicken lumbar DRG neurons revealed high-affinity sites for both molecules with K_d values of 1.55×10^{-11} M and 1.06×10^{-11}, respectively (data not shown). PNT-1, however, displayed a greater number of binding sites (≈2.5-fold) compared with NT-3, suggesting that this chimeric neurotrophin may also be able to interact with high-affinity sites for additional neurotrophins—i.e., NGF and BDNF. The ability of PNT-1 to interact with different neurotrophin-receptor subtypes on cells of both neuronal and nonneuronal origin was investigated by chemical crosslinking in the absence or presence of 50-fold excess of unlabeled PNT-1 or native neurotrophins followed by immunoprecipitation and SDS/PAGE. Crosslinking of iodinated NGF to NIH 3T3 fibroblasts expressing TrkA (3T3-TrkA) or to PC-12 cells was totally prevented by excess unlabeled NGF but was not prevented by BDNF (Fig. 1*A*). NT-3 partially prevented the crosslinking of NGF to 3T3-TrkA but failed to displace NGF from TrkA on PC-12 cells (Fig. 1*A*). PNT-1, however, completely blocked the binding of NGF to TrkA in both fibroblast and PC-12 cells (Fig. 1*A*), indicating that PNT-1, in contrast to NT-3, can also interact with TrkA in a neuronal cell-like context. All neurotrophins prevented crosslinking of NGF to the low-affinity neurotrophin receptor, $p75^{LNGFR}$ (Fig. 1*A*).

Crosslinking of BDNF to 3T3-TrkB or to a mutant PC-12 line expressing TrkB (nnr5-TrkB) was totally prevented by excess unlabeled BDNF but was not prevented by NGF (Fig. 1*B*). NT-3 and PNT-1 also prevented crosslinking of iodinated BDNF to both cell types (Fig. 1*B*), although, in both cases, a residual BDNF binding (5–10%) could still be detected. Interestingly, although both molecules could interact with TrkB receptors on nnr5-TrkB cells, PNT-1, but not NT-3, was able to promote neurite outgrowth from these cells in a concentration-dependent manner (data not shown). Only excess unlabeled NT-3 and PNT-1 were able to prevent crosslinking of iodinated NT-3 to 3T3-TrkC cells or to cell suspensions obtained from dissociated adult rat cerebral cortex (Fig. 1*C*). BDNF partially prevented crosslinking of iodinated NT-3 to cortical cells but not to 3T3-TrkC, presumably by displacing NT-3 bound to cortical TrkB receptors. A residual NT-3 binding (<1%) could still be detected in these cells after competition with unlabeled PNT-1 (Fig. 1*C*).

Biological Activities of PNT-1 in Fibroblasts Expressing Trk Receptors. The ability of PNT-1 to initiate a biological response upon binding to different Trk receptors was investigated with fibroblasts genetically engineered to respond to exogenous neurotrophins (22). Like NT-3, PNT-1 promoted growth and survival of TrkB- and TrkC-expressing fibroblasts but, in contrast to NT-3, it was also active in TrkA-expressing cells (Fig. 2). Although PNT-1 was as efficacious as native neurotrophins in promoting survival and growth of Trk-

