A p75^{NTR} and Nogo receptor complex mediates repulsive signaling by myelin-associated glycoprotein

Scott T. Wong[1], John R. Henley[1], Kevin C. Kanning[2], Kuo-hua Huang[1], Mark Bothwell[2] and Mu-ming Poo[1]

[1] *Division of Neurobiology, Department of Molecular and Cell Biology, University of California, Berkeley, California 94720, USA*

[2] *Department of Physiology and Biophysics, University of Washington, Seattle, Washington 98195, USA*

The first two authors contributed equally to this work.

Correspondence should be addressed to M.P. (mpoo@uclink.berkeley.edu)

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Myelin-associated glycoprotein (MAG), an inhibitor of axon regeneration, binds with high affinity to the Nogo-66 receptor (NgR). Here we report that the p75 neurotrophin receptor (p75^{NTR}) is a co-receptor of NgR for MAG signaling. In cultured human embryonic kidney (HEK) cells expressing NgR, p75^{NTR} was required for MAG-induced intracellular Ca^{2+} elevation. Co-immunoprecipitation showed an association of NgR with p75^{NTR} that can be disrupted by an antibody against p75^{NTR} (NGFR5), and extensive coexpression was observed in the developing rat nervous system. Furthermore, NGFR5 abolished MAG-induced repulsive turning of *Xenopus* axonal growth cones and Ca^{2+} elevation, both in neurons and in NgR/p75^{NTR}-expressing HEK cells. Thus we conclude that p75^{NTR} is a co-receptor of NgR for MAG signaling and a potential therapeutic target for promoting nerve regeneration.

Myelin of the central nervous system contains several inhibitory factors that prevent axon regeneration after injury[1–3]. Among these factors are myelin-associated glycoprotein (MAG)[4,5], Nogo-A[6,7] and oligodendrocyte myelin glycoprotein[8], all of which show high-affinity binding to the Nogo-66 receptor (NgR)[9–12]. As NgR lacks a cytoplasmic domain, MAG is thought to require a co-receptor (as yet unidentified) for transmembrane signaling after binding to NgR. A recent study[13] shows that MAG-dependent inhibition of neurite outgrowth and activation of RhoA is reduced in cultured dorsal root ganglion or cerebellar neurons from p75^{NTR} knockout mice, suggesting the involvement of p75^{NTR} in MAG signaling. As MAG did not directly bind to p75^{NTR}, it was proposed that MAG binding to the ganglioside GT1b may activate p75^{NTR}. However, the subsequent discovery of MAG binding to NgR[10,11] led to the alternative possibility that MAG may activate p75^{NTR} via its interaction with NgR. Based on our previous finding that MAG-dependent repulsion of axonal growth cones requires Ca^{2+} signaling[14], we used intracellular Ca^{2+} signals and growth cone repulsion as functional assays to examine whether p75^{NTR} mediates MAG-dependent cellular actions as a co-receptor of NgR.

In the present study, we first showed that extracellular MAG could rapidly induce a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the growth cone of cultured *Xenopus* spinal neurons. Using cultured HEK-293 cells expressing NgR and p75^{NTR}, either alone or together, we found that MAG-induced $[Ca^{2+}]_i$ elevation required the coexpression of p75^{NTR} and NgR. Co-immunoprecipitation studies showed a direct association of p75^{NTR} and NgR in membrane extracts from HEK-293 cells and E17 rat cerebella. Furthermore, this direct association between p75^{NTR} and NgR can be disrupted by an antibody[15] raised specifically against the ectodomain of p75^{NTR} (NGFR5). Immunostaining of rat embryos also revealed extensive coexpression of p75^{NTR} and NgR in the developing nervous system. The functional significance of the p75^{NTR}/NgR association was demonstrated by the finding that NGFR5, which cross-reacted with *Xenopus* p75^{NTR}, could abolish both the repulsive turning of *Xenopus* growth cones that had been induced by a gradient of MAG and by MAG-induced $[Ca^{2+}]_i$ elevation. Finally, removal of GPI-linked surface proteins, which presumably include a *Xenopus* NgR, abolished the MAG-induced $[Ca^{2+}]_i$ elevation in the growth cone. Taken together, these results identify p75^{NTR} as a co-receptor of NgR for MAG signaling in cultured *Xenopus* spinal neurons and in a heterologous expression system, and suggest that interference of p75^{NTR} signaling may be a useful therapeutic approach in reversing the growth inhibition and repulsive action of MAG and, perhaps, other myelin factors that bind to NgR.

Results

In the presence of a microscopic gradient of guidance factors, isolated growth cones of cultured *Xenopus* spinal neurons show attractive or repulsive turning responses[16]. A gradient of MAG can induce repulsive turning of the growth cone[14,17], and the response depends on the presence of extracellular Ca^{2+}. This suggests that Ca^{2+} influx or $[Ca^{2+}]_i$ elevation may mediate the MAG-induced growth inhibition or repulsive turning responses. Using

