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Receptors in Collaboration with p75^{NTR}

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trkC receptors, which serve critical functions during the development of the nervous system, are alternatively spliced to yield isoforms containing the catalytic tyrosine kinase domain (TK+) and truncated isoforms which lack this domain (TK−). To test for potential differences in their roles during early stages of neural development, TK+ and TK− isoforms were ectopically expressed in cultures of neural crest, the stem cell population that gives rise to the vast majority of the peripheral nervous system. NT-3 activation of ectopically expressed trkC TK+ receptors promoted both proliferation of neural crest cells and neuronal differentiation. Strikingly, the trkC TK− isoform was significantly more effective at promoting neuronal differentiation, but had no effect on proliferation. Furthermore, the trkC TK− response was dependent on a conserved receptor cytoplasmic domain and required the participation of the p75^{NTR} neurotrophin receptor. Antibody-mediated receptor dimerization of TK+ receptors, but not TK− receptors, was sufficient to stimulate differentiation. These data identify a phenotypic response to activation of the trkC TK− receptor and demonstrate a functional interaction with p75^{NTR}, indicating there may be multiple trkC receptor-mediated systems guiding neuronal differentiation.

INTRODUCTION

Neurotrophin-3 has been shown to regulate multiple events in the development of the peripheral nervous system, including the survival and proliferation of neural precursor cells, neural differentiation, and neuronal survival (cf. Davies, 1994; Lewin and Barde, 1996). trkC, the primary high-affinity receptor for NT-3, has been shown to be expressed throughout neurogenesis and neuronal differentiation in the peripheral nervous system (Tessarollo *et al.*, 1993; Zhang *et al.*, 1994; Williams *et al.*, 1993; Henion *et al.*, 1995; Lefcort *et al.*, 1996), yet an analysis of its function has been complicated by the existence of multiple receptor splice forms. In addition to the canonical tyrosine kinase-containing isoform, other isoforms have been identified which contain inserts, or entirely lack the catalytic kinase domain (the "truncated" or TK− isoform) or are missing the last third of the kinase domain (Tsoulfas *et al.*, 1993; Valenzuela *et al.*, 1993; Garner and Large, 1994). The *trkB* gene, the BDNF high-affinity receptor, but not *trkA*, also codes for splice forms which are truncated, or lack the kinase domain (Klein *et al.*, 1990; Middlemass *et al.*, 1991). In both *trkB* and *trkC*, an alternative cytoplasmic domain which is not present in the TK+ splice form is substituted for the kinase domain. While the *trkB* and *trkC* alternative cytoplasmic domains differ from each other, they are each highly conserved across species (Garner and Large, 1994; Biffo *et al.*, 1995; Armanini *et al.*, 1995).

To date, no independent functions have been identified for the kinase deleted isoforms. Rather, they have been assumed to only modulate the activity of the tyrosine kinase isoforms of trk receptors, including sequestering or presenting neurotrophin to TK+ receptors or to act as potential dominant negatives to dampen signaling through the TK+ receptor isoforms. Recent intriguing data from Tessarolo, Parada, and their colleagues have shown that targeted deletion in mice of the entire *trkC* gene results in a more severe phenotype than does elimination of just the kinase-containing isoform of trkC (Klein *et al.*, 1994; Donovan *et al.*, 1996; Tessarollo *et al.*, 1997), suggesting that the truncated isoform of the receptor does play a significant role *in vivo.*

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To directly determine the function of distinct isoforms of *trkC*, we ectopically expressed individual *trkC* isoforms in neural crest cells, since they are the stem cell population that gives rise to the vast majority of the peripheral nervous system. Several growth factors have been demonstrated to influence neural crest cell behavior (cf Stemple and Anderson, 1993) including NT-3 (Pinco *et al.*, 1993; Kalcheim *et al.*, 1992; Henion *et al.*, 1995) although the NT-3 receptors which transduce these activities have not been completely identified. Our data demonstrate that activation of ectopically expressed kinase-containing trkC receptors induces both the proliferation and neural differentiation of neural crest cells, while activation of the truncated, kinase-deleted trkC receptor has no effect on cell proliferation but strikingly induces neural differentiation. Furthermore, we show that this activity mediated by the TK− trkC receptor requires the participation of $p75^{NTR}$.

METHODS

Avian Neural Crest Cultures

Neural tubes were dissected from White Leghorn chickens between Stages 12 and 17 and cultured for up to 24 h as described (Sieber-Blum, 1991). Neural tubes were then removed, and neural crest cells were replated in 8-well Nunc or Falcon glass chamber slides that were coated with fibronectin (50 μg/ml; Gibco). Equal numbers of cells were plated in each well and all experimental conditions were repeated in quadruplicate. Immediately following replating, RCASBP virus, either control or engineered to contain trkC receptor isoforms, was added to each well at a concentration titered to infect at least 90% of the crest cells. NT-3 (rHu, Peprotech, 20–40 ng/ml) was added either at the time of virus application or 24 h later. The total number of cells in each well was determined 2–4 h after plating ($t = 0$) and again at 24 and 48 h by phase-contrast microscopy. Forty-eight hours after exposure to virus, all cells were fixed in 4% paraformaldehyde and stained with a nuclear stain, DAPI (Molecular Probes) and with an antibody (CTC IgG) to the extracellular domain of avian trkC (Lefcort *et al.*, 1996), and an antibody to the 180-kDa neurofilament subunit (kind gift of Dr. B. Granger, Montana State University), and/or p^{19GAG} (AMV-3C2, Developmental Studies Hybridoma Bank) followed by labeling with fluorescently tagged secondary antibodies (Jackson Immunoresearch Labs). To visualize $p75^{NTR}$ and to block p75 activity, we used the anti-avian p75 antibody, CHEX (Weskamp and Reichardt, 1991; kind gift of Dr. L. F. Reichardt). To verify cell surface expression of ectopically expressed trkC, infected, cultured neural crest cells were washed and labeled for 1 h at 4°C with the CTC IgG. Cells were then washed and fixed in 4% paraformaldehyde and labeled for 1 h with secondary antibodies as described. As a control, in some wells, the primary antibody was excluded. To quantify the extent of neuronal differentiation in each culture, the percentage of neurofilament-positive cells of 200–500 cells/well was determined. NF+ cells with a small round or oval phase-bright cell body and long thin neurites were counted as neurons, while larger flattened lamellar NF+ cells with thick, short neurites were counted as "proneurons." In each experiment, all conditions were carried out in quadruplicate and statistical significance was determined by ANOVA and Student–Newman–Keuls method. We noted that in initial experiments, total cell number after 48 h increased under all culture and virus conditions, with the greatest increase in wells infected with the TK+ virus. However, cell numbers in later experiments tended to decrease for all conditions, except for the TK+-infected cultures. This may be due to undefined changes in serum lots or embryo extracts, but did not affect the results since the relative differences among TK+, TK−, Cyto−, or KT-infected cells compared to each other and to control virus-infected cells was constant throughout the study. To determine the labeling index (LI), the percentage of BrdU-positive cells in each well was determined. BrdU (10 μg/ml; Sigma) was added at $t = 24$ h and cells were fixed 18 h later. BrdU+ cells were detected by immunocytochemistry with an anti-BrdU antibody per instructions (Novocastra).