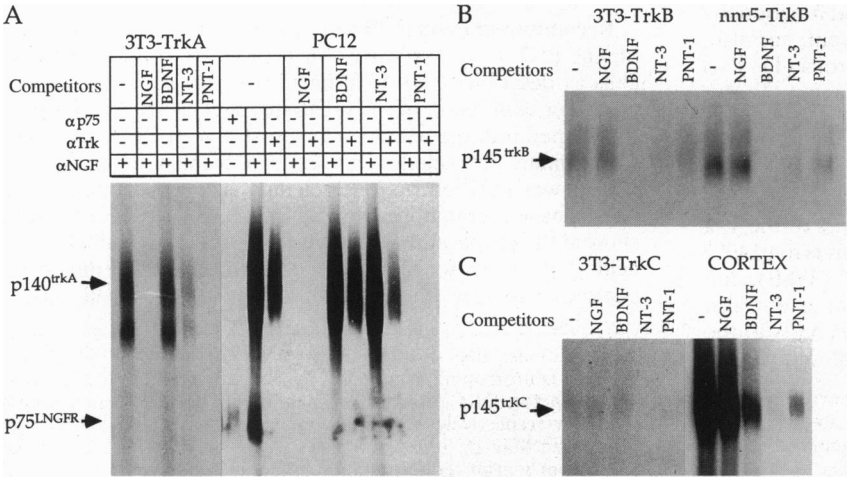

FIG. 1. Interactions between PNT-1 and TrkA, TrkB, and TrkC receptors analyzed by chemical crosslinking. Radiolabeled NGF (*A*), BDNF (*B*), or NT-3 (*C*) was crosslinked to the indicated cells in the presence or absence of 50-fold excess of unlabeled PNT-1 or native neurotrophins. Lysates were immunoprecipitated with the indicated antibodies (*A*) or with α-Trk antibodies (*B* and *C*) and analyzed by SDS/PAGE.

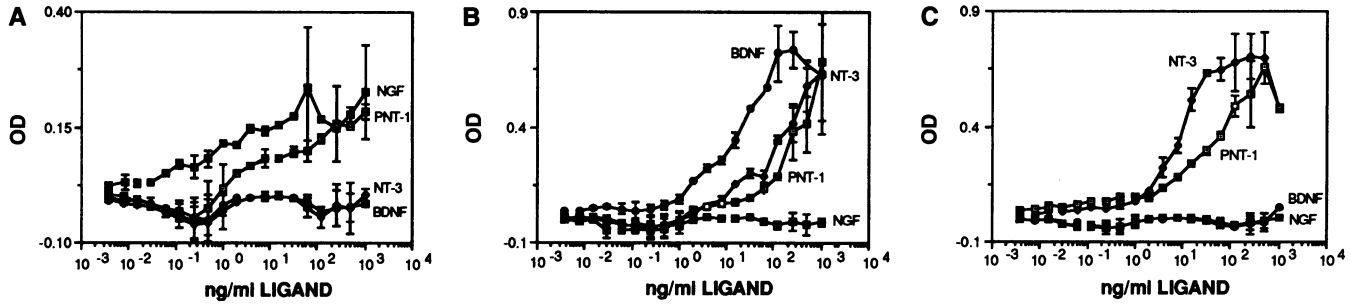

FIG. 2. Biological activities of PNT-1 in MG87 fibroblasts expressing TrkA, TrkB, or TrkC receptors. Dose–response effects of NGF (■), BDNF (●), NT-3 (◆), and PNT-1 (□) on the survival and growth of MG87 fibroblasts constitutively expressing TrkA (*A*), TrkB (*B*), or TrkC (*C*) receptors assessed by metabolic labeling with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Data are expressed as the average optical density (OD) of triplicate wells ± SD.

expressing fibroblasts, its broader specificity was accompanied by a somewhat decreased potency.

Receptor Subtype-Specific Neurotrophic Activities of PNT-1 in Cultured Peripheral Neurons. Saturating concentrations of PNT-1 promoted the survival of greater numbers of chicken E8 DRG neurons than equivalent concentrations of any of the other neurotrophins alone (Fig. 3*A*). The effects of PNT-1 on neuronal survival were comparable to those obtained with a combination of NGF, BDNF, and NT-3. To define the specificity of the responses to PNT-1, receptor-specific neurotrophic activities were studied in a bioassay that assesses the survival of distinct subpopulations of neurons expressing specific Trk mRNAs (21, 22). Ganglion explants or dissociated neurons were cultured in the absence or presence of different neurotrophins, and after 48 hr, total RNA was extracted and analyzed for the presence of either TrkA, TrkB, or TrkC mRNAs by RNase protection analysis. In E11 chicken sympathetic neurons, PNT-1 rescued higher levels of TrkA mRNA than NT-3, its effects being comparable to those of NGF (Fig. 3*B*). The response obtained with NT-3 in developing sympathetic neurons may be due to residual TrkC mRNA expression at this developmental age (26). A substantial number of TrkB mRNA-containing neurons were rescued in dissociated cultures of E8 chicken DRG neurons treated with BDNF (Fig. 3*B*). NGF only partially rescued some of the TrkB mRNA signal, probably due to a small number of cells coexpressing TrkA and TrkB mRNAs. Rat NT-4 had only weak effects, in agreement with its low potency on avian neurons (27). The partial effects of NT-3 on TrkB mRNA-containing DRG neurons could be due to receptor mRNA coexpression, TrkB mRNA up-regulation, or a genuine action of NT-3 through neuronal TrkB receptors. In the presence of PNT-1, however, the recovery of TrkB mRNA was comparable to that obtained with BDNF, suggesting true TrkB-mediated