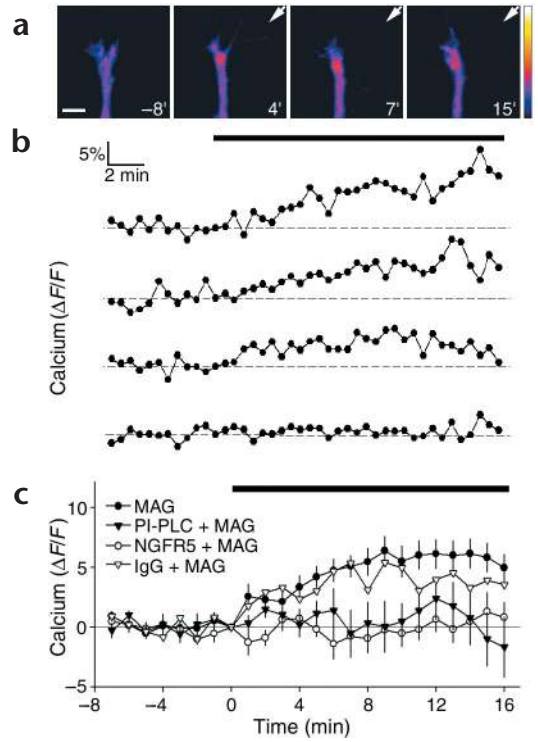

Fig. 1. Calcium signals at the growth cone induced by an extracellular gradient of MAG. (**a**) Fluorescence images of the growth cone of a cultured *Xenopus* spinal neuron, which was co-injected with the Ca^{2+}-sensitive fluorescence indicators Oregon green-BAPTA and Fura Red (see Methods). Images depict green fluorescence at different times (in min) before and after the onset of the MAG gradient. Scale bar, 10 μm. (**b**) Example traces of $[Ca^{2+}]_i$ changes that were observed at the growth cone and were induced by the presence of a MAG gradient (marked by the black bar). The fluorescence values measured at each time point were normalized to the mean value obtained during the 5-min period before the onset of the MAG gradient for each growth cone. Normalized changes in $[Ca^{2+}]_i$ levels at the growth cone are shown by percentage changes in the ratio of Oregon Green and Fura Red fluorescence ($\Delta F/F$) with time. (**c**) Summary of the MAG-induced $[Ca^{2+}]_i$ elevation in the growth cone for all data collected as in (**b** MAG alone). Data points represent mean ± s.e.m. (n = 19). Also shown are data collected from experiments in which a function-blocking antibody against $hp75^{NTR}$ (NGFR5, 5 μg/ml, n = 10) or a mouse IgG control antibody (5 μg/ml, n = 7) was added in the medium (error bars were removed for clarity), or the neurons were treated with PI-PLC (1 unit/ml, n = 12) for >30 min before Ca^{2+} imaging. The differences between baseline Ca^{2+} signals, defined as the 10-min period before MAG application, and that observed during the treatment period, defined as 5–15 min after, were statistically significant for MAG and MAG + IgG (both, $P < 0.001$), but not for MAG + NGFR5 ($P > 0.4$) or MAG + PI-PLC ($P > 0.2$), by ANOVA and Tukey's test.

fluorescence ratio imaging of Ca^{2+}-sensitive indicators (Oregon Green BAPTA–dextran and Fura Red; see Methods), we monitored $[Ca^{2+}]_i$ changes in the *Xenopus* growth cone. A rise in growth-cone $[Ca^{2+}]_i$ was observed within minutes of the onset of the MAG gradient (**Fig. 1**). This finding is consistent with the previous observations[10–12] that NogoA and MAG bind to the same receptor (NgR); the inhibition and collapse of growth cones of dorsal root ganglion neurons induced by myelin-associated inhibitor factor NI-35 (NogoA) is accompanied by a rapid rise of $[Ca^{2+}]_i$ (ref. 18). The rise in $[Ca^{2+}]_i$ after MAG binding to the cell surface suggests that Ca^{2+} signals may provide a sensitive assay for the transmembrane signaling of MAG.

As the NgR is attached to the cell surface via a glycosylphosphatidyl-inositol (GPI) linkage[12], a transmembrane co-receptor of NgR is presumably required for the MAG-NgR binding to trigger the Ca^{2+} signal. A recent report[13] shows that cerebellar and spinal sensory neurons from $p75^{NTR}$ -deficient mice are insensitive to MAG. Although this suggested that an interaction between ganglioside GT1b and the $p75^{NTR}$ complex might be sufficient to render cells responsive to MAG, the study used neurons that express NgR constitutively, leaving open the possibility that MAG exerts its activity through NgR to activate $p75^{NTR}$. To assess the role of $p75^{NTR}$ and NgR in MAG-induced transmembrane signaling, we used $[Ca^{2+}]_i$ elevation as a functional assay for the MAG response. We transfected HEK-293 cells with expression vectors for NgR and human $p75^{NTR}$ ($hp75^{NTR}$), either alone or together, and performed fluorescence ratio imaging of $[Ca^{2+}]_i$ in response to MAG. Baseline $[Ca^{2+}]_i$ was relatively similar among all groups of transfected cells. Ten minutes after bath application of MAG (1 μg/ml), cells expressing vector control, NgR or $hp75^{NTR}$ alone showed no detectable changes in $[Ca^{2+}]_i$, whereas cells co-transfected with $hp75^{NTR}$ and NgR consistently showed a marked elevation of $[Ca^{2+}]_i$. Furthermore, addition of the monoclonal antibody NGFR5 completely abolished MAG-dependent Ca^{2+} signals in co-transfected cells (**Fig. 2a** and **b**). It is unlikely that the NGFR5 effect was due to nonspecific antibody binding to the cell surface, as there was an excess of bovine IgG (19–34 μg/ml) in the standard culture medium for these cells throughout the experiments. The finding that $p75^{NTR}$ can trigger cytoplasmic Ca^{2+} signals is consistent with a previous study[19] showing that $[Ca^{2+}]_i$ rises in response to neurotrophins in fibroblasts transfected with $p75^{NTR}$.