Retroviral Expression of trkC Isoforms

Isolation of the trkC TK+, KT, and TK− cDNAs were described previously (Garner and Large, 1994). The TK− cDNA, which contains most of the cytoplasmic juxtamembrane domain and a conserved 39 amino acid C-tail, was engineered to include the T antigen epitope tag by fusing the sequence WWSSKPPTPPPEPET to the C-terminus. The Cyto− construct was engineered to remove all but the first two amino acids of the TK− cytoplasmic sequence and retaining the C-terminal epitope tag. The cDNAs encoding the *trkC* receptor isoforms were subcloned into the replication competent avian retrovirus RCASBP(B) (Hughes *et al.*, 1987) and propagated in line 0 pathogen-free chick embryo fibroblasts (CEF) as previously described (Garner and Large, 1994).

Immunoblots and Immunoprecipitates

Cell lysates were prepared from infected chick embryo fibroblasts (Garner and Large, 1994) and incubated with the CTC trkC IgG (Lefcort *et al.*, 1996) overnight at 4°C. After reduction, immunoprecipitated proteins were run on an SDS gel, transferred onto polyvinyl difluoride membrane (Sigma), followed by sequential incubation in CTC trkC IgG, protein A–HRP (Amersham), and developed with chemiluminescence detection (Amersham).

RT/PCR of trkC mRNA

Total RNA from embryonic DRG was isolated using RNAzolB (Tel-Test, Inc.) and the reverse transcription and PCR amplification were as described in the GeneAmp RNA PCR kit (Perkin–Elmer Cetus). Specific pairs of 24-mer primers were used to amplify mRNA for trkC TK+ isoforms (Garner and Large, 1), trkC TK− (forward, ACAAGGGGAAGTGTCTGA; and reverse, GCTGAAATAAACACTGACATCCTC) and β-actin. An annealing temperature of 58°C and 30 cycles was used for all three reactions, a number empirically determined to be within the linear phase of amplification of trkC transcripts (Garner and Large, unpublished data). The reactions were separated on a 1.5% agarose gel, denatured, and transferred to Genescreen Plus (NEN, DuPont) and hybridized with internal ^{32}P-labeled cRNA and oligonucleotide probes specific for each product.

RESULTS

Avian neural crest cells were infected *in vitro* with RCAS(BP) retrovirus engineered to express either full-length receptors (TK+), or the kinase-truncated (KT) and

kinase-minus (TK−) isoforms lacking kinase activity (Fig. 1). Immunoblots with an anti-trkC antibody (CTC IgG; Lefcort *et al.*, 1996) confirmed expression of each receptor isoform (Fig. 2A). Within 24 h of infection, between 90 and 100% of the cells expressed the p^{19GAG} viral protein and, except for cells infected with control virus, were strongly immunopositive for trkC (Figs. 2B and 3). In permeabilized, fixed cells, approximately 60% of the uninfected and control virus infected cells were very faintly trkC immunopositive. However, the level of this putative endogenous trkC expression was considerably weaker than that observed in cells infected with the trkC-containing viruses (compare Fig. 2B, a and b to c and d; and compare Fig. 3A to 3B and 3C). These data demonstrate the effectiveness of RCAS-mediated expression in neural crest cells *in vitro* and that all viral constructs could drive expression of their respective *trk* isoform.

To determine whether the trkC immunoreactivity observed corresponded to cell surface expression, we immunolabeled virus-infected, nonpermeabilized, live, cultured neural crest cells at 4°C with the anti-trkC antibody (Fig. 3). This procedure not only confirmed that the TK− and TK+ ectopically expressed trkC receptor was on the cell surface, but also that no trkC immunoreactivity was evident on control virus-infected cells. Thus we conclude that the faint trkC immunoreactivity observed on fixed, permeablized, control virus-infected cells (Fig. 2) reflects either low levels of cytoplasmically localized, and hence unavailable receptor, or background immunoreactivity which can often appear in triton-permeablized, fixed cells.

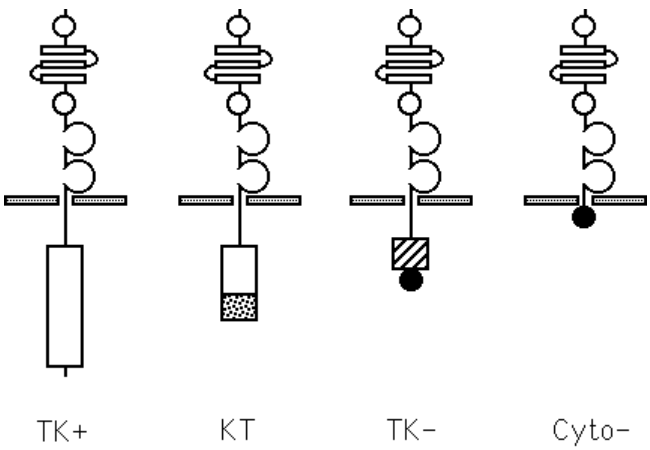
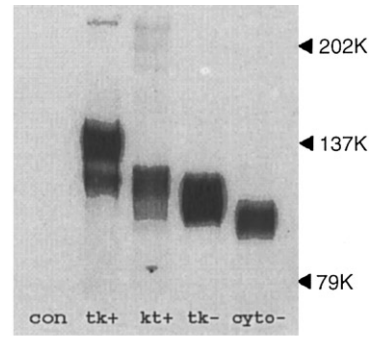