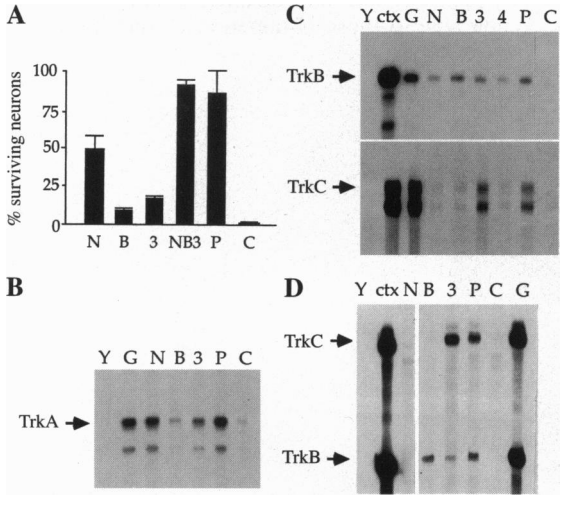

FIG. 3. Receptor subtype-specific neurotrophic activities of PNT-1 in cultured peripheral neurons. (*A*) Survival of dissociated E8 chicken DRG neurons (10,000 cells per 35-mm well) after 48 hr in culture in the presence of NGF (bar N), BDNF (bar B), NT-3 (bar 3), or PNT-1 (bar P) at 20 ng/ml or a combination of NGF, BDNF, and NT-3 (bar NB3) at 20 ng/ml each. Data are expressed as the percentage of surviving neurons in triplicate wells ± SD. RNase protection analysis of TrkA mRNA (*B*) and TrkB and C mRNAs (*C* and *D*) in cultures of explanted E11 chicken sympathetic ganglia (*B*), dissociated E8 chicken DRG neurons (*C*), and explanted E8 chicken nodose ganglia (*D*) 48 hr after treatment with NGF (lane N), BDNF (lane B), NT-3 (lane 3), rat NT-4 (lane 4), or PNT-1 (lane P). Chick cortex poly(A)$^+$ RNA (lane ctx) and RNA from explanted ganglia before culture (lane G) were used as positive controls. Yeast tRNA (lane Y) was used as negative control. Because the treatments affect the amounts of RNA recovered from each sample, each well represents an equivalent amount of ganglia or dissociated cells.

