Development/Plasticity/Repair

# Small, Nonpeptide p75$^{NTR}$ Ligands Induce Survival Signaling and Inhibit proNGF-Induced Death

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**Studies showing that neurotrophin binding to p75$^{NTR}$ can promote cell survival in the absence of Trk (tropomyosin-related kinase) receptors, together with recent structural data indicating that NGF may bind to p75$^{NTR}$ in a monovalent manner, raise the possibility that small molecule p75$^{NTR}$ ligands that positively regulate survival might be found. A pharmacophore designed to capture selected structural and physical chemical features of a neurotrophin domain known to interact with p75$^{NTR}$ was applied to *in silico* screening of small molecule libraries. Small, nonpeptide, monomeric compounds were identified that interact with p75$^{NTR}$. In cells showing trophic responses to neurotrophins, the compounds promoted survival signaling through p75$^{NTR}$-dependent mechanisms. In cells susceptible to proneurotrophin-induced death, compounds did not induce apoptosis but inhibited proneurotrophin-mediated death. These studies identify a unique range of p75$^{NTR}$ behaviors that can result from isolated receptor liganding and establish several novel therapeutic leads.**

*Key words:* **small molecules; nerve growth factor; brain-derived neurotrophic factor; p75; neurotrophin receptor; hippocampal neurons; oligodendrocytes**

## Introduction

Neurotrophins [NGF, BDNF, neurotrophin-3 (NT-3), and NT-4/5] are secreted proteins that have potent effects on neuronal survival, differentiation, and synaptic function (Chao, 2003; Huang and Reichardt, 2003; Teng and Hempstead, 2004; Lu et al., 2005). Mature neurotrophins bind preferentially to tropomyosin-related kinase (Trk) and p75$^{NTR}$, whereas proneurotrophins, which contain an N-terminal domain proteolytically removed in "mature" forms, interact with p75$^{NTR}$ and through their N-terminal domains, with the sorting receptor sortilin (Fahnestock et al., 2001; Harrington et al., 2004; Nykjaer et al., 2004). p75$^{NTR}$ interacts with Trks and modulates Trk signaling but is also independently coupled to various prosurvival and proapoptotic signaling systems (Salehi et al., 2000; Roux et al., 2001; Mamidipudi et al., 2002). Depending on the operative ligands, coexpression of Trk or other receptors, and expression of downstream signaling elements, p75$^{NTR}$ promotes cell survival or death and modulates neurite outgrowth (DeFreitas et al., 2001; Chao, 2003). proNGF induces death of superior cervical ganglion neurons and oligodendrocytes through p75$^{NTR}$, and its concomitant binding to p75$^{NTR}$ and sortilin has been shown to activate death pathways (Lee et al., 2001; Beattie et al., 2002; Nykjaer et al., 2004). In other systems, p75$^{NTR}$ signaling through phosphatidylinositol 3-kinase AKT (PI3K/AKT) or interleukin-1 receptor-associated kinase/nuclear factor κB (IRAK/NFκB) promotes survival (Carter et al., 1996; Roux et al., 2001; Mamidipudi et al., 2002; Reddypalli et al., 2005).

It has been suggested that the unliganded monomeric form of p75$^{NTR}$ is proapoptotic and that homodimerization induced by neurotrophin binding eliminates that effect (J. J. Wang et al., 2000). This is consistent with studies showing no effects on survival of monomeric p75$^{NTR}$ ligands, including monovalent Fabs (Maliartchouk et al., 2000b) and monomeric cyclic peptides (Longo et al., 1997), whereas related bivalent forms in each study promoted cell survival. However, it is unlikely that these monomeric ligands engage the receptor in the same way as the natural ligands. Moreover, although active NGF is a homodimer containing two potential p75$^{NTR}$ binding sites, recent structural evidence suggests it engages only one p75$^{NTR}$ molecule, disallowing the binding of another (He and Garcia, 2004). These findings raise the possibility that small molecules mimicking a single p75$^{NTR}$-

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interacting domain of NGF might be found that are capable of competing with NGF or proNGF binding and/or modifying p75$^{NTR}$ signaling and regulating cell survival.

## Materials and Methods

*Computational modeling, pharmacophore generation, and virtual and functional screening.* Computational studies were performed using the Accelrys (San Diego, CA) Catalyst and InsightII systems. Three-dimensional (3D) multiconformer virtual libraries were generated using catDB and catShape (Accelrys) from molecule files obtained from numerous sources including: The Chapman and Hall/CRC Combined Chemical Dictionary (Boca Raton, FL), the National Cancer Institute library of anticancer compounds, and libraries from Maybridge (Cornwall, UK), Asinex (Moscow, Russia), Interbioscreen (Moscow, Russia), Chemstar (Minneapolis, MN), Comgenex (Budapest, Hungary), Timtec (Newark, DE), and Sigma (rare chemicals dictionary; St. Louis, MO). Molecular dynamics simulations were performed using the Discover module within InsightII, using the CFF91 force field and explicit water solvent. Pharmacophore placement and library searching were performed with Catalyst, as illustrated in Figure 1 and further described in Results.

*Antibodies and proteins.* Polyclonal rabbit anti-NGF antibody (Ab) was obtained from Chemicon (Temecula, CA). Monoclonal anti-phospho-AKT$^{S473}$, polyclonal anti-AKT, polyclonal anti-phospho-NFκB-p65$^{S536}$, and site-specific polyclonal anti-Trk$^{Y490}$ (recognizes TrkB$^{Y515}$) were purchased from Cell Signaling Technology (Beverly, MA). Monoclonal anti-NFκB-p65 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-actin was purchased from Sigma. Polyclonal anti-TrkB antibodies were purchased from Upstate (Charlottesville, VA). p75$^{NTR}$ polyclonal rabbit antibodies 9651 (Huber and Chao, 1995) and 9650 raised against the neurotrophin-binding region (residues 43–161; cysteine repeat regions II, III, and IV) of the extracellular domain of recombinant p75$^{NTR}$ were generously provided by Dr. Moses Chao (Skirball Institute, New York University, New York, NY). Mouse 2.5 S NGF was purchased from Invitrogen (Carlsbad, CA). Recombinant human BDNF was from Sigma. Polyclonal anti-IRAK was obtained from Santa Cruz Biotechnology, and polyclonal anti-p75$^{NTR}$ antibody was from Promega (Madison, WI). p75$^{NTR}$/Fc and TrkA/Fc chimeras were obtained from R & D Systems (Minneapolis, MN). Furin-resistant proNGF was prepared as described previously (Beattie et al., 2002).

*Neuronal bioassays.* Hippocampal neurons were prepared from embryonic day 16 (E16) to E17 mouse embryos as described previously (Yang et al., 2003). Cell survival was examined by microscopic analysis of morphology and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) metabolism, or terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) and 4′,6′-diamidino-2-phenylindole (DAPI) staining. For morphology/MTT studies, low-density cultures were initiated in poly-L-lysine-coated A/2 plates by adding 25 μl of cell suspension (2000 neurons per well; 12,500 cells/cm$^2$), 25 μl of DMEM containing 10% FBS, and different concentrations of recombinant BDNF, NGF, or NGF loop 1 mimic (LM11A) compounds to each well. After 48 h in culture, cell survival was assessed as described previously (Longo et al., 1997) using a combination of standard morphological criteria along with visual determination of whether a given cell converted MTT to its blue formazan product. The number of surviving neurons was determined by counting the total number of cells in each well that were both morphologically intact and filled with blue product. For each neurotrophin or compound concentration, duplicate wells were counted and the resulting values averaged. Activity of each compound was confirmed by blinded counts. Counts were normalized to survival achieved with 0.95 nM BDNF or to baseline survival. Fitting of dose–response curves was performed with SigmaPlot (Systat Software, Richmond, CA).

For TUNEL/DAPI analysis, E16–E17 mouse hippocampal neurons were plated on poly-L-lysine-coated cover glasses in 24-well plates (Fisher Scientific, Pittsburgh, PA) by adding 0.25 ml of cell suspension (20,000 cells/cm$^2$), 0.25 ml of DMEM containing 10% FBS, recombinant BDNF, or NGF, and different concentrations of LM11A compounds to each well. The final concentrations of BDNF and NGF were 1.9 nM. After 48 h in culture, cell death was assessed using the fluorescein-12-dUTP, Dead-End Fluorometric TUNEL system (Promega) and Vectashield plus DAPI (Vector Laboratories Burlingame, CA). Cells were visualized with a fluorescence microscope (DM IRE2; Leica, Nussloch, Germany) using 520 nm (TUNEL) and 460 nm (DAPI) filters. For each condition, images of 24 preselected fields (totaling 300–500 cells) were captured in a nonbiased manner from each of three separate assays. The fraction of dead cells was determined by dividing the number of nuclei exhibiting TUNEL staining by the number of DAPI-positive nuclei. Counting was performed in a blinded manner.

For studies using *p75$^{NTR+/+}$* and *p75$^{NTR-/-}$* neurons, mice carrying a mutation in exon 3 of the p75$^{NTR}$ gene (encoding the extracellular domain) (Lee et al., 1992) were bred onto a B6 congenic background (>10 B6 backcrosses).

For signaling pathway inhibitor studies, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002), 2-(2-diamino-3-methoxyphenyl-4*H*-1-benzopyran-4-one (PD98059) (Calbiochem, La Jolla, CA), and SN50 (Alexis, Lausen, Switzerland) were added to cultures at final concentrations of 25 μM, 50 μM, and 2.5 μg/ml respectively, concomitantly with BDNF, NGF, or LM11A compounds. For antibody inhibition studies, p75$^{NTR}$ antisera and control nonimmune serum were used at a final dilution of 1:100 in the presence of BDNF, NGF, or LM11A compounds. For all studies applying signaling inhibitors, p75$^{NTR}$ antibodies, or *p75$^{NTR-/-}$* neurons, survival was assessed at 48 h.