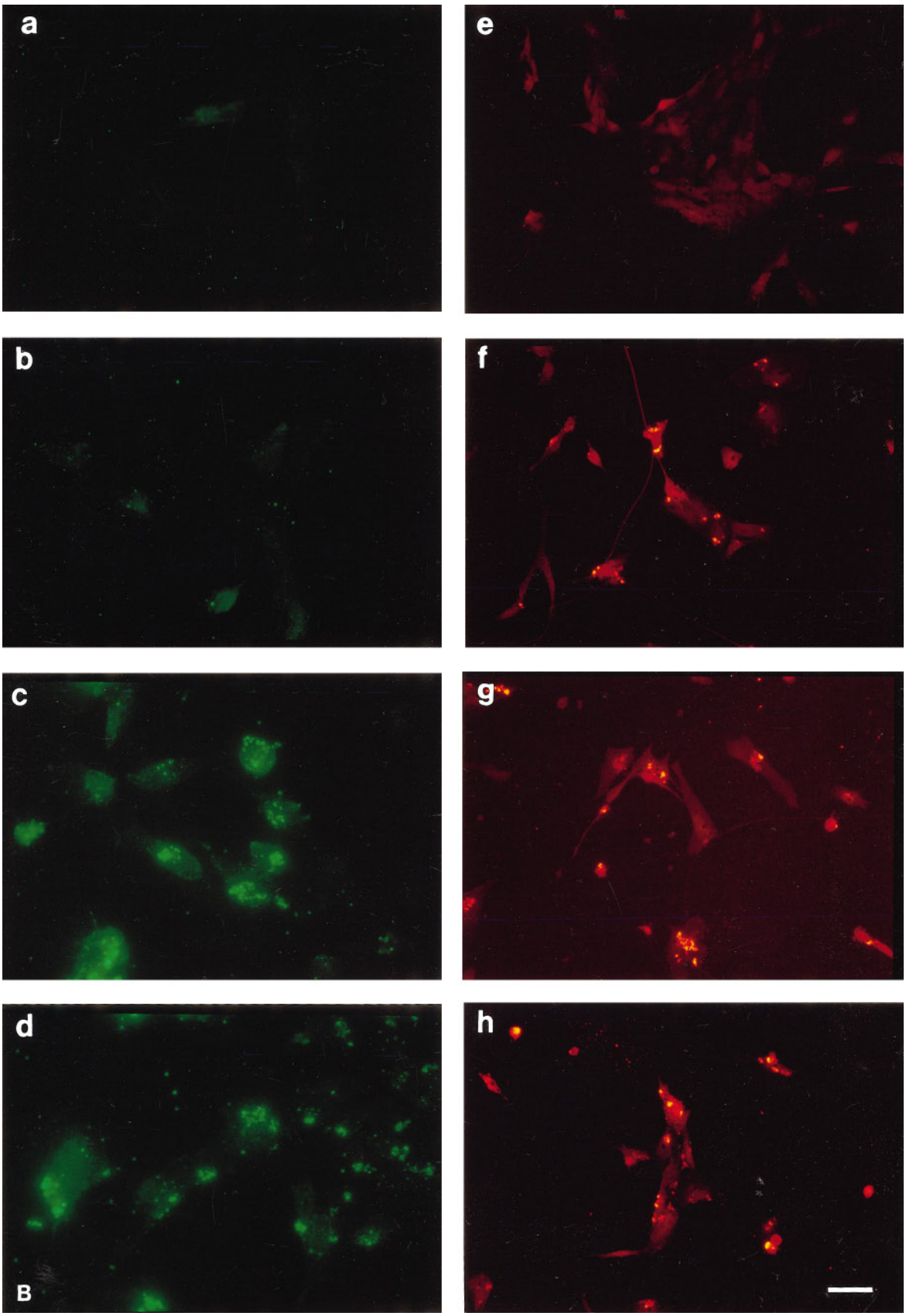
FIG. 1. Four isoforms of the avian trkC receptor, differing in their cytoplasmic domains, were expressed using RCAS(BP) retrovirus. TK+ indicates full-length trkC receptors containing the tyrosine kinase domain (large open box). KT indicates a naturally occurring trkC splice variant that replaces the last two-thirds of the kinase domain with novel sequence (speckled box) and lacks kinase activity ("kinase-truncated": Garner and Large, 1994). TK− indicates a splice variant that replaces the tyrosine kinase domain with a 39-amino-acid domain (hatched box), essentially identical to that found in mammalian trkC TK− isoforms. The black circle represents a 13-amino-acid SV40 T antigen epitope tag. Cyto− indicates a mutated trkC receptor that retains only the first 2 amino acids of the cytoplasmic domain fused to the epitope tag.



A

FIG. 2. (A) Retroviral-mediated expression of trkC isoforms. Extracts from chick embryo fibroblasts infected with either control, TK+, TK−, KT, or Cyto− RCAS viruses were immunoprecipitated with CTC IgG. Immunoprecipitates were reduced and separated on an SDS gel, transferred to PVDF membrane, and immunoblotted with the CTC IgG. The lower-molecular-weight band visible in the TK+ lane is the precursor, underglycosylated form of the full-length trkC protein (Garner and Large, 1994). (B) Recombinant RCAS retroviruses effectively infect chicken neural crest cells *in vitro* and can drive the expression of *trkC*. Cultured neural crest cells were fixed and permeabilized and then immunolabeled either with the CTC IgG (a–d) or the anti- $p19^{GAG}$ antibody (e–h). For detection of trkC expression, all photographic exposure times were of equal duration to compare relative levels of expression. A subset of uninfected cells (a, e) are weakly trkC immunoreactive and exhibit only background staining for $p19^{GAG}$. Control virus-infected cells (b, f) are equally weakly trkC immunoreactive but are brightly labeled with the $p19^{GAG}$ antibody. Both the TK+-infected (c, g) and TK−-infected (d, h) cells are strongly positive for trkC and for $p19^{GAG}$. (Scale bar, 75 μm). In this figure, cells were not double labeled with both antibodies simultaneously to prevent the strong anti-GAG immunoreactivity from spilling through the filter channel used to observe the anti-trkC labeling.

Since trk receptors can regulate mitosis (cf. Bothwell, 1995), we determined whether activation of trkC would influence neural crest cell proliferation (Fig. 4A). In the absence of ligand (NT-3) activation, there were no significant differences in cell number between any of the virus treatments (data not shown). In the presence of NT-3, cultures expressing TK+ receptors contained about 20% more cells ($P \leq 0.0001$) than control virus-infected cells. In contrast, cultures expressing TK− or KT trkC isoforms showed no significant difference in cell number from control cultures. To determine whether the trkC TK+-induced increase was due to enhanced mitotic activity, we measured the labeling index. Cultures expressing TK+ receptors contained significantly more BrdU+ cells than all other conditions and had a significantly higher LI (Table 1). The fact that there were consistently ($n = 16$ separate experi-