To determine whether $p75^{NTR}$ and NgR can physically interact in the membrane, we carried out immunoprecipitation (IP) and western blotting on solubilized membrane fractions from HEK-293 cells transfected with (i) flag-tagged NgR (NgR-flag), (ii) $hp75^{NTR}$ or (iii) both. We used antibodies against the NgR-flag epitope and the $p75^{NTR}$ intracellular domain to avoid potential antibody masking of receptor domains involved in the NgR/$p75^{NTR}$ interaction. Both NgR-flag and $p75^{NTR}$ were strongly expressed in the appropriate cells but not in those transfected with vector control (**Fig. 3a**). Anti-flag or anti-$p75^{NTR}$ antibodies were used for IP of NgR or $p75^{NTR}$, respectively. Anti-flag IPs were probed for the presence of either $p75^{NTR}$ or NgR by western blotting with each respective antibody (**Fig. 3b**). We detected co-IP of $p75^{NTR}$ with NgR-flag in IPs from NgR- and $p75^{NTR}$-coexpressing cells, but not in cells expressing NgR-flag or empty vector. Conversely, NgR-flag was detected in $p75^{NTR}$ IPs from HEK-293 cells in which both NgR and $p75^{NTR}$ were coexpressed. Thus NgR and $p75^{NTR}$ can physically associate with each other. The addition of NGFR5 was able to disrupt the MAG-induced rise in $[Ca^{2+}]_i$ (**Fig. 2a** and **b**), indicating that this antibody binding interferes with the association of the two receptors. Indeed, increasing NGFR5 concentrations reduced the NgR/$p75^{NTR}$ association (**Fig. 3c**), whereas mouse IgG (100 μg/ml) had no effect (**Fig. 3c**, last lane). These results support the idea that the extracellular domains of NgR and $p75^{NTR}$ interact to mediate MAG signaling.

Is there a similar interaction between NgR and $p75^{NTR}$ *in vivo*? Co-IP experiments on freshly dissected cerebella from rat pups (embryonic day E17) showed that $p75^{NTR}$ and NgR indeed associate at endogenous expression levels for these two receptors (**Fig. 4a** and **b**). We further examined, by immunohistochemical

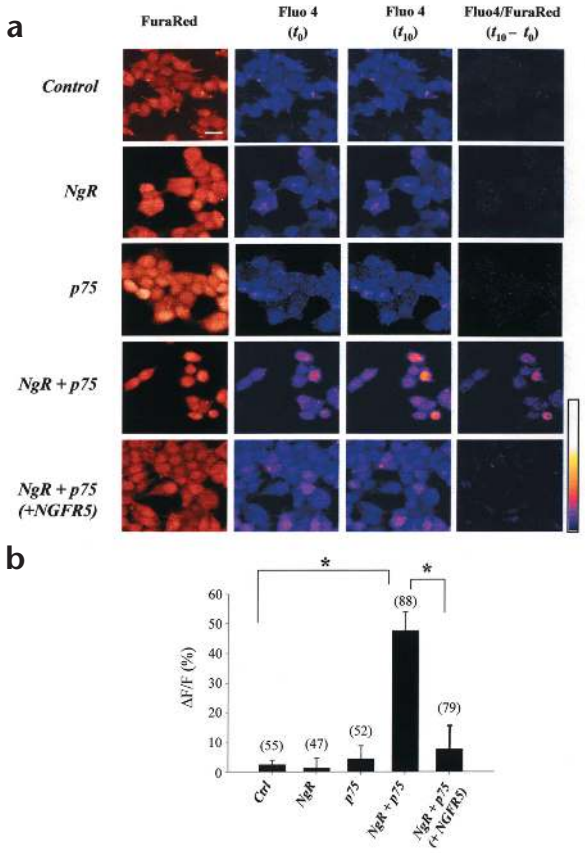

Fig. 2. The p75^{NTR} mediates MAG-dependent Ca^{2+} signaling in HEK-293 cells. (**a**) Fluorescence imaging of $[Ca^{2+}]_i$ in transfected HEK-293 cells in the absence and presence of MAG. Left, Fura Red fluorescence. Middle two panels, Fluo-4 fluorescence before (t_0) and 10 min after (t_{10}) bath application of MAG (1 μg/ml), respectively. Right, difference of the fluorescence ratios (Fluo-4/Fura Red) at t_{10} and t_0. Control, HEK-293 cells transfected with empty expression vector. (**b**) Summary of percentage changes in $[Ca^{2+}]_i$. The mean $[Ca^{2+}]_i$ levels over each cell was measured by the ratio Fluo-4/Fura Red, and the percentage change at t_{10} relative to t_0 was calculated for each cell. Data represent mean ± s.e.m. The number above each bar is the total number of cells measured from at least five separate cultures. *$P < 0.001$, *t*-test.

staining, whether p75^{NTR} and NgR are coexpressed in neurons that are known to be responsive to MAG. In the rat nervous system (E14.5), p75^{NTR} is widely expressed in neuronal and non-neuronal tissues. However, colocalization with NgR was found to be exclusively neuronal, including the soma of dorsal root ganglia, their projections to the dorsal horn of the spinal cord (**Fig. 4c–e**) and spinal cord motor neurons (**Fig. 4f–h**). These results are consistent with the reports that embryonic spinal and sensory neurons are responsive to MAG[20].