*Protein extraction and Western blot analysis.* For assays of Trk levels and Trk, AKT, and NFκB activation, hippocampal neurons derived from E16–E17 mice were cultured in poly-L-lysine-coated six-well plates ($1.5 \times 10^6$ cells per well; Corning, Corning, NY) in DMEM containing 10% FBS, followed by incubation in serum-free DMEM for 2 h before addition of neurotrophins or compounds. At the indicated time points, neurons were harvested in lysis buffer consisting of: 20 mM Tris, pH 8.0, 137 mM NaCl, 1% Igepal CA-630, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 500 μM orthovanadate (Zhou et al., 1995). Lysates were centrifuged, the supernatant collected, and protein concentrations determined using the BCA protein assay reagent (Pierce, Rockford, IL). Western blots were performed as described previously (Yang et al., 2003). Western blot signals were detected using the ECL chemiluminescence system (Amersham Biosciences, Arlington Heights, IL).

*NGF and proNGF displacement from p75$^{NTR}$ or TrkA.* NGF ELISA was performed as described previously (Longo et al., 1999). Briefly, 96-well plates were incubated with 0.1 pmol (at 1 nM) of p75$^{NTR}$/Fc or TrkA/Fc recombinant protein (R & D Systems) overnight at 4°C followed by incubation with blocking buffer for 1 h at room temperature. proNGF at 1.8 nM or different concentrations of NGF and LM11A compounds were diluted in sample buffer, added to wells, and incubated for 6 h with shaking at room temperature. Plates were then washed five times with Tris-buffered saline (TBS) containing 0.05% Tween 20 and incubated with anti-NGF rabbit polyclonal antibody overnight at 4°C. After five washes with TBS, wells were incubated for 2.5 h at room temperature with anti-rabbit IgG horseradish peroxidase (HRP) conjugate and then washed five times again. 3,3′,5,5′-tetramethyl-benzidine substrate was added and optical density measured at 450 nm. Data showing compound-mediated displacement of NGF from p75$^{NTR}$ were fit using the Nonlinear Regression module of SigmaPlot to the following modification of the Gaddum-Schild equation [adapted from Motulsky and Christopoulos (2003)]:

$$\text{signal} = \frac{\text{top} - \text{bottom}}{1 + \left( \frac{10^{\log EC_{50}}[1 + ([C]/10^{\log A_2})]^S}{[\text{NGF}]} \right)^{\text{Hill slope}}} + \text{bottom},$$

where top is the maximal signal, bottom is the basal signal, [C] is compound concentration, *S* is the Schild coefficient, Hill slope is the Hill coefficient, [NGF] is NGF concentration, $EC_{50}$ is the concentration of NGF resulting in 50% maximal (top − bottom) binding, and $A_2$ is the concentration of compound resulting in a doubling of the $EC_{50}$ from the curve obtained in the absence of the compound. In these

studies, the calculated Hill slope ranged from 1.0 to 1.6 and was generally not significantly different from 1.

*p75$^{NTR}$ antibody competition.* NIH3T3 fibroblasts expressing either null vector or p75$^{NTR}$ (Huang et al., 1999) were obtained from Dr. William Mobley (Stanford University, Stanford, CA). Cells were grown in monolayers, harvested in PBS with 2 mM EDTA, pelleted, and resuspended in ice-cold DMEM HEPES with 1 mg/ml BSA. Six to nine $\times 10^6$ cells from one confluent six-well plate were used for each experimental point. For binding analysis, p75$^{NTR}$ antibody (1:100) was allowed to bind in the presence or absence of 100 nM LM11A compounds for 90 min at 4°C with gentle rotation, followed by four washes in PBS. The final cell pellet was resuspended in lysis buffer. Western blots were performed as described above. To detect the presence of p75$^{NTR}$ antibody, blots were probed with horseradish peroxidase-linked goat anti-rabbit IgG (Amersham Biosciences). Signals were detected by the ECL chemiluminescence system. To control for variation in protein loading, the blot was stripped and reprobed with β-actin monoclonal antibody (Sigma).

*IRAK-p75$^{NTR}$ coimmunoprecipitation.* Pheochromocytoma 12 (PC12) cells (generously provided by Dr. W. Mobley) (Beattie et al., 1996) were grown on plates coated with poly-L-lysine (Sigma) in DMEM containing 5% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. NGF (1.9 nM) or LM11A compounds (5 nM) were added to the same medium for 1 min before harvesting. Coimmunoprecipitations were conducted as described previously (Wooten et al., 2001; Mamidipudi et al., 2002), with 400 μg of cell extract incubated overnight at 4°C with 2 μg of polyclonal anti-p75$^{NTR}$ antibody followed by 2 h incubation with 30 μl of protein-A beads (Pierce). After incubation, the beads were washed five times with protein lysis buffer as described above. Protein was eluted by boiling for 10 min in 30 μl of 2× NuPAGE LDS sample buffer. Aliquots were submitted to NuPAGE 4–12% Bis-Tris Gels (Invitrogen) and transferred to polyvinylidene difluoride membranes. Western blots were incubated with antibody against IRAK (1:1000) or p75$^{NTR}$ (1:2000), then with HRP-linked secondary antibodies. Signal was detected using the ECL chemiluminescence system.

*Oligodendrocyte culture, proneurotrophin treatment, and cell death assay.* Cortical oligodendrocytes from rat pups were prepared as described previously (Yoon et al., 1998; Harrington et al., 2002). Cells were treated with recombinant, cleavage-resistant proNGF at 0.05 nM. Controls were treated with equivalent volumes of proNGF purification buffer containing 350 mM imidazole. Twenty-four hours after treatment, cells were fixed and processed for MBP and TUNEL staining as described previously (Beattie et al., 2002). Two hundred to 250 cells were counted per well, for a minimum of 600 cells per experimental condition.

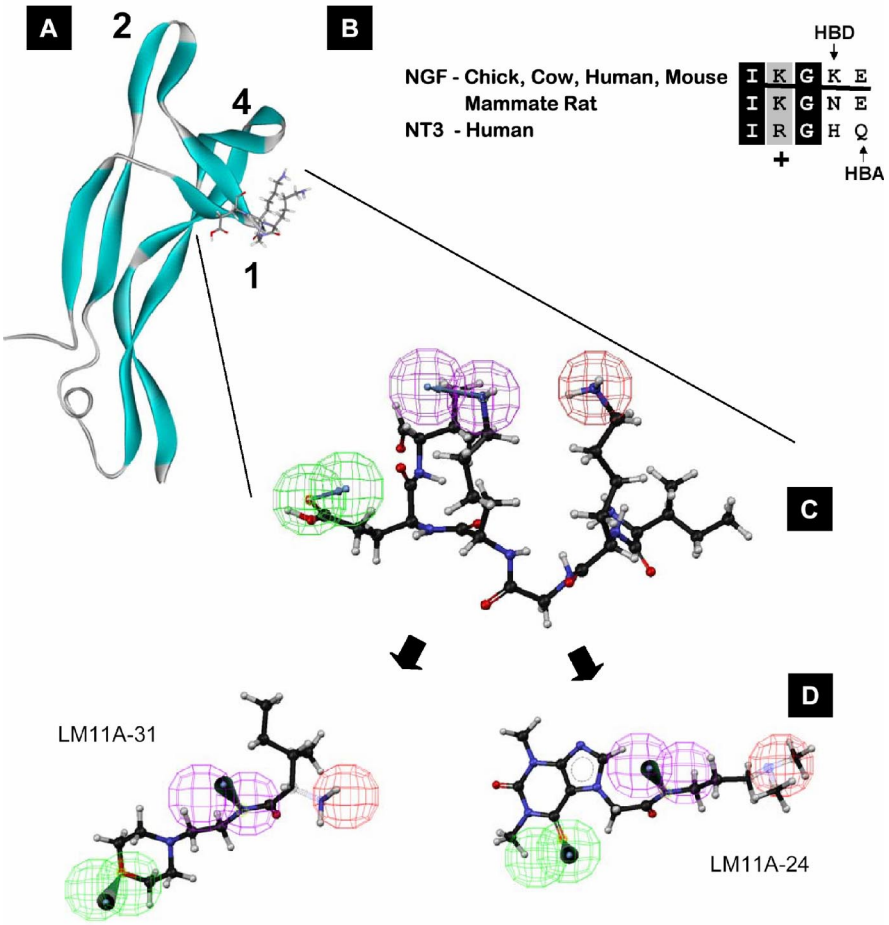


**Figure 1.** LM11A compound selection strategy. ***A***, Ribbon representation of the x-ray crystal structure of human NGF with β-turn loops 1, 2, and 4 designated. Average side-chain positions for loop 1 (illustrated), from an ensemble of samples from loop molecular dynamics simulations, were extracted and used to build a pharmacophoric model. ***B***, Comparison of peptide sequences of loop 1 from NGF and NT-3 from the indicated species and the assignment of pharmacophores. Guidance for the placement of pharmacophore features was obtained from consideration of loop phylogeny and side-chain chemistry. As a first approximation, we assumed that analogous loop 1 peptides from neurotrophins of different species and different neurotrophin family members should bind similarly to p75$^{NTR}$. The primary structure of BDNF loop 1 diverged significantly from NGF and NT-3, and in efforts to reduce pharmacophore complexity, only the latter two were used. The propensity of histidine to act as a hydrogen bond donor (McDonald and Thornton, 1994) suggested the use of that feature at position 34, whereas the K-to-R transition at position 32 suggested the use of a positively ionizable feature there. +, Positively ionizable group; HBD, hydrogen bond donor; HBA, hydrogen bond acceptor. ***C***, Application of the pharmacophoric features to the 3D loop model. Hydrogen bonding features are represented by pairs of spheres with their relative positions indicating the locations of the acceptor and donor. One of the spheres of the pair is centered on a putative acceptor/donor feature in the model, whereas the other indicates the target location of a complementary feature on any potentially interacting molecule. A blue stick bearing a 3D arrowhead extending between the centers of the spheres also indicates the direction of the bond. Red sphere, Positively ionizable group; purple spheres, hydrogen bond donor group; green spheres, hydrogen bond acceptor. The diameter of the spheres represents the spatial tolerance for chemical feature matching in 3D conformer library scans. ***D***, Representative fits to the pharmacophore of two compounds subsequently found to be active and further investigated in this study.