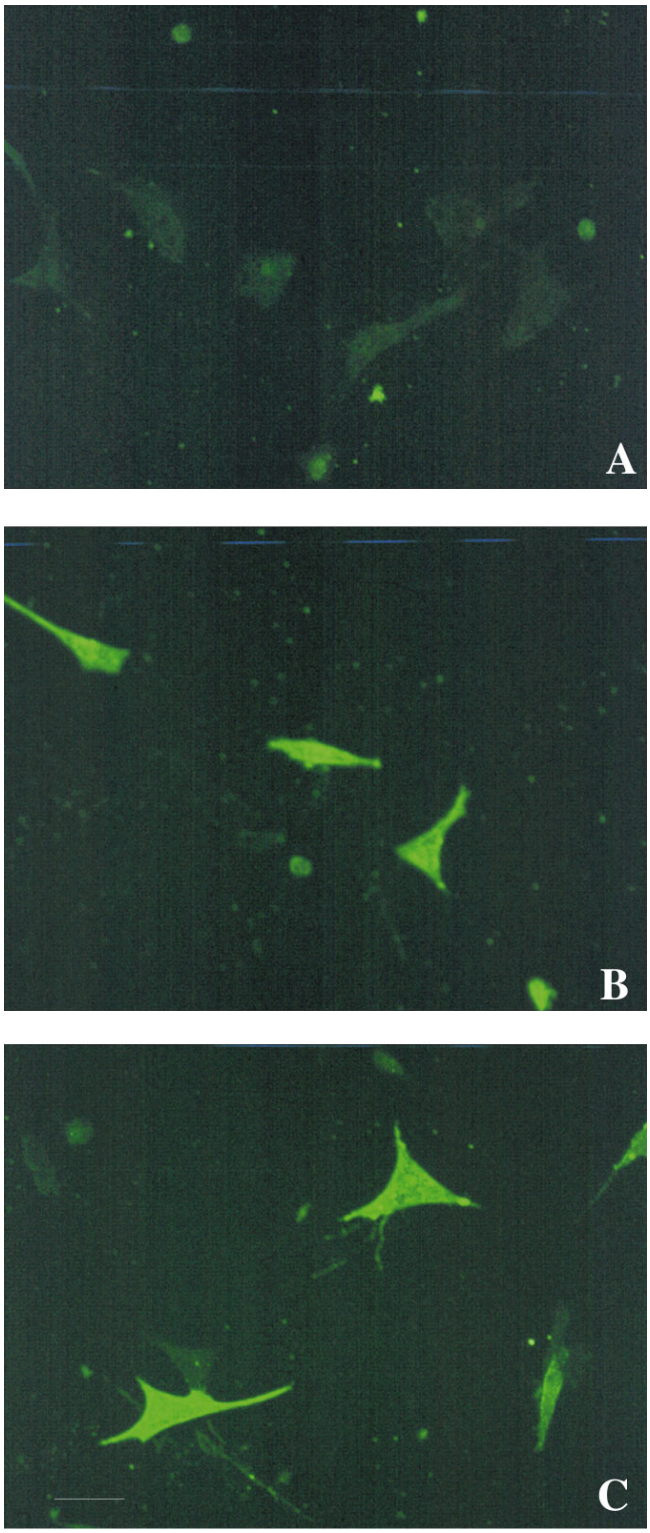


FIG. 3. Immunolabeling with the anti-trkC IgG on live, nonpermeabilized cells reveals no endogenous expression of trkC in control virus-infected cells. Cells infected either with the control virus (A), the TK+ virus (B), or TK− virus (C) were incubated with the CTC IgG at 4°C for 1 h as described under Methods. All photographic exposures were of equal duration to compare intensity levels. Staining live, nonpermeabilized cells reveals that ectopic expression of trkC is on the surface and that control virus-infected cells express no endogenous trkC on their surface (scale bar, 50 μm). Note how overexpression of the TK− receptor alters cell morphology; overexpression of the trkB TK− receptor has also been observed to alter cell morphology (Haapasalo *et al.*, 1997).

ments) more cells at $t = 48$ h than at $t = 0$ is further evidence that activation of the TK+ receptor promotes proliferation in these early cultures.

Previous studies have shown that NT-3 can regulate neural crest differentiation (Pinco *et al.*, 1993; Henion *et al.*, 1995), although the receptors that mediate these activities have not been fully characterized. To examine whether ectopic expression and activation of trkC was capable of inducing neural differentiation, the relative percentages of neurofilament positive, virally infected cells, were determined 48 h after cells were plated. This relatively short culture period resulted in low numbers of neurons, but minimized the potential complication of the survival effects of neurotrophins on crest-derived neurons (Wright *et al.*, 1992). In the absence of NT-3, there were no significant differences in neuronal numbers in any of the infected cultures (data not shown), nor were there any differences between the percentages of neurons that differentiated in the control virus-infected cells in the presence or absence of NT-3 (1.25% vs 1.37%, mean of two separate experiments, quadruplicate wells). As shown in Fig. 4B, in the presence of NT-3, the percentage of cells that differentiated into neurons in the cultures infected with TK+ virus was nearly 2.5-fold greater than that in either control virus-infected or uninfected cultures ($P \leq 0.0001$). Counts of GAG+/NF+ cells confirmed that 125/126 neurons were indeed infected. In addition to the classical, NF+ neuronal morphology, another population of cells was observed in all treatments that was flat, lamellar, and nonpolar in shape with short neurites. Because these cells were also NF+, we refer to these cells as proneurons and their number was also significantly increased in cultures expressing TK+ receptors.

Surprisingly, we found that the cultures containing the greatest percentage of neurons and proneurons were those expressing TK− receptors. TK−-infected cultures contained 3.5 to 4 times the percentage of neurons ($P \leq 0.0001$) and proneurons as in control cultures and significantly more neurons than the TK+ wells (Fig. 4B; $P \leq 0.05$). These results were observed in a total of 16 separate experiments and demonstrate that the kinase-minus isoform of trkC is capable of promoting neural differentiation.

Truncated trk receptors have the capacity to alter cellular activity by heterodimerizing with, and thereby inactivating, full-length tyrosine kinase-containing receptors (Eide *et al.*, 1996). Our immunocytochemical studies demonstrated the lack of cell surface expression of any endogenously expressed trkC receptors (Fig. 3) and hence are