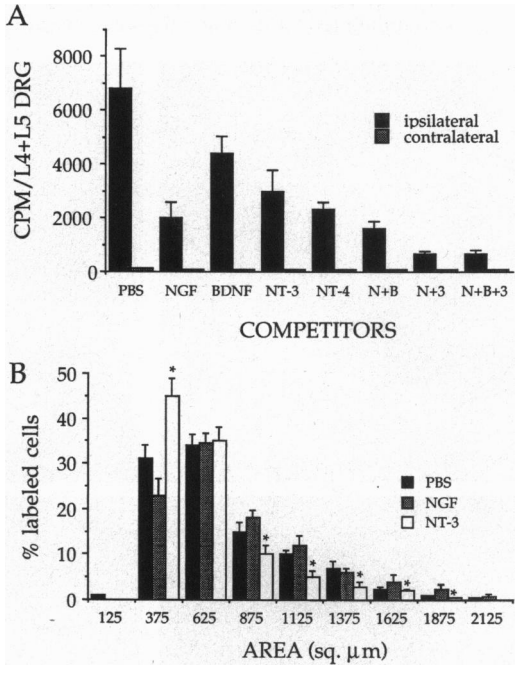

FIG. 4. Retrograde transport of PNT-1 to rat lumbar DRG neurons. (*A*) Average cpm ± SD ($n = 4$) recovered in ipsilateral (solid bars) and contralateral (gray bars) rat L4 and L5 DRG 18 hr after unilateral injection of ^{125}I-labeled PNT-1 together with 32-fold excess of the indicated unlabeled competitors. N, NGF; B, BDNF; 3, NT-3. (*B*) Size–frequency histogram of DRG cells retrogradely labeled by PNT-1. Data represent percentage of labeled cells after injection of ^{125}I-labeled PNT-1 in PBS (solid bars) or together with excess unlabeled NGF (gray bars) or NT-3 (open bars) in different size categories. Values are means ± SD of 10–14 animals. *, $P < 0.05$ (ANOVA, Scheffe test).

biological actions of PNT-1 in DRG neurons. Analysis of TrkC mRNA revealed that only NT-3 and PNT-1 were able to rescue TrkC mRNA-containing neurons in E8 chicken DRG (Fig. 3*C*). In E8 chicken nodose ganglion explants, BDNF and NT-3 were able to rescue only cognate Trk mRNA species, whereas PNT-1 could promote survival of both TrkB and TrkC mRNA-containing subpopulations (Fig. 3*D*). Thus, these data show that PNT-1 can rescue neurons expressing either TrkA, TrkB, or TrkC mRNAs.

Retrograde Transport of PNT-1 to Rat DRG Neurons. To assess neurotrophic specificities *in vivo*, we studied the retrograde transport of PNT-1 to L4 and L5 DRG neurons after injection in the rat sciatic nerve. Iodinated PNT-1 showed robust retrograde transport to rat DRG neurons (Fig. 4*A*). Comparison of the levels of retrograde transport of different neurotrophins on a per molar basis indicated that the transport of PNT-1 was highest, 3.58 ± 0.29 fmol of protein transported per pmol of injected ^{125}I-labeled PNT-1 compared with 2.09 ± 0.20, 0.28 ± 0.02, 0.67 ± 0.12, and 0.59 ± 0.10 fmol of protein transported per pmol of injected ^{125}I-labeled NGF, ^{125}I-labeled BDNF, ^{125}I-labeled NT-3, and ^{125}I-labeled NT-4, respectively. Coinjection with unlabeled NGF decreased the accumulation of iodinated PNT-1 in L4 and L5 DRG by 70%. In addition, unlabeled NT-3 and BDNF decreased PNT-1 transport to 60% and 40%, respectively (Fig. 4*A*), indicating that all neurotrophins can block the uptake and transport of PNT-1 to various degrees. Coinjection with a combination of unlabeled NGF and NT-3 reduced PNT-1 transport by 90% (Fig. 4*A*). Combination of NGF, NT-3, and BDNF gave no further inhibition, probably because of the high extent of coexpression of TrkB with other Trks in adult DRG neurons (28).