## Results

### Tandem *in silico* and *in vitro* screening yields chemically distinct neurotrophic compounds with high efficiency

Initial modeling of active dimeric cyclic peptides mimicking NGF β-hairpin loop 1, a domain that interacts with p75$^{NTR}$ (Longo et al., 2005), suggested that energetic and structural constraints would disallow simultaneous β-hairpin folding of both peptide subunits, and this implied that the peptides might act in a monomeric manner (S. M. Massa, unpublished data). Based on early virtual screening of 3D conformer libraries, it also seemed unlikely we would find many functionally dimeric molecules that were nonpeptides and <500 Da molecular weight. We therefore focused on finding compounds emulating a single loop 1 structure. Compounds were selected for screening in cell survival assays by virtual screening of a compound library with a pharmacophoric loop 1 model, as outlined in Figure 1. An average of 35 conformers of each of >800,000 compounds were screened,

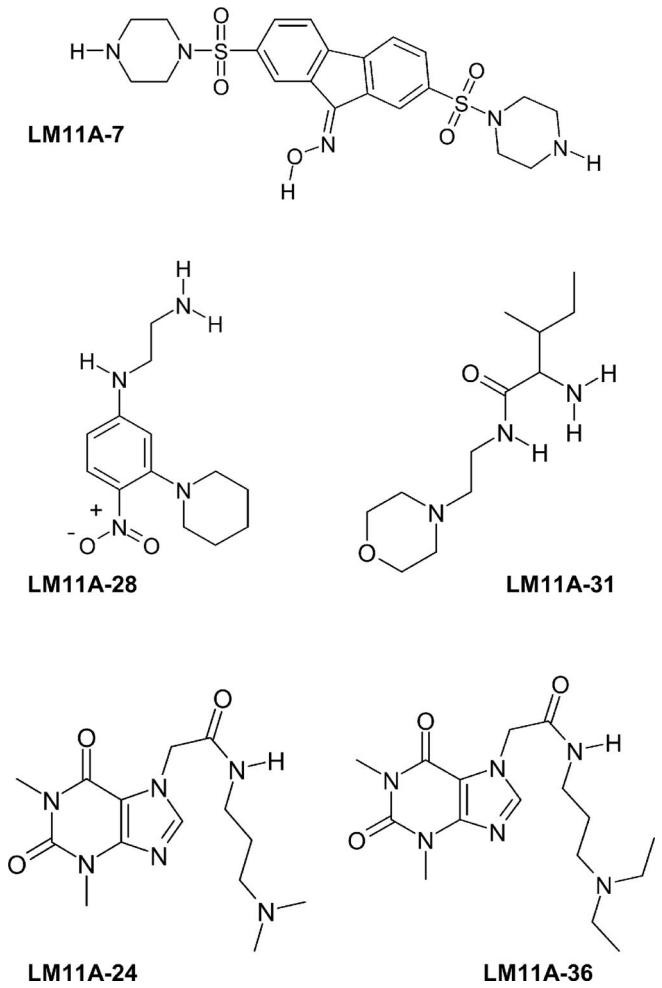


**Figure 2.** LM11A compound structures.

yielding ~800 compounds with conformers that fit with a calculated internal energy of <10 kcal/mol. This number was further reduced to ~60 compounds by visual inspection on the bases of likely steric compatibility with a hypothetical shallow receptor binding pocket and maximal flexibility of the functional groups. Thirty-five compounds were obtained from commercial sources, of which 23 were soluble in water. In preliminary studies using a chicken DRG neuronal survival assay, 4 of 23 compounds tested showed significant neurotrophic activity (Massa et al., 2002, 2003).

### Neurotrophic effects of NGF and LM11A compounds are similar in hippocampal neuron cultures

To extend the evaluation of the compounds from chick DRGs to mammalian CNS neurons, further biologic analysis of the compounds was performed using embryonic mouse hippocampal neurons. Under conditions of low density and lack of glial support, the survival of these neurons is dependent, in part, on the addition of neurotrophins (Lindholm et al., 1996). Screening of the 23 previously tested compounds in these cultures demonstrated activity of three of the compounds (LM11A-7, -24, and -31) previously identified as having activity in DRG cultures and identified a fourth active compound, LM11A-28 (structures in Fig. 2).

To begin to understand the mechanisms of action of these compounds and test the conjecture that they work via the targeted receptor p75$^{NTR}$ we examined the dose–response relationships of the survival-promoting activities of LM11A compounds compared with NGF and BDNF using embryonic hippocampal neurons in culture conditions in which NGF promotes neuronal survival. Similar cultures have been reported to express little or no TrkA (Ip et al., 1993; Marsh et al., 1993; Brann et al., 1999), but consideration of differences in species, timing, media, and the presence or absence of a glial feeder layer prompted examination of Trk expression in the cultures used in the present studies (Fig. 3*A*). Western analysis demonstrated abundant TrkB and no detectable TrkA expression. Although the expression of TrkA at trace levels cannot be entirely ruled out, these findings suggest that in these cultures, neurotrophic activity is mediated by BDNF principally through TrkB and p75$^{NTR}$, whereas NGF functions primarily through p75$^{NTR}$.

Addition of LM11A-7, -24, -28, and -31 increased the number of GAP-43-positive neurite-bearing cells, consistent with increased neuronal survival (Fig. 3*B*, photomicrographs). Dose–response profiles of the active compounds (Fig. 3*C*) demonstrated $EC_{50}$ values in the range of 100–300 pM and intrinsic activities 80–100% of the NGF response. Maximal NGF and compound responses were less than that of BDNF. Although it seems likely this lower level of efficacy was caused by the simultaneous activation of TrkB and p75$^{NTR}$ within a cell by BDNF that does not occur with the other ligands, it remains possible that it is attributable to differential effects on neuronal subpopulations expressing different complements of receptors. Independently synthesized preparations of LM11A-24 and LM11A-31 gave similar results (data not shown). LM11A-36, a compound structurally similar to LM11A-24 (Fig. 2), showed little or no neurotrophic activity. Using TUNEL/DAPI staining, a similar profile of effects on preventing cell death were found for BDNF, NGF, and LM11A-24, -31, and -36 (Fig. 4*A*,*B*), thus confirming the findings of the morphological/MTT assays. Of note, in both assays, at compound concentrations >5 nM, survival response was reduced (Figs. 3*C*, 4*B*). This reduction could have resulted either from mechanisms unrelated to p75$^{NTR}$ or from modulation of receptor multimer formation and survival signaling at higher receptor occupancies, even by a compound without neurotrophic activity (e.g., LM11A-36). Neurotrophic responses similar to that of NGF in this hippocampal neuron system are consistent with activation of survival signaling through the NGF binding region of p75$^{NTR}$.

Of the four active compounds initially identified, two (LM11A-24, a derivative of caffeine, and LM11A-31, an amino acid derivative), which were predicted to have "drug-like" character by Lipinski criteria (Lipinski, 2000) and blood–brain barrier calculations (Clark, 1999; Fu et al., 2001), were chosen for more detailed mechanistic study. We further prioritized LM11A-31, because preliminary studies (data not shown) indicated that it exhibits significant oral uptake and blood–brain barrier penetration. The relatively inactive compound LM11A-36 was chosen as a negative control because of its structural similarity to LM11A-24 (Fig. 2). The hypothesis that the LM11A compounds work through p75$^{NTR}$ was examined by investigating the influence of the compounds on (1) the binding of ligands or antibodies to p75$^{NTR}$, (2) neuronal survival with blockade or in the absence of p75$^{NTR}$, (3) maximal cell survival mediated by NGF, and (4) the activation of signaling systems associated with p75$^{NTR}$-mediated survival. To further explore possible functional differences between the compounds and neurotrophins, we examined

the effects of compounds in oligodendrocyte cultures, preparations in which neurotrophins cause, rather than prevent, cell death.