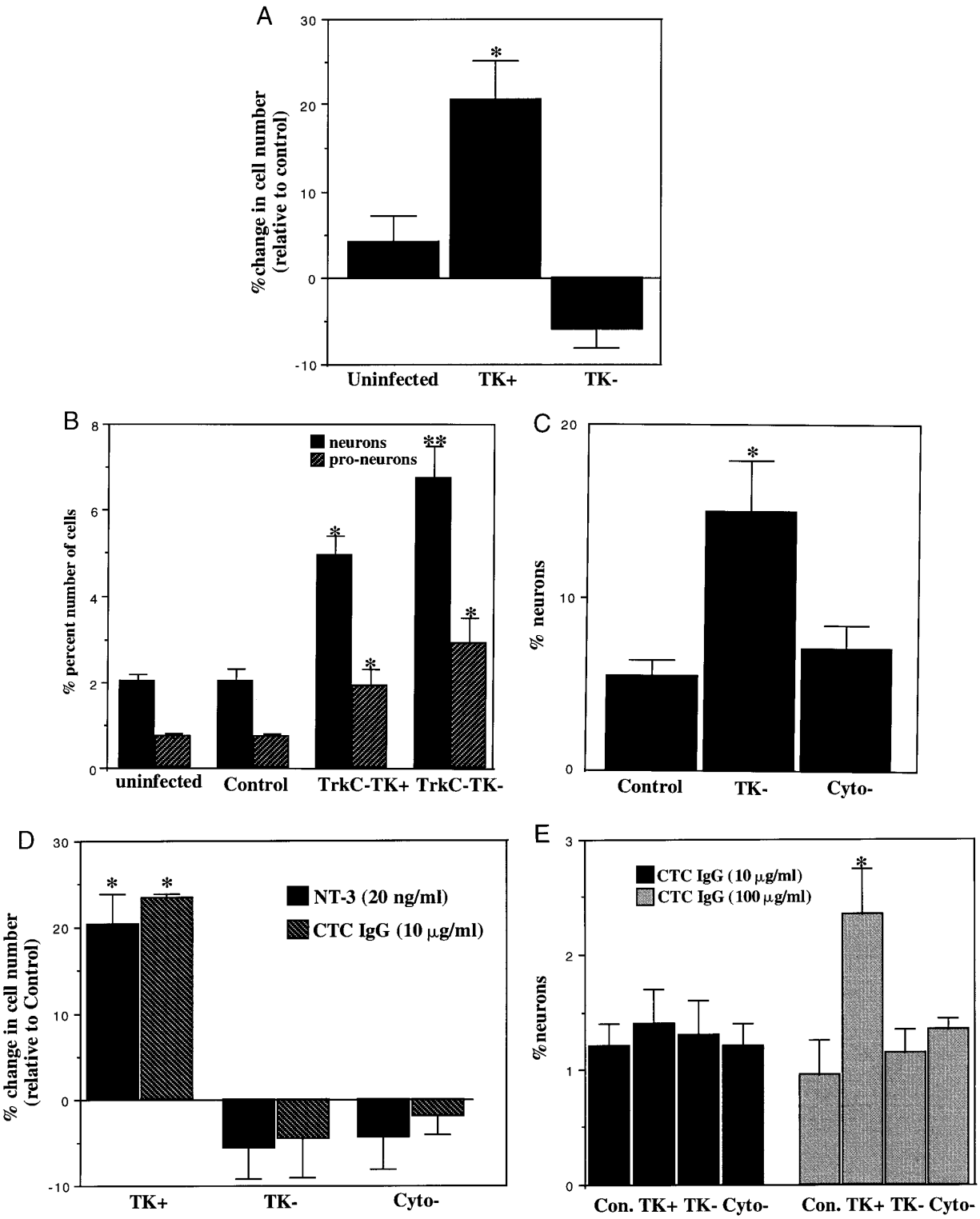


FIG. 4. (A) Expression and activation of trkC TK− promotes neural crest proliferation. Neural crest cells were either uninfected or infected with control, TK+, or TK− viruses and total cell number determined both 2 and 48 h after plating. Viruses were added at the time of plating, and NT-3 (40 ng/ml) was added 24 h after plating. Each experiment was conducted in quadruplicate. Values represent the mean change in cell number for each treatment (number of cells at t = 48 h minus number of cells at t = 2 h) relative to the change in number of control virus-infected cells over the same period, plus SEM from three separate experiments. ANOVA and Student–Newman–Keuls Method; $P \leq$

TABLE 1

Proliferation Rate (Labeling Index) for Virus-Infected Cultured Neural Crest Cells

Virus treatment	Labeling index
Control (no NT-3)	43.8%
Control (NT-3, 40 ng/ml)	42.1
trkC-TK+ (NT-3, 40 ng/ml)	54.9*
trkC-TK− (NT-3,40 ng/ml)	46.7
Cyto− (NT-3,40 ng/ml)	42.3

* ANOVA, $P \leq 0.025$. The proliferation rate for TK+-infected cells was significantly greater than that for control virus or Cyto−-infected cells (Student–Newman–Keuls, $P \leq 0.05$).

inconsistent with a dominant negative mechanism underlying the TK−-induced neural differentiation. To further examine this question, we engineered and expressed a novel receptor construct that contained the entire extracellular and transmembrane domain of trkC, but replaced the conserved cytoplasmic domain of the truncated trkC receptor with an epitope tag (Cyto−; Fig. 1). We verified that both the TK− and Cyto− receptors could significantly suppress TK+ tyrosine autophosphorylation in chick embryo fibroblasts and hence could function in a dominant negative manner (see Discussion; and Boeshore and Large, unpublished). As shown in Fig. 4C, cultures expressing the Cyto− receptor did not contain a significantly greater percentage of neurons than control cultures arguing against a mechanism involving a "dominant negative" effect on TK+ receptor signaling. The failure of the trkC KT isoform to promote neural differentiation (data not shown) is also consistent with this interpretation and suggests that the conserved cytoplasmic tail specific to the TK− receptor is required for stimulating neural differentiation.

Since no data exist that explain mechanisms for how the trkC TK− receptor might function, we tested whether receptor dimerization was sufficient. Previous studies have demonstrated that neuronal survival, in the absence of neurotrophins, can be promoted by incubation with polyclonal bivalent antibodies directed against the extracellular domain of trk receptors (Clary *et al.*, 1994; Lefcort *et al.*, 1996). Activation of the TK+ isoform with 10 μg/ml of bivalent trkC IgG (CTC IgG) mimicked the effect of NT-3 (Fig. 4D), resulting in a 22% increase in TK+ cells relative to control virus-infected cells ($P \leq 0.0001$). Conversely, NT-3 or trkC IgG treatment of cultures expressing TK− and Cyto− receptors had no significant effect on cell proliferation (Fig. 4D). Although a higher concentration of CTC IgG (100 μg/ml) was required to stimulate neural differentiation for TK+-infected cells (Fig. 4E), the bivalent antibody was unable to promote neural differentiation in the TK− and Cyto−-infected cells. Analysis of phosphotyrosine levels on trkC−TK+ immunoprecipitated from CEF cells confirmed a higher level of autophosphorylation after stimulation with 100 versus 10 μg/ml of CTC IgG (data not shown). These data indicate that low levels of TK+ receptor activation are sufficient for promoting proliferation, yet higher levels of TK+ receptor stimulation are required for neural differentiation (cf. Verdi and Anderson, 1994; Lillien, 1995). Furthermore, there appear to be at least two distinct receptor pathways that can promote neural differentiation: one pathway stimulated by dimerization and hence activation of the TK+ receptor and a second pathway mediated by the TK− receptor, in which its dimerization is insufficient for activation.

The results for the TK− receptor could be explained by the involvement of an additional receptor capable of interacting with NT-3. One candidate, the common neurotrophin receptor, $p75^{NTR}$, has been identified on avian neural crest cells (Bernd, 1985; Heuer *et al.*, 1990; Rifkin and Lefcort, unpublished observation). RCAS(BP)-infected neural crest cells were immunolabeled *in vitro* with an anti-

0.0001). (B) Expression and activation of either trkC TK− or trkC TK− induces neural differentiation. Numbers represent mean percentage of cells that were neurons or proneurons, plus SEM, from quadruplicate cultures from three separate experiments. Cells were cultured for 48 h total, as above (A). ANOVA and Student–Newman–Keuls method of analyses demonstrated that activation and expression of both the TK+ and TK− isoforms of trkC induces significantly more neurons and proneurons than in control virus-infected or uninfected cells ($P \leq 0.0001$). The TK− isoform induces significantly more neurons and proneurons than the TK+ isoform ($P \leq 0.05$). (C) Expression of the cytoplasmic tail of the trkC TK− isoform is required for TK−-induced neural differentiation. Numbers represent the mean percentage of neurons which differentiated after 48 h in culture and are the results of quadruplicate cultures from three separate experiments. There are not significantly more neurons in the Cyto−-infected wells than control virus-infected wells. Rather, activation of TK− isoform promotes significantly more neural differentiation than both the control and Cyto− isoform. Analyses as above, $P \leq 0.003$. (D) Dimerization of trkC TK+ by the anti-trkC IgG is sufficient to promote proliferation. Numbers represent the change in cell number, relative to control virus-infected cells, between the time of plating and fixation (48 h). Activation of only the TK+ isoform of trkC by either NT-3 or CTC IgG causes a net increase in cell number ($P \leq 0.0001$). (E) A higher level of TK+ activation is required for neural differentiation than for proliferation while receptor dimerization is insufficient for TK−-induced neural differentiation. Neural crest cells were infected with control, TK+, TK−, or Cyto− viruses and cultured in the presence of CTC IgG at either 10 or 100 μg/ml and neuronal numbers were determined after 48 h. While CTC IgG at 10 μg/ml was incapable of inducing TK+-induced neural differentiation, CTC IgG at 100 μg/ml could promote TK+-induced neural differentiation (ANOVA, $P \leq 0.014$) while still being insufficient for the promotion of TK−-induced neural differentiation.