Emulsion autoradiography confirmed accumulation of iodinated PNT-1 in cell bodies of sensory neurons and further showed that cells of all sizes were labeled (Fig. 5*A*). The labeling of small-sized neurons was preferentially inhibited by coinjection with unlabeled NGF (Fig. 5*B*), whereas coinjection with unlabeled NT-3 competed against the labeling in large-sized neurons (Fig. 5*C*). In addition, the overall mean areas of ^{125}I-labeled PNT-1-labeled cells differed significantly (ANOVA, Student–Newman–Keul test, $P < 0.05$) after coinjection with PBS only (718 ± 7.3 μm^2, SE) compared with coinjection with unlabeled NGF (781 ± 25.6 μm^2, SE) or unlabeled NT-3 (608 ± 23.1 μm^2, SE). A more detailed analysis showed that approximately half of the neurons labeled after coinjection with unlabeled NT-3 had an area of 250–500 μm^2, compared with only 25% after coinjection with unlabeled NGF (Fig. 4*B*). Conversely, while 20% of the neurons labeled after coinjection with unlabeled NGF had an area of 750–1000 μm^2, only half as many neurons of this size class were labeled after coinjection with unlabeled NT-3 (Fig. 4*B*). These results are consistent with the general notion that NGF and NT-3 are preferentially transported by small and large sensory neurons, respectively (24). Coinjection with unlabeled NGF and NT-3 prevented the transport of PNT-1 to DRG neurons of both size classes (Fig. 5*D*). In this case, a few labeled cells could still be found in some sections, indicating transport via a pharmacologically distinct system, possibly BDNF-sensitive.

DISCUSSION

In this paper we characterize a synthetic multispecific neurotrophic factor for peripheral sensory and sympathetic neurons. PNT-1 is a chimeric neurotrophin that combines active determinants from NGF, BDNF, and NT-3. Saturation binding experiments indicated that DRG neurons have high-affinity binding sites for PNT-1 in greater numbers than for its parental molecule NT-3. This result was probably due to the ability of PNT-1 to bind all neurotrophin receptors in neuronal, as well as nonneuronal cellular contexts, as demonstrated by chemical crosslinking. PNT-1 rescued greater numbers of DRG neurons in culture than any one neurotrophin alone, and its effects were comparable to those obtained after simultaneous addi-