### LM11A compounds interact with p75$^{NTR}$ but not TrkA

To assess the interactions of the LM11A compounds with neurotrophin receptors, we examined the effects of increasing concentrations of compounds on NGF binding to the recombinant chimeric proteins p75$^{NTR}$-Fc and TrkA-Fc. These chimeric proteins contain the extracellular regions of the p75$^{NTR}$ and TrkA receptors and thus provide a useful tool for determining whether compounds affect neurotrophin binding. LM11A-31 (Fig. 5*A*) and LM11A-24 (Fig. 5*B*), but not LM11A-36 (Fig. 5*C*), shifted the NGF/p75$^{NTR}$-Fc binding curve significantly to the right. The inhibition of NGF binding caused by each active compound was reversed with increasing NGF concentration, consistent with a mechanism that is, at least in part, competitive in nature. When these data were fit to the Gaddum–Schild equation that describes ligand binding in the presence of an inhibitor (Motulsky and Christopoulos, 2003; Neubig et al., 2003) (see Materials and Methods), the resulting Schild coefficients were significantly $<1.0$ for both active compounds (LM11A-31, $0.58 \pm 0.04$; LM11A-24, $0.26 \pm 0.01$), suggesting a more complex model (e.g., multiple sites within a ligand interacting with a receptor) (Lutz and Kenakin, 1999; Neubig et al., 2003). Gaddum–Schild analysis also yields a measure of potency that is independent of the mechanism of inhibition known as $A_2$ (i.e., the concentration of compound that shifts the $EC_{50}$ twofold to the right). The derivation of the $K_d$ (that is, the equating of $A_2$ with $K_d$) for an inhibitor in Gaddum–Schild analysis requires a competitive interaction between the antagonist and the ligand, as indicated by a Schild coefficient of 1.0. When this is not the case, $A_2$ nonetheless remains a useful quantitative measure for comparing compounds within a given system and provides a basis for comparison with other parameters such as biologic potency. For example, LM11A-24 ($A_2$, 31.6 + 1.3 nM) and LM11A-31 ($A_2$, 1192 + 1.2 nM) have similar biologic potencies (Fig. 3) despite the ~35-fold greater potency of LM11A-24 in the NGF displacement assay; this suggests that properties in addition to their ability to disrupt NGF binding contribute to the biological activity of each ligand. It could also suggest different mechanisms of action between the compounds.

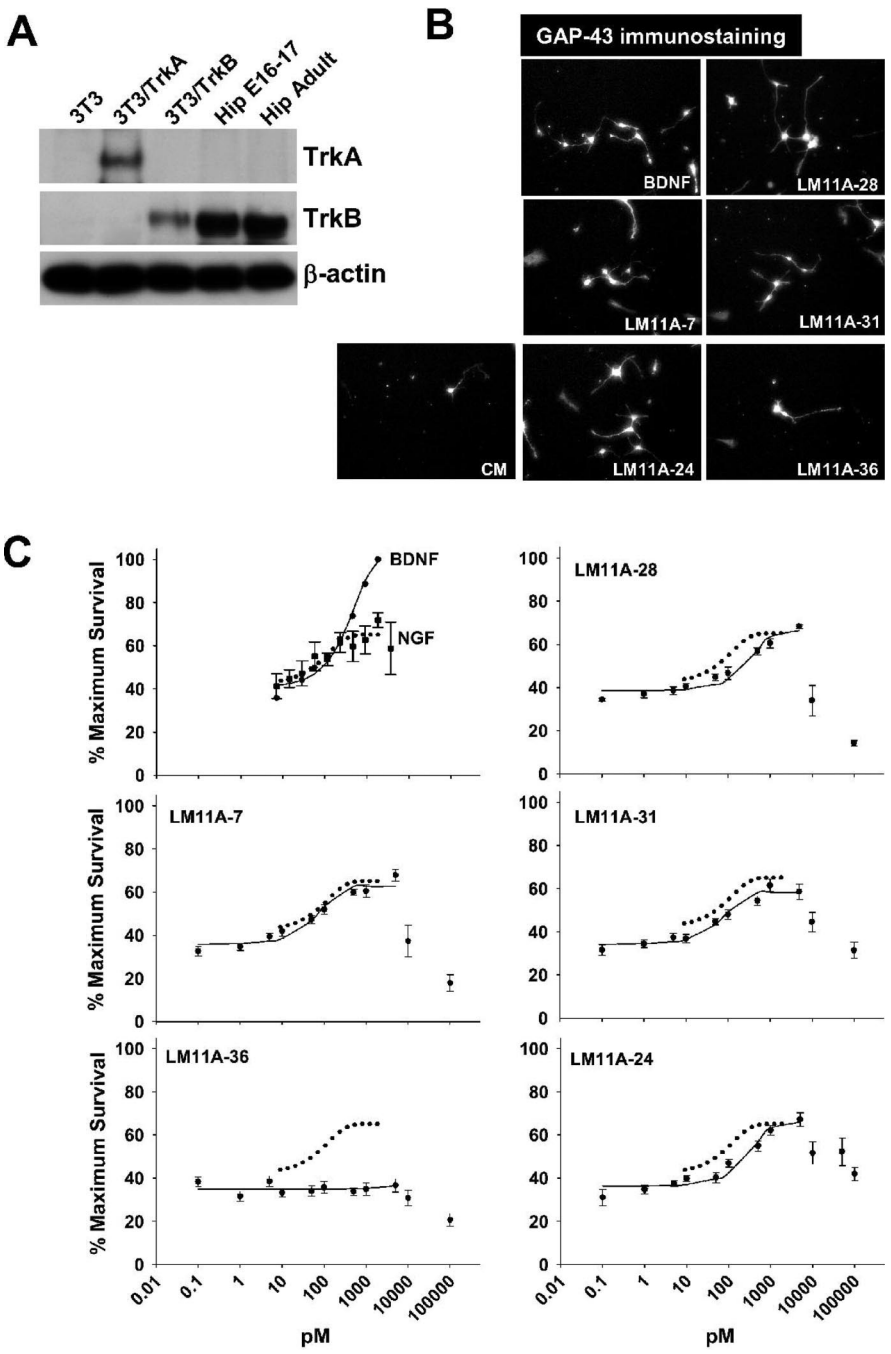


**Figure 3.** LM11A compounds promote hippocampal neuron survival. ***A***, Western blot analysis for TrkA and TrkB expression of 3T3 cells expressing TrkA or TrkB, E16–E17 mouse hippocampal (Hip) neuronal cultures, and adult hippocampus. TrkA is undetectable in hippocampal cultures and tissues, whereas TrkB expression is readily apparent. ***B***, Representative fluorescence photomicrographs of E16–E17 mouse hippocampal neuronal cultures treated with culture medium only (CM) or medium containing BDNF or LM11A compounds and stained for expression of the neuron-specific, growth-associated protein GAP-43 at 48 h after treatment. ***C***, Neuron survival dose–response curves of BDNF, NGF, and the LM11A compounds, showing similar potency and maximal responses, between NGF and LM11A 7, 24, 28, and 31 up to 5 nM, with no response to LM11A-36. BDNF has similar potency but a higher maximal response. All LM11A compounds showed a decrementing response above 5 nM. Survival was determined as the total number of cells in each well that were both morphologically intact and filled with blue formazan MTT-conversion product. Counts were normalized to survival achieved with 0.95 nM BDNF or to baseline survival. *n* is 4–18 for all determinations. The symbols and bars indicate mean ± SE, and lines are fits of a single-exponential rise model to the data. The dotted lines in each graph represent the fitted NGF response.

Additional support for the interaction of LM11A-31 with p75$^{NTR}$ was obtained through the analysis of LM11A compound effects on Ab 9651 binding to p75$^{NTR}$-expressing fibroblasts. Ab 9651 was raised against the extracellular cysteine-rich repeat regions II, III, and IV, implicated in neurotrophin binding (Huber