As the NGFR5 antibody abolished MAG-induced Ca^{2+} signaling in HEK-293 cells transfected with both NgR and hp75^{NTR}, we further examined the effect of NGFR5 on the MAG-induced Ca^{2+} signaling and repulsive turning of *Xenopus* growth cones. First, we determined whether the anti-hp75^{NTR} antibody NGFR5 cross-reacts with *Xenopus* p75^{NTR} (xp75^{NTR}). We transfected HEK-293 cells with xp75^{NTR}, hp75^{NTR} or trkA and tested whether NGFR5 could recognize the xp75^{NTR} in membrane fractions from these cells by western blotting. Indeed, we found that NGFR5 identified both xp75^{NTR} and hp75^{NTR} in the latter membranes, but not in membranes from cells transfected with vector control or trkA (**Fig. 5a**). Recognition of xp75^{NTR} by NGFR5 is further supported by the finding that NGFR5 strongly immunostained the surface of unpermeablized HEK-293 cells expressing xp75^{NTR}, and that this staining was abolished by the ectodomain peptide of hp75^{NTR} (**Fig. 5b**). Having established the cross-reactivity of NGFR5 with xp75^{NTR}, we further examined whether xp75^{NTR} is expressed in the *Xenopus* growth cones at the same developmental stage at which we performed our Ca^{2+} imaging experiments (**Fig. 1**). NGFR5 immunostaining of *Xenopus* spinal neurons revealed a high level of xp75^{NTR} expression in the growth cone (**Fig. 5c**).

Furthermore, we found that NGFR5 binding to *Xenopus* spinal neurons could disrupt MAG signaling in the growth cones of these neurons. Bath addition of NGFR5 completely abolished MAG-induced elevation of $[Ca^{2+}]_i$ in these growth cones, whereas similar treatment with a nonspecific control IgG (5 μg/ml) had no significant effect (**Fig. 1c**). Although we have shown that *Xenopus* neurons express a p75^{NTR}, it remains unclear whether

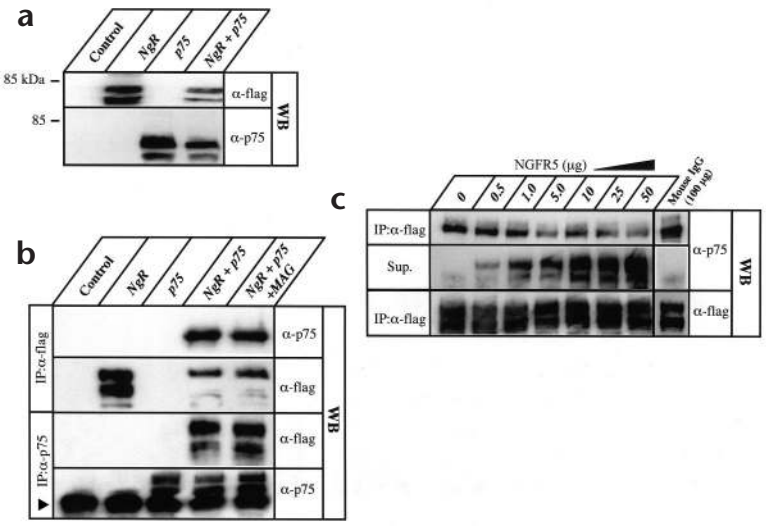

Fig. 3. Association of NgR with hp75^{NTR}. (**a**) Western analysis of total cell lysates from HEK-293 cells transfected with empty vector, NgR-flag, hp75^{NTR} or NgR-flag + hp75^{NTR}. Immunoblots (WB) were made with M2 mAb to flag (upper) or anti-p75^{NTR} antibody raised against cytoplasmic domain of p75^{NTR} (lower). (**b**) Co-immunoprecipitation (IP) with p75^{NTR} and flag antibodies on membrane preparations made from HEK-293 cells transfected with empty vector, NgR-flag, hp75^{NTR} or NgR-flag + hp75^{NTR} and immunoblotted with either hp75^{NTR} or flag antibodies. Arrowhead, IgG heavy chain. (**c**) NGFR5 pretreatment can disrupt the NgR and p75^{NTR} association. HEK-293 cells co-transfected with both NgR-flag and human p75^{NTR} were incubated for 1 h with indicated concentrations of NGFR5 or a mouse IgG control antibody (far right lane). Subsequent immunoprecipitation with immobilized, flag-antibody Sepharose revealed a decreasing association of p75^{NTR} with NgR with increasing antibody concentration (top row), whereas there was no significant change in the amount of NgR-flag that was bound to the resin (bottom row). The amount of free p75^{NTR} increased with higher NGFR5 concentrations in the IP supernatants (middle row). The depicted blots are representative of at least three independent experiments for each study.