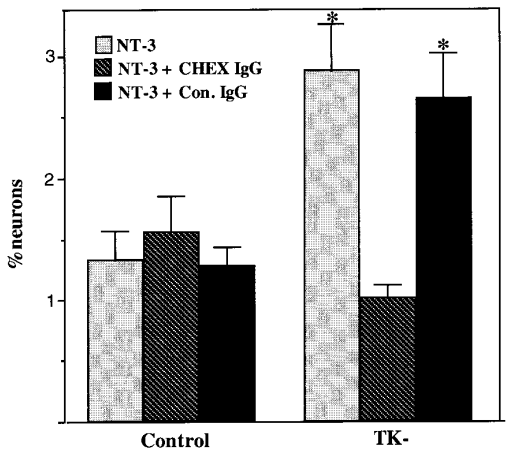


FIG. 5. Blockade of p75 inhibits neural differentiation induced by activation of the TK− receptor. Numbers represent mean percentage of neurons which differentiated in cultures of neural crest cells after 2 days *in vitro* (of 300–500 cells counted total/well). Cells were infected with viruses (Control or trkC TK−) immediately after plating and incubated with 20–40 ng/ml NT-3. In addition, some wells received the anti-$p75^{NTR}$ antibody, CHEX (100 μg/ml; gift of Dr. L. F. Reichardt) or control rabbit IgG (Cappel, 100 μg/ml). Mean and SEM of quadruplicate wells from two separate experiments. $*P \leq 0.05$.

body to $p75^{NTR}$ (CHEX; Weskamp and Reichardt, 1991) to determine the number of $p75^{NTR}$+/NF+ and NF− cells. We found that all neurons and proneurons were $p75^{NTR}$+ (145/145 neurons; 73/73 proneurons) and that in each well approximately 60% of the cells expressed $p75^{NTR}$. We then tested the requirement of $p75^{NTR}$ in the neurogenic response induced by the ectopically expressed TK− receptor and found that antibodies to $p75^{NTR}$ completely blocked TK−-induced neural differentiation (Fig. 5) but had no affect on TK+-induced neural differentiation (2.2 ± 0.3% in the absence of CHEX vs 2.1 ± 0.2% in the presence of 100 μg/ml CHEX IgG). These data indicate that $p75^{NTR}$ collaborates with the trkC TK− receptor to stimulate the neurogenic response.

DISCUSSION

Our studies are the first to identify a behavioral response to activation of the tyrosine kinase-deleted, truncated isoform of the trkC receptor. Furthermore, they show that while neural differentiation can be induced by activation of either the full-length kinase-containing receptor (TK+) or the truncated receptor (TK−), the underlying mechanisms differ considerably. Only in the case of the TK+ receptor is dimerization of the receptor sufficient to elicit neural differentiation. In contrast, neural differentiation induced by activation of the truncated receptor requires the participation of $p75^{NTR}$. Not only can activation of the TK+ receptor induce neural differentiation, but also cell proliferation, whereas activation of the truncated receptor has no mitogenic affect. These results indicate that two very different behavioral repertoires can be elicited by two distinct isoforms of the same receptor and demonstrate the potentially signficant role truncated tyrosine kinase receptors could play in developing systems. If each receptor isoform can influence a discrete behavior, then expression of multiple receptor isoforms may provide a cell with an effective mechanism to modulate its response to environmental signals.

All three relevant receptors described here, the truncated TK− isoform, the TK+ isoform, and $p75^{NTR}$, are expressed in the developing peripheral nervous system (cf. Davies, 1994). By RT–PCR, we have demonstrated that mRNAs for both *trkC* receptor isoforms are present in the immature chick DRG *in ovo* at an age (E4.5) corresponding to the peak period of neurogenesis (data not shown; Carr and Simpson, 1978; Pannesi, 1974). We have also shown that $p75^{NTR}$ protein is widely expressed on both dividing precursor cells and nascent neurons in the immature chick DRG (Rifkin *et al.*, 1996; Rifkin and Lefcort, unpublished observations). Using our antibody directed against the extracellular domain of trkC (CTC IgG), we have shown that individual cells (including a subset of dividing precursor cells) in the nascent DRG (E4.5) coexpress both trkC protein and $p75^{NTR}$ protein, although we can not determine the identity of the trkC receptor isoform with this antibody. Furthermore, using the CTC IgG, we have demonstrated that ca. 5% of migrating chick trunk neural crest cells *in vivo* express trkC protein and the vast majority of them express $p75^{NTR}$ (Rifkin *et al.*, 1996; Rifkin and Lefcort, unpublished observations). Interestingly, Henion *et al.* (1995) have shown that only the *trkC* mRNA-expressing subpopulation of neural crest cells have neurogenic potential *in vitro.* Not only is it now important to determine whether a single cell in the neural crest or DRG at any stage of its development coexpresses both receptor isoforms, but also to elucidate the mechanisms leading from trkC activation to neuronal differentiation.

The biological function and significance of truncated tyrosine kinase receptors has been a topic of considerable interest since their discovery. Based on their structure, they have been proposed to act as modulators of the full-length, tyrosine kinase-containing receptors by potentially heterodimerizing with them and acting as "dominant negatives" or perhaps by sequestering neurotrophin and thereby regulating the amount of neurotrophin available to the TK+ receptor isoforms (cf. Bothwell, 1995). Given their lack of a tyrosine kinase domain, they have not been thought to transduce any signals by themselves. Strikingly though, their alternative cytoplasmic domains are extremely well conserved through evolution: 36/39 amino acids of the trkC alternative cytoplasmic domain are entirely conserved between chicken and mammals (see Garner and Large, 1994) and the conservation between the truncated alternative trkB cytoplasmic domains is also maintained across species

(Armanini *et al.*, 1995; Biffo *et al.*, 1995). Recent evidence indicates that TK− isoforms of the trkB receptor can stimulate cytoplasmic signal transduction using an as yet unknown mechanism (Baxter *et al.*, 1997). Furthermore, Tessarollo and his colleagues have shown that targeted deletion of the entire gene for *trkC* results in mice with a more severe phenotype in both the nervous system and other tissues than mice in which just the TK+ isoform has been deleted (Donovan *et al.*, 1996; Liebl *et al.*, 1997; Tessarollo *et al.*, 1997). Recently they have shown that transgenic mice overexpressing the truncated trkC receptor exhibit severe defects in their nervous systems (L. Tessarollo, personal communication).