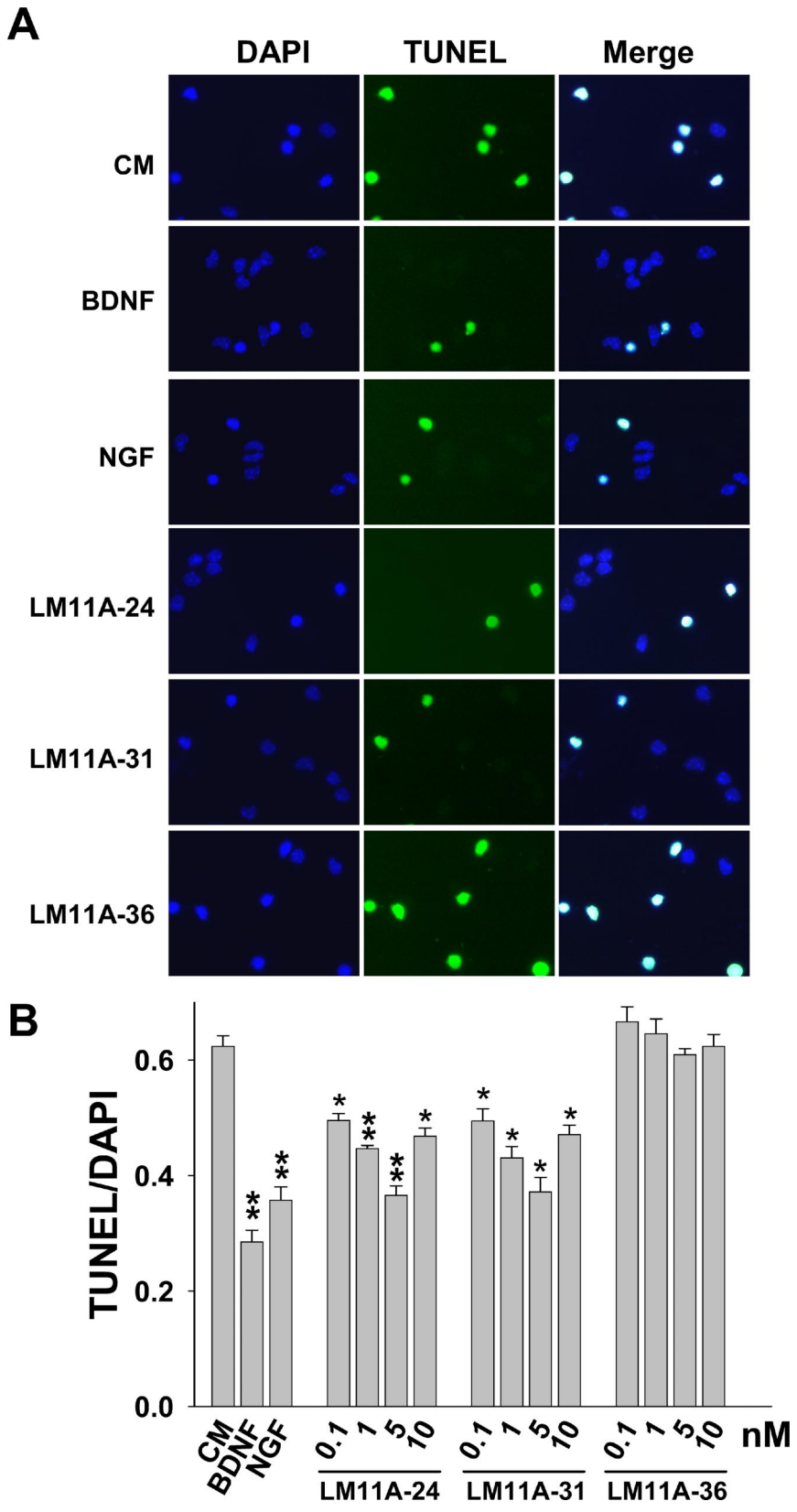


**Figure 4.** LM11A compounds inhibit hippocampal culture DNA damage. ***A***, Representative fluorescence photomicrographs of TUNEL and DAPI staining and merged images of E16–E17 hippocampal cultures treated with neurotrophins at 1.9 nM or LM11A compounds at 5 nM. CM, Culture medium only. ***B***, Quantitation of the fraction of DAPI-staining cells that are also TUNEL positive. Cultures were treated with 1.9 nM neurotrophins or LM11A compounds at the indicated concentrations. $n = 3$ separate assays (with >100 cells counted per assay) for each condition. Error bars represent mean + SE. $*p < 0.01$, $**p < 0.001$ by Student's $t$ test for comparisons with culture medium only (CM). At 5 nM, LM11A-24 and -31 inhibit TUNEL staining to a degree similar to that of NGF. At 10 nM, cell-death-preventing activity of compounds is less than that found at 5 nM. LM11A-36 had no detectable effect.

and Chao, 1995). Ab 9651 binding to NIH3T3 cells was dependent on the expression of p75$^{NTR}$ (Fig. 5*D*). In p75$^{NTR}$-expressing cells, coincubation of a high concentration of LM11A-31 (100 nM) with antibody, in the absence of NGF, at 4°C (which blocks receptor uptake) (Schlessinger et al., 1978), caused a significant decrease in antibody binding, whereas LM11A-36 (the analog inactive in binding or functional assays) had no effect (Fig. 5*D*). The specificity of LM11A compounds for p75$^{NTR}$ was supported by additional receptor binding studies showing that the active compounds had no effect on NGF binding to TrkA (Fig. 5*E*,*F*). Together, these results suggest that the compounds interact with the extracellular part of p75$^{NTR}$ in a way that can interfere with the function of the ligand-binding domain.

### LM11A compound activity is p75$^{NTR}$ dependent

We then determined whether compound survival-promoting effects were dependent on the presence of p75$^{NTR}$. Ab 9651, previously shown to block neurotrophic activity of NGF and NGF loop 1 peptide mimetics in mouse dorsal root ganglion neurons (Longo et al., 1997), partially blocked the neurotrophic activity of BDNF and NGF (Fig. 6*A*) and completely blocked the neurotrophic activity of LM11A-24 and LM11A-31, whereas nonimmune antibody had no effect. Another independently derived rabbit polyclonal anti-p75$^{NTR}$ antibody (Ab 9650) gave virtually identical results (data not shown), further corroborating the specificity of p75$^{NTR}$ blockade. Neither of the antibodies produced changes in baseline survival in these cultures. In addition, we examined the response of *p75*$^{NTR\ -/-}$ cells to neurotrophins and the LM11A compounds (Fig. 6*B*). Baseline survival under these culture conditions was similar in *p75*$^{NTR+/+}$ and *p75*$^{NTR-/-}$ cells. *p75*$^{NTR-/-}$ cultures demonstrated decreased responsiveness to BDNF and the LM11A compounds in a pattern similar to that found in the p75$^{NTR}$ antibody studies, but differed in that the response to NGF was completely lost. These results are consistent with BDNF acting through both TrkB and p75$^{NTR}$, whereas the LM11A compounds act primarily through p75$^{NTR}$. The ability of p75$^{NTR}$ antibody to completely disrupt activity of LM11A compounds while only partially disrupting that of NGF may be a result of differences between NGF and compounds in ligand-receptor affinity or steric and/or al-

losteric mechanisms. Finally, treatment with 5 nM LM11A-24 or -31 along with 1.9 nM NGF, concentrations of each which induce a maximal response, produced no additive effect on survival (data not shown), further supporting the hypothesis that LM11A compounds act through p75$^{NTR}$.

If the LM11A compounds work through interactions with p75$^{NTR}$, we expect that they should affect its ability to signal. One of the most proximal NGF-induced effects on p75$^{NTR}$ associated with cell survival is the rapid recruitment to the receptor of IRAK (Mamidipudi et al., 2002, 2004). In PC12 cell cultures, addition of NGF has been shown to induce, within 1 min, the recruitment of IRAK, with subsequent downstream activation of NFκB (Mamidipudi et al., 2002). We found that LM11A-24 and -31, but not LM11A-36, induced recruitment of IRAK to p75$^{NTR}$ in PC12 cells (Fig. 7*A*).

Despite the observation that LM11A-24 and LM11A-31 did not affect NGF-TrkA/Fc binding, it remained possible that LM11A compounds might activate TrkB, the principal Trk expressed by these hippocampal neurons, or the nominally expressed TrkA, as their primary mechanism for promoting survival. Also, ligand binding to p75$^{NTR}$ might influence Trk activation. With these considerations, it was of interest to determine whether LM11A compounds would promote Trk activation. LM11A-24 and LM11A-31 were assessed for their ability to activate Trk autophosphorylation, as indicated by TrkA$^{Y490}$ (or the analogous TrkB$^{Y515}$) phosphorylation, a well established marker of Trk activation. In hippocampal cultures, BDNF exposure resulted in robust Trk activation (Fig. 7*B*), whereas no activation could be detected with NGF or LM11A compounds. The lack of signal with NGF confirms that these cultures produce little or no TrkA and supports the idea that the trophic effects of NGF are mediated principally by p75$^{NTR}$. In 3T3–TrkA cells, NGF exposure produced the expected TrkA autophosphorylation response, whereas the LM11A compounds again showed no activity (Fig. 7*C*). These results suggest that activation of Trk receptors does not play a primary role in promotion of cell survival by LM11A compounds. In addition, they argue against a mechanism in which the compounds produce their neurotrophic effects by inducing secretion of endogenous neurotrophins. In particular, if compounds induced BDNF secretion, we would expect to detect activation of TrkB in compound-treated hippocampal cultures, which was not observed (Fig. 7*B*,*C*). The possibility that the compounds selectively induce secretion of other factors remains although seems unlikely.