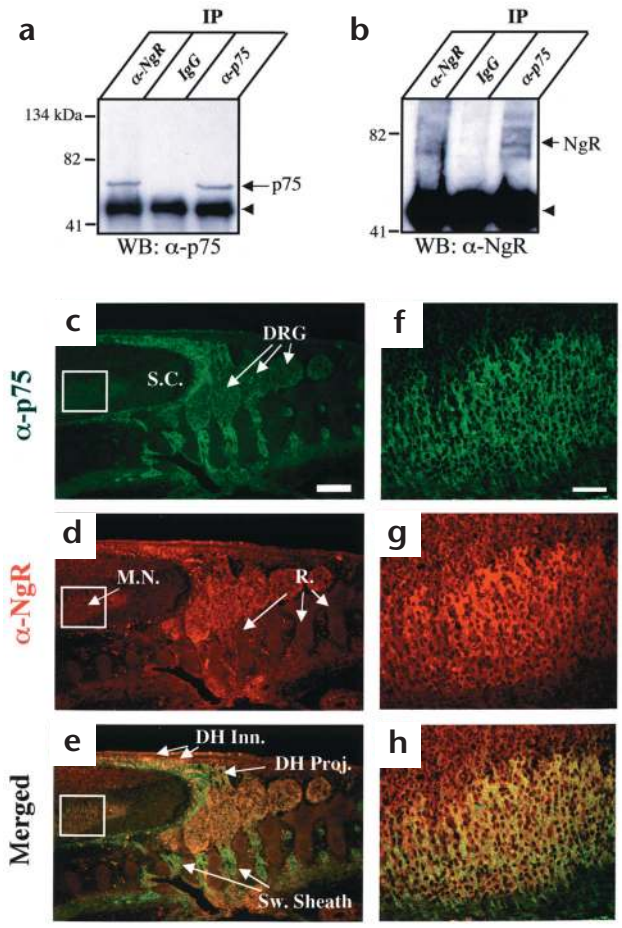

Fig. 4. Nogo Receptor and $p75^{NTR}$ are coexpressed in the developing nervous system. (**a**, **b**) Co-immunoprecipitation of $p75^{NTR}$ and NgR in E17 rat cerebellum. Using a specific antibody against the NgR, $p75^{NTR}$ is co-immunoprecipitated with the NgR (**a**, lane 1), but not with a mouse IgG control (**a**, lane 2). Immunoprecipitation with a $p75^{NTR}$ antibody shows the reciprocal interaction with NgR (**b,** lane 3), suggesting that the two proteins interact *in vivo*. Cerebellar extracts were immunoprecipitated with the denoted antibodies (marked above lanes) and then western blotted (WB) with the indicated antibodies. Arrowhead, IgG heavy chain. (**c**–**e**) Immunohistochemistry of a sagittal section of an E14.5 rat embryo shows that the expression of $p75^{NTR}$ (**c**) and Nogo R (**d**) is colocalized in motor neurons of the spinal cord (SC) and dorsal root ganglia (DRG). Note that $p75^{NTR}$ is expressed independently of NgR in non-neural cells such as Schwann cells surrounding sensory axon tracts (Sw. sheath), but colocalizes extensively in neurons (**e**). Scale bar, 250 μm; dorsal is up, anterior is left. (**f**–**h**) Confocal images of spinal motor neurons (boxed areas in **c**–**e**) are shown at a higher magnification. Scale bar, 50 μm. A clear overlap between $p75^{NTR}$ (**f**) and NgR (**g**) shown in the merged image (**h**). MN, motor neurons; R, developing ribs; DH inn., axons from the DRG innervating the dorsal horn; DH Proj., projection from DRG into dorsal horn of spinal cord.

an endogenous NgR ortholog is present in these cells. However, as it is well characterized that these neurons are sensitive to MAG[14,16,21] and that in mammals MAG interacts with high affinity to the NgR[10,11] to exert its effects on $p75^{NTR}$, it is likely that *Xenopus* neurons express a NgR or NgR-like homolog. Consistent with the fact that NgR is a GPI-linked protein, we found that treatment of the *Xenopus* neurons with the phosphoinositide-specific phospholipase C (PI-PLC), an enzyme that releases GPI-anchored proteins from the cell surface, abolished MAG-induced $[Ca^{2+}]_i$ elevation (**Fig. 1c**). Taken together, these results strongly support the idea that $p75^{NTR}$ and a GPI-linked surface component, presumably *Xenopus* NgR, are essential components for MAG-induced Ca^{2+} signaling in neurons.

To determine whether $p75^{NTR}$-dependent MAG signaling is functionally important in axonal growth regulation, we examine the role of $p75^{NTR}$ in the growth cone turning response induced by an extracellular gradient of MAG. In the standard turning assay[22], a gradient of MAG was established across the growth cone by pulsatile application of picoliters of concentrated solution of MAG (100 μg/ml) from a micropipette, which was positioned at a distance of 65 μm from the center of the growth cone, at an angle of 45° with respect to the original direction of growth cone extension. Within one hour after the onset of the gradient, a great majority of *Xenopus* spinal neurons showed repulsive turning of the growth cone (**Fig. 5d**), and no consistent turning was observed when MAG was omitted in the pipette solution (**Fig. 5e**). However, addition of NGFR5 (5 μg/ml) in the bath abolished the repulsive turning induced by the MAG gradient (**Fig. 5f**), whereas addition of control mouse IgG (5 μg/ml) had no effect. The average turning angle in the MAG gradient was significantly negative, and was reduced to near zero by the presence of NGFR5. The average neurite extension did not differ significantly under the various conditions (**Fig. 5g** and **h**). These results showed that interfering with the interaction between NgR and $p75^{NTR}$ by specific binding of NGFR5 to $p75^{NTR}$ (**Fig. 3c**) indeed blocked MAG-induced repulsive growth cone turning.

Discussion

The $p75^{NTR}$ is a membrane protein that signals in response to the binding of proneurotrophins or to a lower-affinity binding of mature neurotrophins[23–25]. It may convey transmembrane signals either independently or as a co-receptor of the Trk family of tyrosine receptor kinases, which are preferentially activated by mature neurotrophins[26]. There is growing evidence that $p75^{NTR}$ has a key role in axon guidance during the development of the nervous system[25,26]. Axon pathfinding errors of $p75^{NTR}$-expressing neurons are prominent among the phenotypes observed in *$p75^{NTR}$* mutant mice, including mistargeting of sympathetic and cortical subplate axons[27,28]. Absence of the $p75^{NTR}$ alters the pattern of sympathosensory sprouting in the trigeminal ganglia of mice overexpressing nerve growth factor[29]. It remains to be determined whether these $p75^{NTR}$-mediated behaviors are predominantly regulated by neurotrophins or by myelin-associated factors such as MAG and NogoA.