In our study, we do not believe that the TK− receptor is inducing neural differentiation by modifying signaling through any endogenously expressed TK+ trkC receptor. Not only do we see no expression of any endogenous trkC in nonpermeabilized, live cells using an antibody directed against the extracellular domain of the receptor, but also neither the KT isoform of the receptor nor the Cyto− construct was able to induce neural differentiation in the same neural crest cell cultures. Furthermore, we have verified that the Cyto− construct and the TK− receptor were equivalent in their ability to act as dominant negatives by coexpressing each isoform with the TK+ receptor in an approximately 1:1 ratio in chick fibroblasts, and the extent of tyrosine phosphorylation on the TK+ isoform was determined. We found that both receptors, either the TK− or Cyto−, were able to decrease the amount of TK+ tyrosine phosphorylation to the same extent and with the same time course (when coexpressed with TK− receptor, TK+ phosporylation was reduced by 83 ± 6.5%; when coexpressed with Cyto− receptor construct, TK+ phosphorylation was reduced by 78 ± 4%, Boeshore and Large, unpublished observations). Thus the capacity of the TK− isoform to induce neural differentiation cannot simply be due to a potential "dominant negative" activity.

How might activation of the truncated trkC receptor alter cell behavior? The trkC TK− isoform contains the juxtamembrane KFG motif necessary for activation of SNT/FRS2 (Peng *et al.*, 1995; Kouhara *et al.*, 1997), a cytoplasmic adapter protein involved in neurotrophin-stimulated differentiation. However, the inability of the KT isoform which also contains the KFG motif to stimulate differentiation suggests that the critical signaling motif resides in the highly evolutionarily conserved 39 amino acid C-terminal domain (Garner and Large, 1994). This domain contains a tyrosine residue, although it is not phosphorylated in response to NT-3 (Boeshore and Large, unpublished observations). The inability of the trkC extracellular antibodies alone to stimulate differentiation and the blockade of differentiation by the anti-$p75^{NTR}$ antibodies indicates the response also requires the $p75^{NTR}$ receptor. Conceivably, independent signaling through trkC TK- and $p75^{NTR}$ receptors may combine to generate a differentiative signal. Alternatively, trkC TK- receptor may heterodimerize with the $p75^{NTR}$ receptor to activate signaling pathways leading to differentiation, or modify $p75^{NTR}$ signaling such that cellular responses are shifted from apoptosis (Dechant and Barde, 1997; Casaccia-Bonnefil *et al.*, 1996) to differentiation.

Our data implicate both kinase-containing and kinase-minus isoforms of trkC and $p75^{NTR}$ in the process of peripheral neuronal differentiation. One pathway requires only stimulation of the TK+ receptor, while the other pathway requires the joint participation of the TK− receptor and $p75^{NTR}$. Indeed, both TK+ and TK− mRNA are present in the immature (E4.5) DRG, which contains both proliferating precursor cells and newly differentiated neurons (data not shown; Carr and Simpson, 1978). Hence, the neuronal deficit observed in mice in which both the TK− and TK+ isoforms of *trkC* are deleted (Liebl *et al.*, 1997; Tessarollo *et al.*, 1997) might be due, in part, to the inability of a subset of cells to differentiate. Similarly, it is conceivable that in transgenic mice that overexpress the TK− isoform, neural progenitor cells are prematurely driven out of the cell cycle and differentiate, ultimately leading to a net reduction in neuronal numbers (L. Tessarollo, personal communication; Farinas *et al.*, 1996). In light of our data implicating $p75^{NTR}$ in neurogenesis, it will be important to determine if the neuronal deficit observed in DRG of $p75^{NTR}$ knock-out mice is partially due to an inhibition of neuronal differentiation.

The fact that only a small subset of neural crest cells were driven toward a neuronal fate by overexpression of the TK− receptor may speak not only to our culture conditions (media and time course), but also to the heterogeneity of neural crest cells and perhaps to the narrow temporal window in which these differentiative decisions can be modulated. Our data extend the range of activities in which TK− receptors may perform, from merely inhibiting TK+ receptors, or sequestering ligand, to promoting neural differentiation, and identify a novel functional interaction of this receptor with $p75^{NTR}$. Thus, given that all three receptors are expressed in the immature dorsal root ganglion during a period of active proliferation and differentiation, the decision by a precursor cell to proliferate or differentiate may be significantly regulated by the relative expression levels and activation of the TK+ and TK− isoforms of trkC and of $p75^{NTR}$ receptors.

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REFERENCES

Armanini, M. P, McMahon, S. B, Sutherland, J., Shelton, D. L., and Phillips, H. S. (l995). Truncated and catalytic isoforms of trkB are co-expressed in neurons of rat and mouse CNS. *Eur. J. Neurosci.* **7,** 1403–1409.

Baxter, G. T., M. J Radeke, Kuo, R. C., Makrides, V., Hinkle, B., Hoang, R., .Medina-Selby, A., Coit, D., Valenzuela, P., and Feinstein, S. C. (l997). Signal transduction mediated by the truncated trkB receptor isoforms, trkB.T1 and trkB.T2. *J. Neurosci.* **17**(8), 2683–2690.

Bernd, P. (l985). Appearance of nerve growth factor receptors on cultured neural crest cells. *Dev. Biol.* **112,** 145–156.

Biffo, S., Offenhauser, N., Carter, B. D., and Barde , Y. A. (l995). Selective binding and internalization by truncated receptors restrict the availability of BDNF during development. *Development* **121,** 2461–2470.

Bothwell, M. (l995). Functional interactions of neurotrophins and neurotrophin receptors. *Annu. Rev. Neurosci.* **18,** 223–253.

Bronner-Fraser, M. E. (l993). Segregation of cell lineage in the neural crest. *Curr. Opin. Genet. Dev.* **3,** 641–647.

Carr, V. M., and Simpson, S. B. (l978). Proliferative and degenerative events in the early development of chick dorsal root ganglia. *J. Comp. Neurol.* **182,** 727–740.

Casaccia-Bonnefil, P., Carter, B. D. , Dobrowsky, R. T., and Chao, M. V. (l996). Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. *Nature* **383**(6602), 716–719.

Clary, D. O., Weskamp, G., Austin, L. A. R., and Reichardt, L. F. (l994). TrkA crosslinking mimics neuronal responses to nerve growth factor. *Mol. Biol. Cell* **5,** 549–563.

Davies, A. M. (1994). The role of neurotrophins in the developing nervous system. *J. Neurobiol.* **25**(11),1334–1348.

Dechant, G., and Barde, Y. A. (l997). Signalling through the neurotrophin receptor p75NTR. *Curr. Opin. Neurobiol.* **7**(3), 413–418.

Donovan, M. J., Hahn, R., Tessarollo, L., and Hempstead, B. L. (1996). Identification of an essential nonneuronal function of neurotrophin 3 in mammalian cardiac development. *Nat. Genet.* **14**(2), 210–213.

Eide, F. F., Vining, E. R., Eide, B. L., Zang, K., Wang, X. Y., and Reichardt, L. F. (l996). Naturally occurring truncated trkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling. *J. Neurosci.* **16**(10), 3123–3129.

Farinas, I., Yoshida, C. K., Backus, C., and Reichardt, L. F. (l996). Lack of neurotrophin-3 results in death of spinal sensory neurons and premature differentiation of their precursors. *Neuron* **17,** 1065–1078.

Garner, A. S., and Large, T. H. (1994). Isoforms of the avian trkC receptor: a novel kinase insertion dissociates transformation and process outgrowth from survival. *Neuron* **13,** 457–472.