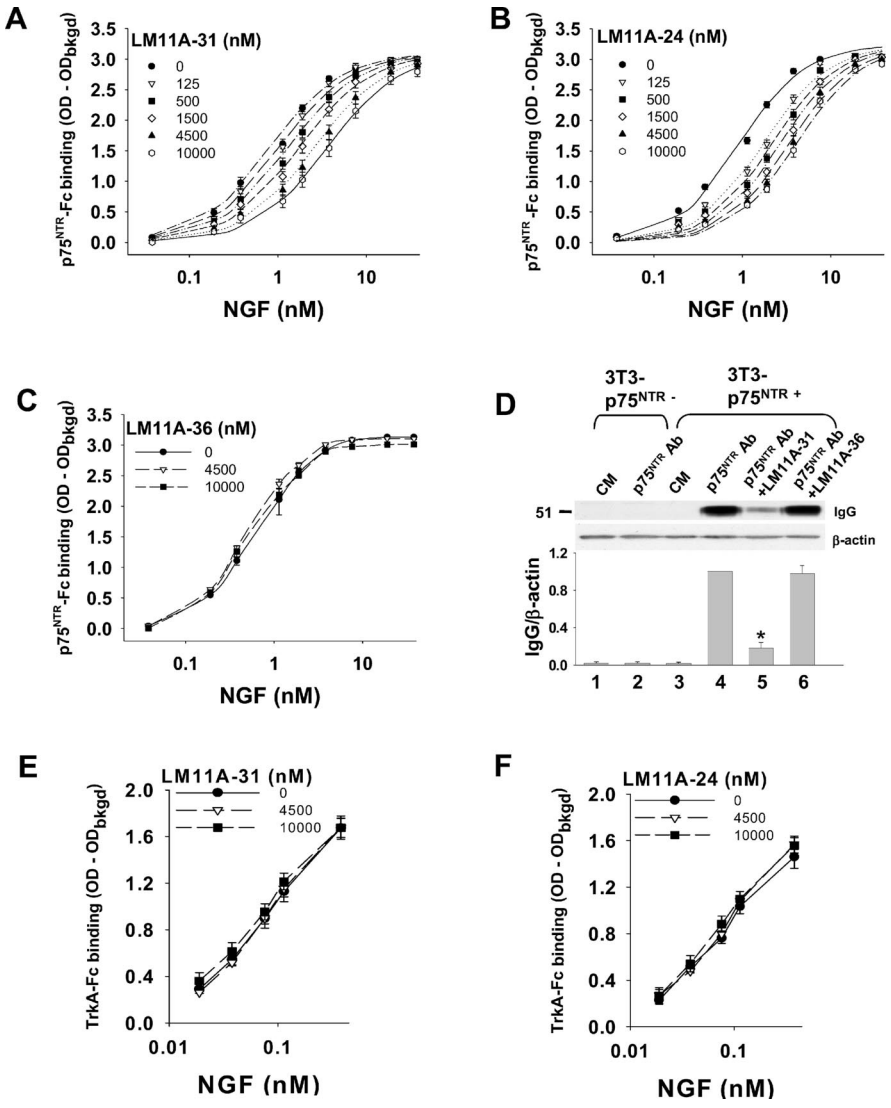


**Figure 5.** LM11A compounds interact with p75$^{NTR}$. ***A***, ***B***, NGF/p75$^{NTR}$-Fc binding curves, in the presence of increasing concentrations (designated in the legend) of LM11A-31 (***A***) or LM11A-24 (***B***), as detected by NGF ELISA. Symbols are mean ± SE; $n > 10$ for all determinations. The lines represent fitting to a modified Gaddum-Schild equation (see Materials and Methods), with overall $r^2$ values of 0.93 for LM11A-31 and 0.96 for LM11A-24. Also, $p < 0.0001$ by ANOVA with *post hoc* Bonferroni/Dunn testing for comparisons between binding curves at 0 nM compound and curves with ≥500 nM compound for LM11A-31 and ≥125 nM for LM11A-24. $K_d$ for NGF in the absence of compounds was 0.8–0.9 nM, consistent with previous reports of ~1 nM (Nykjaer et al., 2004). ***C***, NGF/p75$^{NTR}$-Fc binding curves in the presence of increasing concentrations of LM11A-36, showing no significant effect up to 10,000 nM. Symbols are mean ± SE; $n > 10$ for all determinations. ***D***, Representative Western blot showing displacement of anti-p75$^{NTR}$ Ab 9651 from p75$^{NTR}$-expressing 3T3 cells by LM11A-31, but not LM11A-36. Top, IgG heavy chain; bottom, β-actin; graph, quantitation. Bars represent mean + SE, normalized to bound antibody (lane 4). $n = 4$ for each condition. *$p <$ 0.0005, for comparison with binding in the absence of compound, by Student's *t* test. Antibody and compound treatments are designated above each lane. Ab 9651 did not bind to p75$^{NTR}$-negative cells (lanes 1 and 2). Ab 9651 bound to p75$^{NTR}$-positive cells (lane 4) and was significantly displaced by LM11A-31 (lane 5), whereas LM11A-36 had no effect (lane 6). CM, Culture medium only. ***E***, ***F***, NGF/TrkA-Fc binding curves in the presence of increasing concentrations of LM11A-31 (***E***) or LM11A-24 (***F***), showing no compound effects up to 10,000 nM. Symbols are mean ± SE; $n > 4$ for all determinations.

### Signaling intermediates activated by LM11A compounds

Prosurvival signaling pathways with well established associations with p75$^{NTR}$ actions include activation of NFκB (Carter et al., 1996; Foehr et al., 2000; Gentry et al., 2000; Mamidipudi et al., 2002; Reddypalli et al., 2005) and PI3K/AKT (Roux et al., 2001; Lachyankar et al., 2003; Reddypalli et al., 2005). These signaling intermediates have been shown to be capable of being independently regulated by Trk and p75$^{NTR}$ through pathways with varying degrees of overlap and cross-talk and with different

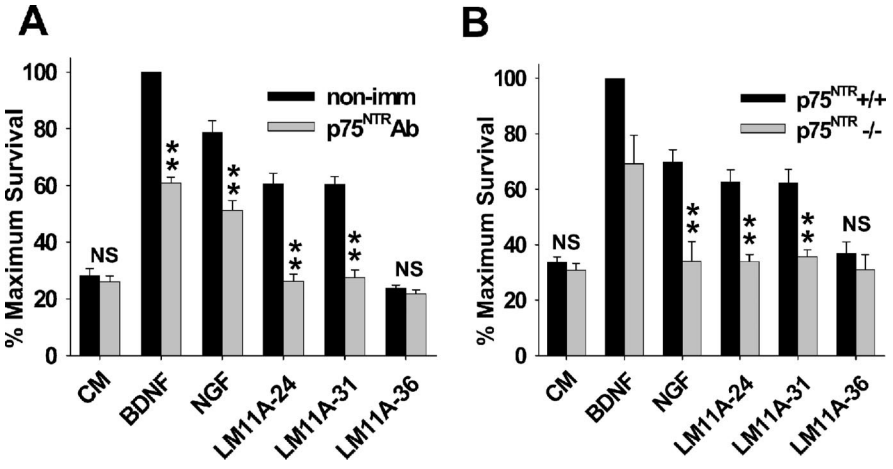


**Figure 6.** LM11A compounds are p75 $^{NTR}$ dependent. ***A***, Ab 9651 has no effect on baseline survival [culture medium only (CM)], partially inhibits BDNF and NGF, and completely inhibits LM11A-24 and -31 promotion of hippocampal neuron survival. Black bars, Nonimmune (non-imm) serum treatment; gray bars, Ab 9651 treatment. Bars represent mean + SE; $n > 11$ for each condition. $^{**}p < 0.0002$ (for comparisons between Ab 9651 and nonimmune); NS, not significant by Student's *t* test. Survival in the presence of BDNF or NGF plus Ab 9651 is significantly greater than CM plus Ab 9651 ( $p < 0.000001$), whereas the differences between CM and LM11A compounds in the presence of antibody are not significant. ***B***, p75 $^{NTR}$ deficiency partially inhibits BDNF and completely inhibits NGF, LM11A-24, and -31 promotion of hippocampal neuron survival. Neurotrophins were applied at 1.9 nM, and compounds were applied at 5 nM. Black bars, $p75^{NTR+/+}$ cells; gray bars, $p75^{NTR-/-}$ cells. Bars represent mean + SE; $n > 3$ for neurotrophins and $n > 6$ for compounds. $^{**}p < 0.005$; NS, not significant (for comparisons between knock-out and wild type) by Student's *t* test. In $p75^{NTR-/-}$ cultures, BDNF treatment produced greater survival than NGF ( $p < 0.05$) or any LM11A compound ( $p < 0.01$). There was no significant difference in baseline survival between the genotypes.

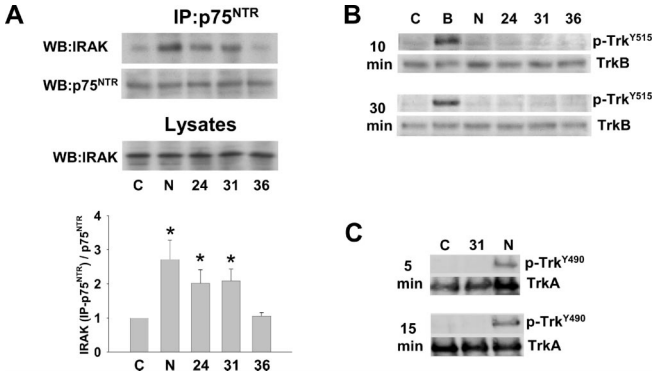


**Figure 7.** LM11A compounds induce proximal signaling events associated with p75 $^{NTR}$ but not Trks. ***A***, LM11A compounds induce rapid association of IRAK with p75 $^{NTR}$. Top, Cell lysates were immunoprecipitated (IP) with p75 $^{NTR}$ antibody followed by Western blotting (WB) with either IRAK or p75 $^{NTR}$ antibody as indicated. Bottom, Cell lysate Western blotting, showing no significant differences in IRAK levels between treatments. Graph, ratios of IRAK coimmunoprecipitated with p75 $^{NTR}$ to total p75 $^{NTR}$. Bars indicate mean + SE; $n = 7$ for each condition. $^{*}p <$ 0.05 for comparison with control media, Student's *t* test. ***B***, Western blots of hippocampal neuron cultures using anti-phosphorylated Trk $^{Y490}$ (which recognizes TrkB $^{Y515}$) compared with total TrkB. BDNF activated TrkB, whereas NGF and the LM11A compounds resulted in no detectable activation at 10 or 30 min. ***C***, Western blots of TrkA-expressing 3T3 cells using anti-phosphorylated Trk $^{Y490}$ compared with total TrkA. NGF activated TrkA, whereas LM11A-31 produced no detectable activation. Results of two additional independent assays for TrkB and TrkA activation were identical. C, Control media; N, NGF; B, BDNF. 24, 31, and 36 represent the corresponding LM11A compounds.

kinetics. NFκB activation was implicated in the survival-promoting activities of the LM11A compounds by the finding that application of an inhibitor of NFκB translocation, SN50 (Lin et al., 1995), at a relatively low concentration targeted to minimize non-NFκB interactions (Kolenko et al., 1999) significantly reduced cell survival promoted by both LM11A compounds and the neurotrophins, with no effect on baseline survival (Fig. 8*A*). Trichodion, a compound that inhibits NFκB activation through the stabilization of IκB (Erkel, 2000), gave nearly identical results (data not shown). The PI3 kinase inhibitor LY294002, which inhibits AKT activation, markedly decreased survival in all cases, including baseline survival under conditions of no treatment or exposure to LM11A-36 (Fig. 8*A*). In addition, the ERK (extracellular signal-regulated kinase) inhibitor PD98059 significantly decreased BDNF-stimulated survival and had a small but significant effect on NGF activity but produced no significant decrement in survival promoted by either LM11A-24 or LM11A-31 (Fig. 8*A*). Although these inhibitors are not completely specific, these studies suggested that ERKs were likely not significantly involved in LM11A compound activity, although the possibility of their action through the PI3K/AKT and NFκB pathways warranted further characterization.