The co-distribution of immunoreactivity for $p75^{NTR}$ and NgR in motor neurons of ventral embryonic rat spinal cord and in primary sensory neurons of dorsal root ganglia suggests that functional $p75^{NTR}$/NgR complexes are likely to be present in these embryonic neurons. The presence of $p75^{NTR}$ in these neuronal populations throughout embryonic development is consistent with previous reports (see ref. 30). Although expression of NgR in the rodent nervous system has mainly been found in neurons of adult animals[31], the presence of functional NgR in embryonic rodent spinal cord neurons and dorsal root ganglion neurons is indicated by reports that embryonic spinal neurons[32] and newborn dorsal root ganglion neurons[5] are responsive to MAG. Neurite outgrowth of these embryonic neurons is stimulated by MAG, which has an inhibitory effect on mature neurons. We posit, however, that participation of a distinct receptor system need not be

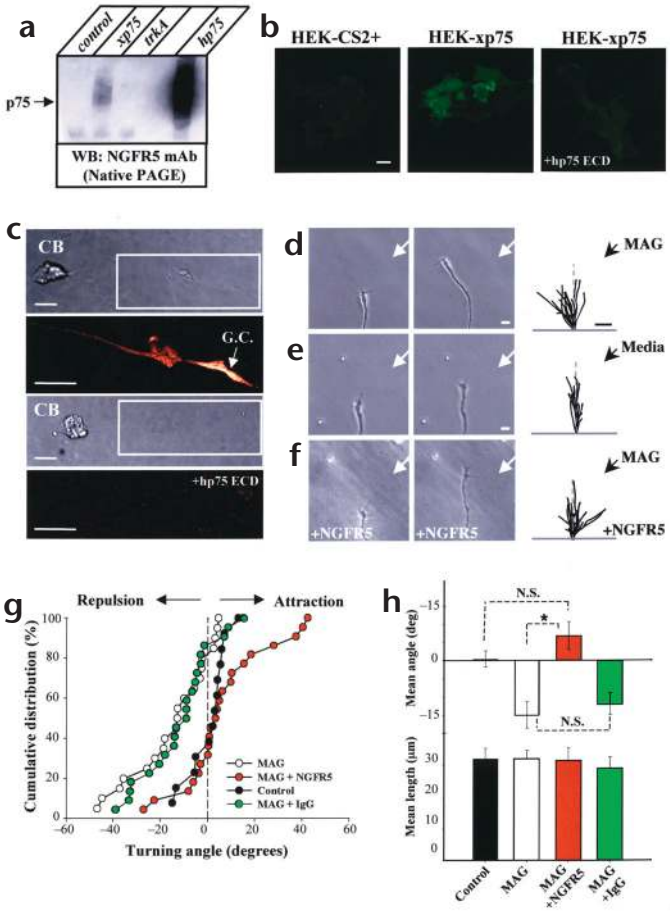

Fig. 5. MAG-induced growth cone repulsion is abolished by the p75^{NTR} antibody NGFR5. (**a**–**c**) The NGFR5 recognizes xp75^{NTR}. (**a**) Native gel electrophoresis shows that *Xenopus* p75^{NTR} (xp75) and human p75^{NTR} (hp75) were recognized by NGFR5 in membrane preparations derived from HEK cells expressing each ortholog of p75^{NTR}, whereas membranes from control or trkA-expressing cells were not recognized. (**b**) HEK cells expressing xp75^{NTR} were specifically immunostained with the NGFR5 antibody (middle), whereas vector control cells (CS2+) were not (left). Preincubation with a hp75^{NTR} ectodomain fragment (hp75 ECD, 10 μg/ml) effectively neutralized the immunoreactivity (right). Scale bar, 20 μm. (**c**) NGFR5 recognized p75^{NTR} in *Xenopus* spinal neurons. A representative *Xenopus* spinal neuron (top two panels) was immunostained with NGFR5 and analyzed for p75^{NTR} immunoreactivity. White box indicates the area magnified (×2) in the panel directly below to show the growth cone (GC) fluorescence. Preincubation of the antibody with the hp75^{NTR} ectodomain fragment neutralized the staining in these neurons (lower pair), suggesting a specific interaction of NGFR5 with xp75^{NTR}. Scale bars, 20 μm; CB, cell body. (**d**) Images of the growth cone of a cultured *Xenopus* spinal neuron at the onset (left) and the end (right) of 1-h exposure to a MAG gradient (marked by the arrow). Superimposed traces depict samples of the trajectory of neurite extension during the 1-h period. The origin was the center of the growth cone at the onset of the assay and the original direction of extension was aligned vertically. Scale bars, 10 μm. (**e**) Similar to (**d**), except that MAG was omitted from the pipette solution. (**f**) Similar to (**e**), except that the NGFR5 mAb (5 μg/ml) was added in the culture before the MAG gradient was applied. (**g**) The distribution of turning angles is shown in a cumulative percentage plot. For each experimental condition, angular positions of all growth cones at the end of 1-h exposure to the MAG gradient are plotted. The data points represent the percentage of growth cones with turning angles ≤ a given angular value. Data are from all experiments similar to those illustrated in (**d**–**f**) and from control experiments in which mouse IgG (5 μg/ml) was added in the culture instead of NGFR5. The differences in the distribution of turning angles between data for the MAG gradient in the presence or absence of the NGFR5 were statistically significant ($P < 0.01$; Kolmogorov-Smirnov test), whereas the differences in the distribution between the NGFR5 antibody treatment and the media control were not significant (n.s., $P > 0.5$). (**h**) Summary of average turning angles and neurite extension for the same data set as that in (**g**).

invoked because elevated cAMP levels can switch repulsive/inhibitory responses to attractive/stimulatory responses induced by MAG[14,33].