Haapasalo, A., Saarelainen, T., Wong, G., and Castren, E. (l997). Expression of truncated trkB receptor induces a morphological change in mouse neuroblastoma cells. *Soc. Neurosci. Abstr.* **23,** 58.

Henion, P. D., Garner, A. S., Large, T. H., and Weston, J. A. (l995). TrkC-mediated NT-3 signalling is required for the early development of a subpopulation of neurogenic neural crest cells. *Dev. Biol.* **172,** 602–613.

Heuer, J. G., Fatemie-Nainie, S., Wheeler, E. F., and Bothwell, M. (1990). Structure and developmental expression of the chicken NGF receptor. *Dev. Biol.* **137**(2), 287–304.

Hughes, S. H., Greenhouse, J. J., Petropoulos, C. F., and Sutrave, P. (l987). Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. *J. Virol.* **61,** 3004–3012.

Kalcheim, C., Carmeli, C., and Rosenthal, A. (l992). Neurotrophin-3 is a mitogen for neural crest cells. *Proc. Natl. Acad. Sci. USA* **89,** 1661–1665.

Klein, R., Silos-Santiago, I., Smeyne, R. J., Lira, S. A., Brambilla, R., Bryant, S. ,Zhang, L., Snider, W. D., and Barbacid, M. (l994). Disruption of the NT-3 receptor gene TrkC eliminates Ia muscle afferents and results in abnormal movements. *Nature* **368,** 249–251.

Kouhara, H., Hadari, Y. R., Spivak-Kroizman, T., Schilling, J., Bar-Sagi, D., Lax, I., and Schlessinger, J. (l997). A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. *Cell* **89,** 693–702.

Lamballe, F., Tapley, P., and Barbacid, M. (1993). TrkC encodes multiple neurotrophin-3 receptors with distinct biological properties and substrate specificities. *EMBO J.* **12,** :3083–3094.

LeDouarin, N. M. (l982). "The Neural Crest." Cambridge Univ. Press, Cambridge, UK.

Lefcort, F., Clary, D. O., Rusoff, A. C., and Reichardt, L. F. (l996). Inhibition of the NT-3 receptor TrkC, early in chick embryogenesis, results in severe reductions in multiple neuronal subpopulations in the dorsal root ganglia. *J. Neurosci.* **16**(11), 3704–3713.

Lewin, G. R., and Barde, Y. A. (l996). Physiology of the neurotrophins. *Annu. Rev. Neurosci.* **19,** 289- 317.

Liebl, D. J., Tessarollo, L., Palko, M. E., and Parada, L. F. (l997). Absence of sensory neurons before target innervation in brain derived neurotrophic factor, neurotrophin-3, and TrkC-deficient embryonic mice. *J. Neurosci.* **17**(23), 9113–9121.

Lillien, L. (l995). changes in retinal cell fate induced by overexpression of EGF receptor. *Nature* **377,** 158–162.

Middlemas, D. S., Lindberg, R. A., and Hunter, T. (l991). TrkB, a neural receptor protein- tyrosine kinase: evidence for a full length and two truncated receptors. *Mol. Cell Biol.* **11,** 143–153.

Ninkina, N., Adu, J., Fischer, A., Pinon, L. G. P., Buchman, V. L., and Davies, A. M. (l996). Expression and function of TrkB variants in developing sensory neurons. *EMBO J.* **15,** 6385–6393.

Pannese, E. (1974). The histogenesis of the spinal ganglia. *Adv. Anat. Embryol. Cell. Biol.* **47**(5), 7–97.

Peng, X., Greene, L. A., Kaplan, D. R., and Stephens, R. M. (l995). Deletion of a conserved juxtamembrane sequence in trk abolishes NGF-promoted neuritogenesis. *Neuron* **15,** 395–406.

Pinco, O., Carmeli, C., Rosenthal, A., and Kalcheim, C. (l993). NT-3 affects proliferation and differentiation of distinct neural crest cells and is present in the early neural tube of avian embryos. *J. Neurobiol.* **24,** 1626–1641.

Rifkin, J. T, Danielson, K., and Lefcort, F. (l996). Dynamic expression of trk receptors during sensory neuron differentiation. *Soc. Neurosci. Abstr.* **22,** :294.

Sieber-Blum, M. (l991). Role of neurotrophic factors BDNF and NGF in the commitment of pluripotent neural crest cells. *Neuron* **6,** 949–955.

Stemple, D. L., and Anderson, D. J. (l993). Lineage diversification of the neural crest: *In vitro* investigations. *Dev. Biol.* **159,** 12–23

Tessarollo, L., Tsoulfas, P., Donovan, M. J., Parklo, M. E., Flynn, J. B., Hempstead, B. L., and Parada, L. F. (l997). Targeted deletion of all isoforms of the trkC gene suggests the use of alternate receptors by its ligand NT-3 in neuronal development and

implicates trkC in normal cardiogenesis. *Proc. Natl. Acad. Sci. USA* **94,** 14776–14781.

Tessarollo, L., Tsouflas, P., Martin-Zanca, D., Gilbert, D. J., Jenkins, N. A., Copeland, N. G., and Parada, L. F.(l993). trkC, a receptor for NT-3 is widely expressed in the developing nervous system and in non-neuronal tissues. *Development* **118,** 463–475.

Tsoulfas, P. Soppet, D., Escandon, E., Tessarollo, L., Mendoza-Ramirez, J. L., Rosenthal, A., Nikolics, K., and Parada, L. (l993).The rat trkC locus encodes multiple neurogenic receptors that exhibit differential response to NT-3 in PC 12 cells. *Neuron* **10,** 975–990.

Valenzuela, D. M., Maisonpierre, P. C., Blass, D. J., Rojas, E., Nunez, L., Kong, Y., Gies, D. R, Stitt, T. N., Ip, N. Y., and Yanacopoulos, G. D. (l993). Alternative forms of rat TrkC with different functional capabilities. *Neuron* **10,** 963–974.

Verdi, J. M., and Anderson, D. J. (l994). Neurotrophins regulate sequential changes in neurotrophin receptor expression by sympathetic neuroblasts. *Neuron* **13**(6),1359–1372.

Weskamp, G., and Reichardt, L. F. (l991). Evidence that biological activity of NGF is mediated through a novel subclass of high affinity receptors. *Neuron* **6**(4), 649–663.

Weston, J. A. (l991). Sequential segregation and fate of developmentally restricted intermediate cell populations in the neural crest lineage. *Curr. Top. Dev. Biol.* **25,** 133–153. l991.

Williams, R., Backstrom, A., Ebendal, T., and Hallbook, F. (l993). Molecular cloning and cellular localization of trkC in the chicken embryo. *Dev. Brain Res.* **75,** 235–252.

Wright, E. M., Vogel, K., and Davies, A. M. (l992). Neurotrophic factors promote the maturation of developing sensory neurons before they become dependent on these factors for survival. *Neuron* **9,** 139–150.

Zhang, D. M., Yao, L. H. and Bernd, P. (l994). Expression of trk and neurotrophin mRNA in dorsal root and sympathetic ganglia of the quail during development. *J. Neurobiol.* **25,** 1517–1532.

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