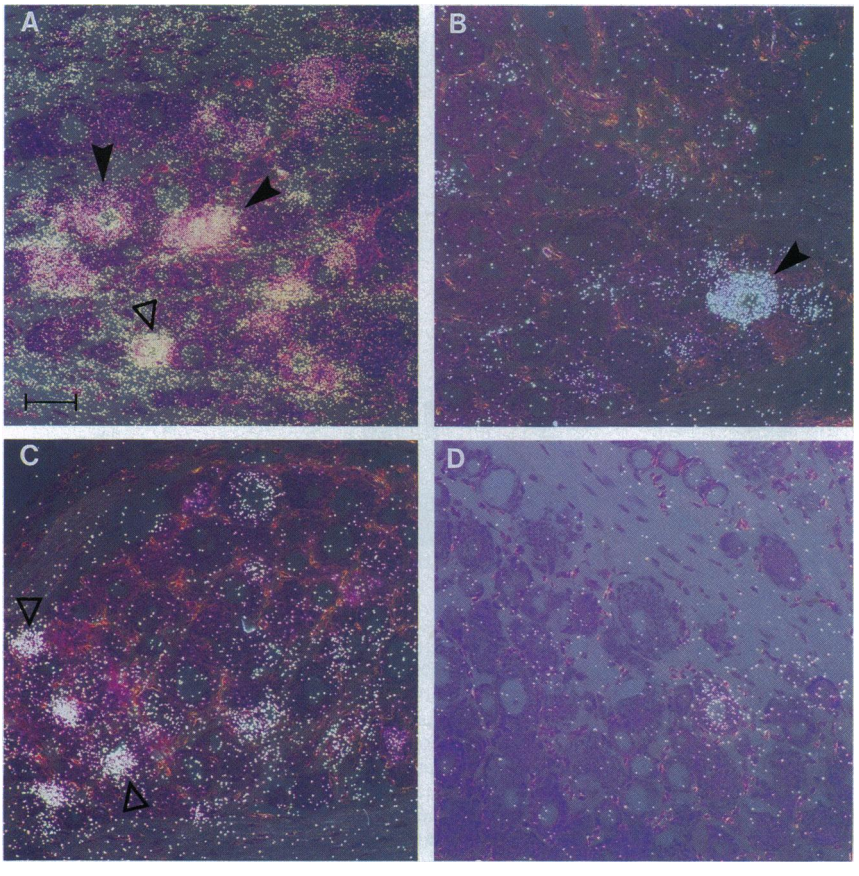

FIG. 5. Emulsion autoradiography of lumbar rat DRG neurons labeled by retrograde transport after coinjection of ^{125}I-labeled PNT-1 with PBS (*A*) or with excess unlabeled NGF (*B*), unlabeled NT-3 (*C*), or unlabeled NGF and NT-3 (*D*). Note that after coinjection with unlabeled NGF or NT-3, only large-sized (solid arrowheads) or small-sized (open arrowheads) neurons, respectively, remained labeled by ^{125}I-labeled PNT-1. (Bar = 100 μm.)

tion of NGF, BDNF, and NT-3. This result was most likely due to the fact that PNT-1 was capable of promoting survival of neurons expressing either TrkA, TrkB, or TrkC mRNAs. In all cases, cell rescue with PNT-1 (as measured by RNase protection) was equivalent to that obtained with cognate native neurotrophins, indicating that this chimeric molecule has multispecific neurotrophic activities in peripheral neurons. Interestingly, PNT-1 appeared to be more potent in neurons than in Trk-expressing fibroblasts, suggesting that lower levels of receptor activation may suffice to promote full biological activity in neurons compared to nonneuronal cells. The fact that PNT-1 retained the ability to bind to the low-affinity neurotrophin $p75^{LNGFR}$ (Fig. 1*A*), which is not expressed by fibroblasts, suggests this receptor may somehow potentiate the effects of PNT-1 in neuronal cells, as recently demonstrated for native neurotrophins (29, 30). Although PNT-1 was not very different from NT-3 in TrkB-expressing fibroblasts, it was clearly superior to NT-3 in promoting survival of TrkB mRNA-expressing neurons from embryonic DRG and nodose ganglia. This result suggests that, although both molecules bind to TrkB, PNT-1, unlike NT-3, may be able to cause conformational changes in TrkB required for biological activity.

The pan-specific nature of PNT-1 *in vivo* was demonstrated by its retrograde transport to rat DRG neurons of different size classes. Both small (putative nociceptive) and large (proprioceptive) neurons showed robust transport, which could be specifically inhibited by excess unlabeled NGF and NT-3, respectively. In a recent report, NGF administration was shown to prevent the behavioral and biochemical manifestations of experimental diabetic sensory neuropathy (31). NGF treatment, however, failed to prevent the reduction of conduction velocity predominantly associated with degeneration of larger, faster-conducting axons from larger-fiber sensory neurons that normally respond to NT-3 but not to NGF. This, as well as other studies, shows that a molecule such as PNT-1, acting broadly on sensory neurons of various sizes and modalities, may be of great advantage for the treatment of peripheral nerve injuries and neuropathies, where neurodegeneration affects heterogeneous subpopulations of neurons with different neurotrophic requirements.

How can PNT-1 accommodate specific structural requirements for binding to and activation of multiple receptors in a single polypeptide chain? The structural similarities among the neurotrophins suggest that they evolved from a common ancestor and then diverged functionally, perhaps by acquiring residues that prevented binding to inappropriate receptors (2). As demonstrated by this and previous studies, binding determinants to the Trk receptors can be manipulated independently in a rational fashion to create neurotrophin analogues with distinctive ligand-binding properties (20, 21). In this way, a divergent evolutionary process may be reversed by the construction of chimeric molecules in which fully crossreactive binding is restored. PNT-1 may thus function by mimicking a hypothetical ancestral neurotrophin molecule in which inhibitory binding determinants are absent.

In conclusion, PNT-1 is an example of an engineered neurotrophin that recruits the functions of several different members of the family in a single polypeptide chain. Besides its applications in the study of neurotrophic interactions, PNT-1 may constitute a valuable therapeutic tool for the treatment of peripheral neuropathies and nerve damage.

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