Studies of *Xenopus* spinal neurons in culture[14,16] show that an extracellular gradient of MAG can trigger either attractive or repulsive turning of the growth cone, depending on the cytoplasmic level of cAMP, with high levels of cAMP favoring attraction and low levels favoring repulsion. Growth attraction or repulsion usually reflects an asymmetric filopodial extension or retraction at the growth cone, respectively, so it may depend on mechanisms that are similar to those that regulate growth stimulation or inhibition. Indeed, elevation of cAMP can also overcome neurite growth inhibition by myelin *in vitro*[33] and promote axon regeneration *in vivo*[34,35]. Similar cAMP-dependent conversion of turning responses[21,36] and reversal of growth inhibition[37] were also observed in cultured neurons in response to neurotrophins. It is possible that the repulsive/inhibitory action of both MAG and neurotrophins is mediated through p75^{NTR}, and the efficacy of p75^{NTR} signaling is regulated by cAMP or its downstream effectors.

Many adaptor proteins that bind to the cytoplasmic domain of p75^{NTR} have been identified[25], including RhoA, a Rho-family GTPase known to regulate cytoskeletal structures[38] and to mediate neurite growth inhibition and growth cone collapse[39–43]. Inactivation of the Rho signaling pathway apparently promotes CNS axon regeneration[40]. As neurotrophin binding to p75^{NTR} can also inactivate RhoA and promote axonal outgrowth[43], p75^{NTR} may serve as a bi-directional switch in setting the growth cone behavior in response to external stimuli, depending on its downstream actions on Rho GTPases. For example, MAG-NgR interaction with p75^{NTR} may activate RhoA, leading to growth inhibition/repulsion, whereas neurotrophin-trk binding to p75^{NTR} may result in inactivation of RhoA and growth stimulation/attraction. Although it remains unclear how cytosolic Ca^{2+} participates in MAG signal transduction, the present study has defined a receptor complex required for MAG-induced Ca^{2+} signaling and repulsive growth cone turning, which is likely to involve RhoA-dependent cytoskeletal rearrangements[13,43]. As a co-receptor of NgR, p75^{NTR} now emerges as a key player not only for regulating neuronal development[26] and apoptosis[44], but also for regulating the inhibition of axon regeneration induced by myelin-associated factors.

Methods

Culture preparations. Cultures of *Xenopus* spinal neurons were prepared from the neural tube tissue of 1-d old *Xenopus* embryos by methods previously described[45,46] and used for experiments between 14–20 h after plating at room temperature (20–22°C). All animals were used according to approved protocols (Animal Care and Use Committee, UC Berkeley). The culture medium consisted of 86% (v/v) Leibovitz medium (GIBCO, Gaithersburg, Maryland) containing 4 mM Hepes, 0.8% (v/v)

fetal bovine serum (HyClone, Logan, Utah) and 13.2% (v/v) saline solution (10 mM D-glucose, 5 mM Sodium pyruvate, 1.26 mM $CaCl_2$, 1.34 mM Na_2HPO_4, 0.44 mM NaH_2PO_4 and 4 mM HEPES; pH 7.5). The experiments on *Xenopus* neurons were carried out in serum-free culture medium. The HEK cell cultures were maintained in D-MEM (Gibco) with 10% FBS. MAG-Fc was purchased from R&D Systems (Minneapolis, Minnesota). The human $p75^{NTR}$ ectodomain (p75 ECD) was isolated from A875 cell conditioned media and purified by affinity chromatography over NGFR5 mAb-Sepharose. Bound ectodomain was eluted with glycine (pH 2.2) and quickly neutralized in Tris-HCl (pH 7.4). The concentration of purified $p75^{NTR}$ ECD was determined using the Bradford Protein Assay (Pierce Biotechnology, Rockford, Illinois). The integrity of $p75^{NTR}$ ECD was confirmed by western blot using NGFR5 and visualized by ECL as a single band.

Calcium imaging. For cultured *Xenopus* spinal neurons, the cells were microinjected with Oregon green 488 BAPTA-1 conjugated to dextran, as well as with Fura Red (Molecular Probes, Eugene, Oregon). Calcium imaging was carried out using a Leica confocal imaging system (TCS SP) by methods described previously[47,48]. Excitation was at 488 nm, and the Oregon Green BAPTA–dextran and Fura Red emission signals were collected at 500–560 nm and 605–700 nm, respectively. Fluorescence and transmission images (128 × 128 pixels; voxel size, 207 nm) were collected simultaneously every 30 s and analyzed using Image J software (National Institutes of Health; http://rsb.info.nih.gov/ij/). The mean fluorescence intensity was measured over a fixed square area that covered the entire growth cone throughout the measurement period. Ratios of the normalized Oregon Green and Fura Red fluorescence data were used to control for fluctuations in growth cone thickness or changes in focal plane that may have occurred during experiments. HEK-293 cells were transfected with NgR, $p75^{NTR}$ or both with lipofectamine 2,000 (Invitrogen Life Technologies, Carlsbad, California) according to the supplier's recommendations and plated onto imaging dishes (Mattek, Ashland, Massachusetts). The transfected cells were loaded with the fluorescence Ca^{2+} indicators Fluo4 and Fura Red. Images were collected for 15 min before and 25 min after the addition of MAG (1 μg/ml) at 30-s intervals and analyzed by ratio imaging as described above for the *Xenopus* growth cone. A function-blocking monoclonal antibody against the ectodomain of $p75^{NTR}$ (NGFR5, Lab Vision, Fremont, California) was added to the bath where indicated.