Consistent with the recruitment of IRAK to p75 $^{NTR}$ induced by the compounds (Fig. 7*A*) and the effect of SN50, treatment of hippocampal neurons with 20 nM LM11A-24 or LM11A-31 (a concentration in the plateau range for acute signaling activation; see below) led to an ~1.5-fold increase in NFκB-p65 phosphorylation (Fig. 8*B*), indicative of activation of the NFκB pathway (Sakurai et al., 1999), similar in extent and time course to that induced by both neurotrophin proteins. LM11A-36 at the same concentration did not induce NFκB-p65 phosphorylation. Examination of compound dose-activation response (Fig. 8*C*) demonstrated that LM11A-31 induced activation of NFκB over a concentration range of 0.5–3 nM, which is similar to that required for promotion of survival and concordant with a role for this signaling mechanism in mediating compound-induced survival. In studies of AKT activation, LM11A-24 and -31 at 20 nM showed similar degrees of activation to that found with NGF at 30 min, whereas BDNF induced a substantially greater response. In addition, the onset of activation stimulated by LM11A-24 and -31 was slower than that of NGF (Fig. 8*D*). Similar to the observations of NFκB, the half-maximal response of AKT activation to LM11A-31 was at ~1 nM (Fig. 8*E*). Finally, we found that NGF and LM11A compound failed to activate AKT in cultures of p75 $^{NTR-/-}$ neurons (Fig. 8*F*). Together with evidence for the p75 $^{NTR}$ dependence of compound-induced survival (Fig. 6*A*,*B*), these findings suggest that LM11A compounds induce survival of hippocampal neurons in culture, at least in part, through interactions with p75 $^{NTR}$ that produce activation of survival-promoting signaling pathways involving AKT and NFκB. The possibility that LM11A compounds promote survival by blocking death-promoting activity of endogenous proneurotrophins that might be present in hippocampal cultures is made less likely by the finding that baseline survival of hippocampal neurons was not affected by the absence of p75 $^{NTR}$ (Fig. 6*B*).

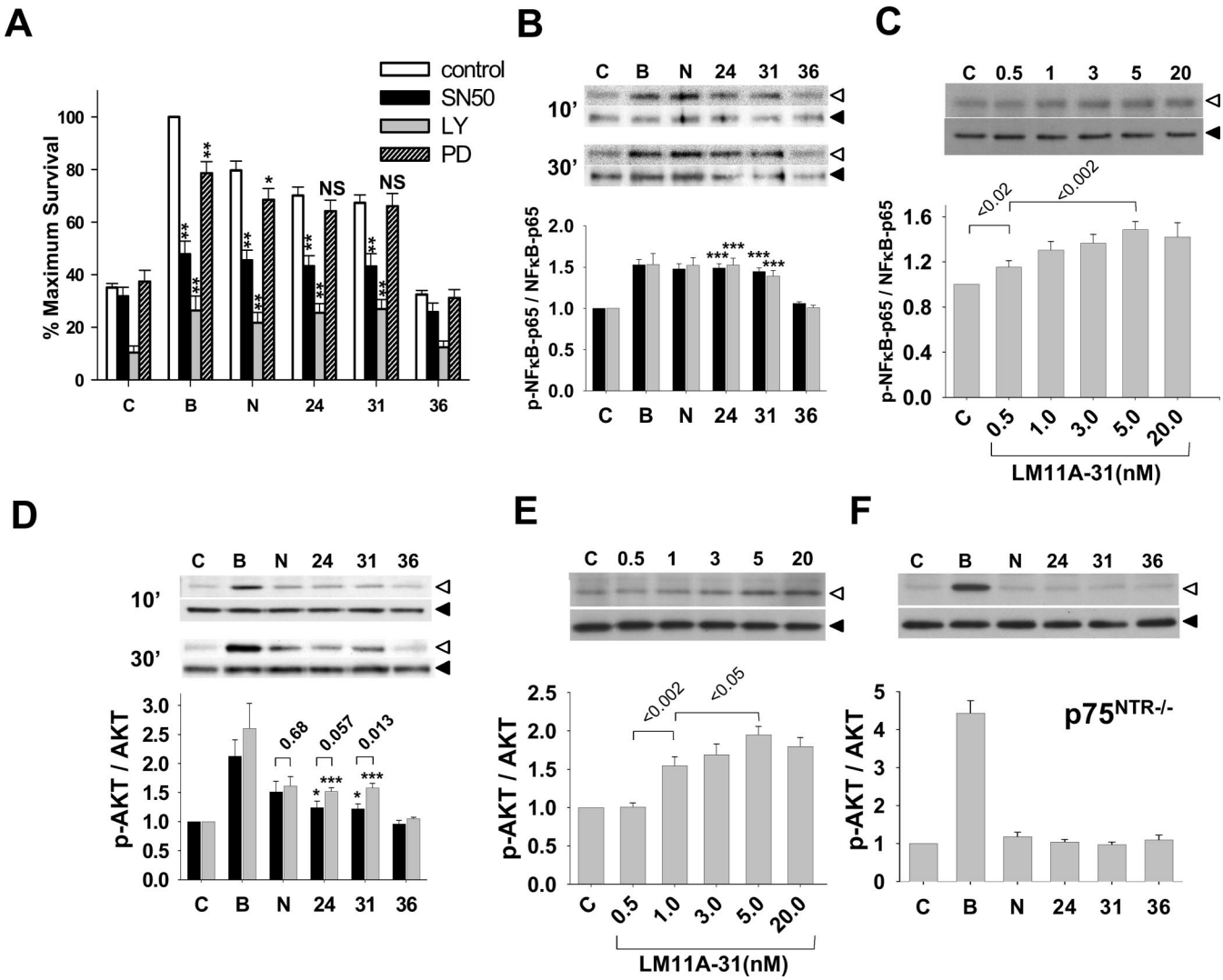


**Figure 8.** LM11A compounds induce survival signaling similar to, but distinct from, neurotrophins. ***A***, Survival of hippocampal neurons in cultures treated with signaling pathway inhibitors and BDNF (0.95 nM), NGF (0.95 nM), or LM11A compounds (5 nM), showing substantial inhibition by NFκB and PI3K pathway inhibitors, small effects of ERK inhibition on BDNF and NGF activity, and no effect of ERK inhibition on LM11A compound activity. SN50, An NFκB translocation inhibitor; LY, LY294002, a PI3K inhibitor; PD, PD98059, an ERK inhibitor. $n = 18$ for each bar, showing mean + SE; NS, not significant; $^{*}p < 0.05$, $^{**}p < 0.001$ for comparison with control (no inhibitor) in each group, Student's *t* test. ***B–F***, Western blot analysis of extracts of hippocampal cultures. Top, Representative bands corresponding to phosphorylated signaling factors (open arrowheads) and the corresponding total factor (filled arrowheads); graphs, quantitation of the ratio of phospho- to total factor, indicating degree of activation. ***B***, NFκB-p65 activation analysis, showing similar activation kinetics for all biologically active treatments. Cells were treated with culture media (C), BDNF (B) at 1.9 nM, NGF (N) at 1.9 nM, and LM11A compounds at 20 nM. The bars indicate mean + SEM. Black bars, Sampling at 10 min; gray bars, 30 min. $n = 6$ independent blots for each determination. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$ for comparison with culture medium only by Student's *t* test. ***C***, LM11A-31 dose–response of NFκB pathway activation. Bars indicate mean + SE; $n > 6$ for each condition. *p* values as indicated, Student's *t* test. Activation is detected between 0 and 0.5 nM and reaches a plateau at 5 nM. ***D***, AKT activation analysis as in ***B***, showing a small lag in activation by the active compounds relative to NGF. ***E***, LM11A-31 dose–response of AKT pathway activation as in ***C***. Activation is detected between 0.5 and 1 nM and reaches a plateau level at 5 nM. ***F***, AKT activation by growth factors and compounds in p75$^{NTR-/-}$ cells. $n > 9$ for each condition. There are no significant differences between culture medium alone and NGF or the LM11A compounds.

### LM11A-31 and LM11A-24 do not promote death of mature oligodendrocytes but inhibit proNGF-mediated death

Although NGF and the LM11A compounds promoted survival in the hippocampal cultures used in these studies, liganding of p75$^{NTR}$ by mature NGF or by proNGF has been associated with cell death rather than promotion of survival in certain cell types (Casaccia-Bonnefil et al., 1996; Lee et al., 2001). To determine whether LM11A compounds would promote survival or cause death in systems in which neurotrophins promote cell death, we examined the survival of mature oligodendrocytes treated with LM11A compounds and proNGF. Mature oligodendrocytes express p75$^{NTR}$ but not TrkA and undergo apoptotic death on treatment with NGF or proNGF (Casaccia-Bonnefil et al., 1996; Yoon et al., 1998; Beattie et al., 2002). Unlike NGF or proNGF, LM11A-24, -31, and -36 alone did not promote cell death (Fig. 9*A*). In addition, proNGF-induced cell death was significantly inhibited by LM11A-24 and -31 over a concentration range of 1–10 nM, but not by LM11A-36, which appeared to decrease survival somewhat at 10 nM (Fig. 9*A*).

To determine whether LM11A compounds block proNGF binding to p75$^{NTR}$, proNGF binding to p75$^{NTR}$ was assessed over a concentration range of 1500–10,000 nM. LM11A-24 and -31 inhibited proNGF binding equally, up to a ~30% decrement at the highest concentration (Fig. 9*B*).