Immunoprecipitation (IP) and western blot analysis. HEK-293 cells, transfected with $p75^{NTR}$, NgR-flag or both, were harvested in ice-cold homogenization buffer (50 mM Tris, 150 mM NaCl, 5% sucrose; pH 7.6). Cells were homogenized, and the lysates were cleared of nuclei and unbroken cells by centrifugation for 10 min at 500*g*. The supernatants were centrifuged at 100,000*g* for 25 min, and pelleted membranes were solubilized in RIPA buffer (Amersham Biosciences, Piscataway, New Jersey). Protein A Sepharose (Amersham) was preincubated with a polyclonal $p75^{NTR}$ antibody (PAS-75, Promega, Madison, Wisconsin). The clarified RIPA extracts were then subjected to IP for 2 h at 4°C with either PAS-75 or agarose covalently conjugated to the M2 monoclonal flag epitope antibody (Sigma, St. Louis, Missouri). The pellets were washed twice with ice-cold RIPA, extracted with boiling Laemmeli buffer, and then separated over a 7.5% SDS-PAGE gel. Proteins were transferred to PVDF (Millipore, Bedford, Massachusetts), and the presence of $p75^{NTR}$ or NgR was examined by western blotting by ECL (Amersham) according to the manufacturer's recommendation. Native PAGE was carried out as above except that SDS and DTT were omitted from the sample and running buffers. *Xenopus* $p75^{NTR}$ in CS2+ has been described previously[49].

For *in vivo* IP experiments, cerebella from eight E17 rat pup brains were dissected. Membranes were prepared as described for HEK-293 cells. Specific antibodies against NgR (Alpha Diagnostics International, San Antonio, Texas) and $p75^{NTR}$ (Promega) were used to perform IP and western blots on this preparation. For the NGFR5 titration experiments, the denoted concentrations of NGFR5 or a control mouse IgG antibody were incubated with HEK cells transfected with both NgR-flag and $hp75^{NTR}$ for 1 h in HEK-293 cell culture media. Cells were washed twice with ice-cold PBS and then prepared and analyzed as described above. Supernatant fractions were normalized for protein and analyzed directly by western blot for $p75^{NTR}$.

Immunohistochemistry. Bisected rat embryos were immersion-fixed in 10% formalin, processed through a 30% sucrose gradient, embedded in OCT, and cryostat-sectioned at 14 μM. Sections were blocked in 5% milk/TBS/0.2% triton for 1 h and immunostained with 2.5 μg/ml IgG192 (mouse anti-rat $p75^{NTR}$) and 2.5 μg/mL rabbit anti human NgR (Alpha Diagnostics) at room temperature for 3 h. After extensive washing in TBS, primary antibodies were detected using Alexa 488 goat anti-mouse (rat pre-adsorbed, Molecular Probes) diluted 1:750, and CY3 donkey anti-rabbit (Jackson ImmunoResearch, West Grove, Pennsylvania) diluted 1:500.

Immunocytochemistry. Isolated *Xenopus* spinal neurons from stage 22 embryos growing in culture for 14 h or HEK-293 cells transfected with CS2+ or $xp75^{NTR}$ were fixed for 20 min with serum-free culture medium containing 2% (v:v) formaldehyde and 4% (wt:v) sucrose. Cells were then rinsed and incubated with serum-free culture medium containing 5 μg/ml NGFR5 mAb to $hp75^{NTR}$ alone or after pre-incubating with 10 μg/ml p75 ectodomain. Staining was detected using a goat anti-mouse antibody coupled to Alexa 488 (2 μg/ml, Molecular Probes). Cells were then mounted in ProLong antifade reagent (Molecular Probes) and subjected to confocal microscopy.

Growth cone turning assay. Microscopic gradients of MAG were produced by methods previously described[22,50]. Repetitive ejection of picoliters of solution containing MAG (100 μg/ml) was made by a micropipette (tip opening ~1 μm). A standard pressure pulse (3 psi, duration 20 ms) was applied to the pipette at 2 Hz with a pulse generator (SD9, Grass Instruments, Quincy, Massachusetts). Phase-contrast microscopic images of neurites were recorded with a CCD camera (Toshiba IK-541RA). For assaying growth cone turning, the tip of the micropipette was placed 65 μm away from the center of the growth cone and at an angle of 45° with respect to the direction of initial direction of neurite extension, which was determined by the last 10-μm segment of the neurite. The entire trajectory of the neurite at the end of the 1-h period was measured with a digitizer. The turning angle was defined by the angle between the original direction of neurite extension and a straight line connecting the positions of the center of the growth cone at the beginning and the end of assay. Only those growth cones with extensions that were >5 μm over the 1-h period were used for analysis.

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Competing interests statement

The authors declare that they have no competing financial interests.

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