## Discussion

Using knowledge of NGF loop 1 peptide activities (Longo et al., 1990, 1997), along with available sequence, mutation analysis,

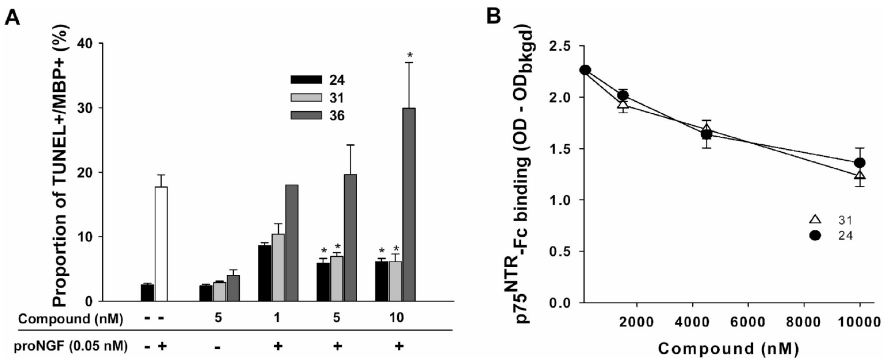


**Figure 9.** LM11A-24 and LM11A-31 do not promote death of mature oligodendrocytes and inhibits proNGF-induced death. ***A***, Mature oligodendrocytes were treated as indicated and cell death assessed by determining the proportion of MBP-positive cells that are also TUNEL positive. In the absence of proNGF, compounds did not promote cell death. In the presence of 0.05 nM proNGF, LM11A-24, and LM11A-31, but not LM11A-36, blocked cell death. The bars represent mean + SE; $n > 2$ for each condition except for 1 nM LM11A-36 with proNGF, which had a single determination. *$p < 0.05$, by Student's *t* test for comparisons with proNGF treatment without compounds. ***B***, proNGF displacement from p75$^{NTR}$ by LM11A-24 and -31. proNGF (1.8 nM) was incubated with the indicated concentrations of compounds and detected by ELISA. $n = 4$ for each condition. The symbols indicate mean ± SE. The signals from all compound-treated samples were significantly less than proNGF alone, with $p < 0.01$ by Student's *t* test.

and x-ray crystallographic structure, we developed a pharmacophoric virtual screening method that efficiently identified [four active of 23 tested compounds (17%)] several chemically diverse small molecules with potent neurotrophic activity that interact with p75$^{NTR}$. Although attempts to identify nonpeptide small molecule inhibitors of protein–protein interactions have recently been fruitful (Berg, 2003), there are relatively few reports of small molecules, interacting with receptors whose principal ligands are proteins, that achieve one or more ligand-like effects. Activators of receptors for thrombopoietin (c-Mpl) (Kimura et al., 1998), erythropoietin (Goldberg et al., 2002), insulin (Zhang et al., 1999), and the granulocyte colony stimulating factor (Tian et al., 1998) were identified by empiric, high throughput screening. In addition, Maliartchouk et al. (2000a) and Zaccaro et al. (2005) have developed low-molecular-weight TrkA agonists through progressive modification of small cyclic peptides emulating a $\beta$-turn. Virtual (also called *in silico*) screening of chemical libraries based on the structure of protein–protein interaction sites, ligand–receptor interaction sites, or a series of known small ligands is a potentially powerful approach for the identification of novel lead compounds (for review, see Kurogi and Guner, 2001; Mason et al., 2001; Guner, 2002; Singh et al., 2002; Guner et al., 2004). To date, in the area of modulation of protein–protein interactions, virtual screening has yielded only inhibitors, including compounds inhibiting the interactions of Bak with Bcl-2 (J. L. Wang et al., 2000), Bak with Bcl-x (Enyedy et al., 2001), major histocompatibility complex class II proteins with CD4 (Li et al., 1997), and very late antigen-4 with vascular cell adhesion molecule-1 (Singh et al., 2002). As in the present work, each of these screening studies was predicated on identification of an oligopeptide with the desired function. The results reported here differ from all previous studies in that we have identified compounds with agonist-like activity. Although binding anywhere within a pocket may lead to inhibition, it seems likely the structural requirements for emulating a functional protein ligand–receptor interaction will generally be more stringent.

Several lines of evidence support the hypothesis that LM11A compounds work principally through direct interactions with p75$^{NTR}$: (1) active compounds fail to elicit prosurvival activity and AKT signaling in hippocampal neurons from *p75$^{NTR-/-}$* mice; (2) active compounds appear to interact with p75$^{NTR}$, as demonstrated by their ability to displace NGF from p75$^{NTR}$-Fc but not from TrkA-Fc; and (3) Ab 9651, an antibody targeted to the neurotrophin binding domain of p75$^{NTR}$, inhibits its compound prosurvival effects and is displaced from cells by active LM11A compounds. Additional support for this contention is provided by the findings that active compounds induce rapid recruitment of IRAK to p75$^{NTR}$, that they do not activate Trks, and that at maximum doses, NGF and active compounds have no additive effects. Thus, p75$^{NTR}$ appears to be necessary for LM11A compound activity; however, it remains possible that the compounds also work in some measure through low-level or alternative modes of Trk activation, or through other mechanisms that interact with p75$^{NTR}$. Finally, it was of interest to note that although they are ostensibly related only by the property of containing a pharmacophore designed to mimic the NGF loop 1 p75$^{NTR}$ binding domain, the otherwise chemically diverse LM11A compounds have similar neurotrophic activities and potencies. This observation is also consistent with the possibility that these compounds act through p75$^{NTR}$.

The apparent monomeric nature of these compounds is consistent with our preliminary computational studies suggesting that peptide dimerization might promote the proper folding of only one of the peptide subunits. Furthermore, these findings suggest that cyclic monomeric peptides (Longo et al., 1997) may be inactive as a result of an inability to maintain a proper binding conformation. The subsequent finding that NGF may be disallowed from simultaneous interaction with two p75$^{NTR}$ molecules (He and Garcia, 2004) is consistent with this possibility as well.

The hypothesis that compound activity occurs through binding to p75$^{NTR}$ raises the question of whether that mechanism is consistent with the finding that a measure of the ligand-displacement potency, $A_2$, of LM11A-31 is nearly four orders of magnitude greater than the $EC_{50}$ for its biologic effect (compare Figs. 3*C*, 5*A*). We suggest that this finding is not unexpected, because large differences between biologic potency of small molecules and apparent binding affinity, as estimated by ligand displacement, are common (Lutz and Kenakin, 1999). Potential causes of these differences include: differences between receptor states in binding versus functional assays; postreceptor signal amplification, such that maximal biologic effects are seen at low receptor occupancies (Lutz and Kenakin, 1999); and partial displacement of a macromolecular ligand by a smaller antagonist. In Gaddum–Schild analysis, a Schild coefficient different from 1.0 suggests a noncompetitive interaction between the ligand and the antagonist; this may occur if the mechanism of antagonism is through allosteric effects, or if there are multiple ligand binding sites available on which the antagonist has differential effects. The latter situation can occur if there are multiple distinct receptors for the ligand, or, as we propose for the present case, the ligand binds at several sites on the same receptor. The current data are consistent with a model in which the compounds are relatively ineffective in displacing

NGF from p75NTR because they interfere at only one of multiple sites of interaction between the neurotrophin and receptor. This mechanism is also consistent with recently reported crystallographic structure (He and Garcia, 2004) of a complex between NGF and p75NTR, suggesting that there are at least two interacting patches, with one centered on NGF loop 1. A purely allosteric model seems less likely (although it is not ruled out), given the virtually complete reversal of the compound binding at high levels of NGF. The incomplete displacement of NGF by the compounds is similar to that observed in the analysis of small chemokine receptor antagonist binding (Sabroe et al., 2000).

The high concentrations required to inhibit proNGF binding compared with those blocking proNGF-induced death of oligodendrocytes suggest the possibilities that: (1) in the cell-based assay, with native receptor conformation in the presence of coreceptors (e.g., sortilin), the proNGF-p75NTR interaction may be more susceptible than in the *in vitro* p75NTR-Fc assay to disruption by the compounds; (2) at low concentrations, the compounds qualitatively alter proNGF binding to decrease the induction of cell death but do not decrease the total amount of proNGF binding; or (3) that the compounds induce preferential activation of prosurvival signaling by p75NTR without affecting proNGF binding. Preferential survival pathway activation by LM11A compounds could result from differences in the ways the compounds modulate receptor structure, as well as lack of binding to the sortilin coreceptor. Indeed, previous studies suggest that engagement of both sortilin and p75NTR by proNGF promotes efficient ligand binding, receptor complex activation, and apoptotic actions (Nykjaer et al., 2005). Future analyses of signaling in these cells in response to compounds in the presence or absence of proNGF may help to further elucidate mechanisms by which compounds block proNGF-induced oligodendrocyte death.

The potential for differences between activities generated by a native ligand and small molecule mimetics of a specific domain of that ligand has important therapeutic implications. By targeting only one member of a group of receptors that interact with a given ligand, we may expect to generate compounds that activate a pattern of receptor-mediated effects that may or may not occur naturally. In the present case, in targeting p75NTR, we seem not to activate Trk, but likely activate pathways involving NFκB and PI3K. Such differences in signaling patterns may eventually prove clinically useful. For example, the LM11A compounds may promote survival under conditions in which neurotrophins promote death (as in oligodendrocyte death) and may be less likely to induce excessive sympathetic fiber sprouting and upregulation of pain transmission occurring, likely via Trk signaling, with neurotrophin treatments (Bergmann et al., 1998; Walsh et al., 1999).

Future systematic studies of these compounds with respect to the structural details of the binding interface, kinetics of activation, and pathways involved should enhance our general understanding of neurotrophin action and promote further development of specific neurotrophin system modulators for therapeutic use. Recent evidence suggests that p75NTR, alone or interacting with other proteins such as proNGF, might mediate cell death in various neuropathological conditions including Alzheimer's disease, spinal cord trauma, axotomy, and retinal dystrophy (Nykjaer et al., 2005). This raises the possibility that LM11A compounds will constitute important leads for therapeutic development. In current studies in our laboratories, the structural diversity of active LM11A compounds has provided a basis for synthesizing novel compounds designed to incorporate optimal medicinal chemistry properties.

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