The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI

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Published online 14 April 2003; doi:10.1038/nn1045

The neurotrophin receptor p75^{NTR} is involved in the regulation of axonal elongation by neurotrophins as well as several myelin components, including Nogo, myelin-associated glycoprotein (MAG) and myelin oligodendrocyte glycoprotein (OMgp). Neurotrophins stimulate neurite outgrowth by inhibiting Rho activity, whereas myelin-derived proteins activate RhoA and thereby inhibit growth. Here we show that direct interaction of the Rho GDP dissociation inhibitor (Rho-GDI) with p75^{NTR} initiates the activation of RhoA, and this interaction between p75^{NTR} and Rho-GDI is strengthened by MAG or Nogo. We also found that p75^{NTR} facilitates the release of prenylated RhoA from Rho-GDI. The peptide ligand that is associated with the fifth α helix of p75^{NTR} inhibits the interaction between Rho-GDI and p75^{NTR}, thus silencing the action mediated by p75^{NTR}. This peptide has potential as a therapeutic agent against the inhibitory cues that block regeneration in the central nervous system.

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The neurotrophin receptor p75 (p75^{NTR}) mediates a strikingly diverse set of biological effects[1], including cell death, Schwann cell migration, modulation of synaptic transmission and functional regulation of sensory neurons and calcium currents. Recent work also implicates p75^{NTR} in the regulation of axon elongation. Nerve growth factor (NGF) stimulates neurite outgrowth from embryonic rat hippocampal neurons and chick ciliary neurons[2], both of which express p75^{NTR} as the sole NGF receptor. These effects can be accounted for by p75^{NTR}-mediated modulation of Rho activity[2]. Rho is a small GTPase that regulates the state of actin polymerization. In its active GTP-bound form, Rho rigidifies the actin cytoskeleton, thereby inhibiting axonal elongation and causing growth cone collapse[3,4]. Neurotrophin binding to p75^{NTR} inactivates RhoA in HN10e cells as well as cerebellar neurons, whereas the overexpression of RhoA in transfected 293 cells results in the activation of RhoA, suggesting that p75^{NTR} elicits bi-directional signals[2]. Indeed, myelin-associated glycoprotein (MAG), a glycoprotein derived from myelin, activates RhoA by a p75^{NTR}-dependent mechanism, thus inhibiting neurite outgrowth from postnatal sensory neurons and cerebellar neurons[5]. Furthermore, Nogo and OMgp, the other myelin-derived inhibitors of the neurite outgrowth, act on neurons via p75^{NTR} (ref. 6). p75^{NTR} in complex with the Nogo receptor is suggested to form a receptor for all the myelin-derived inhibitors found so far[6,7]. However, the precise mechanism underlying the regulation of Rho activity by p75^{NTR} remains to be elucidated.

RhoA interacts with p75^{NTR}, as shown by the yeast two-hybrid system and co-immunoprecipitation[2]. The wild-type form of RhoA, which is predominantly in a GDP-bound form, interacts with p75^{NTR}, whereas the constitutively active form of RhoA does not. This suggested that the activation of RhoA depends on a direct interaction of RhoA and p75^{NTR}. Rho proteins in the GDP-bound form interact with Rho-GDI, which is involved in inhibiting nucleotide dissociation as well as the shuttling of Rho proteins between the cytoplasm and membranes[8]. Rho-GDI prevents Rho-family proteins from being converted to the active, GTP-bound form that is translocated to the membrane. In addition, after the active form of Rho proteins are converted to the inactive form at the membrane, Rho-GDI forms a complex with Rho and translocates it to the cytosol. The Rho-GDI family comprises at least three isoforms: Rho-GDIα, β and γ. Rho-GDIα is ubiquitously expressed and binds to all of the Rho family proteins thus far examined, whereas Rho-GDIβ and γ show unique tissue expression patterns, and their substrate specificities have not been exactly determined.

Here we report the precise mechanism of the regulation of Rho activity by p75^{NTR}. p75^{NTR} displaced the GDP-bound form of RhoA from Rho-GDIα. A peptide (referred to here as Pep5) that specifically associates with p75^{NTR} efficiently inhibited the signal mediated by p75^{NTR}, and might be a useful therapeutic agent in reversing the growth inhibition elicited by myelin-derived inhibitors.

Results

p75^{NTR} associates with Rho-GDI

We first asked whether the complex of RhoA and Rho-GDI associates with the intracellular domain of p75^{NTR}. 293T cells, which express Rho-GDI but not p75^{NTR} endogenously, were transfected with FLAG-tagged p75^{NTR} and HA-tagged wild-type RhoA. In the p75^{NTR} precipitates, the anti-Rho-GDI antibody revealed

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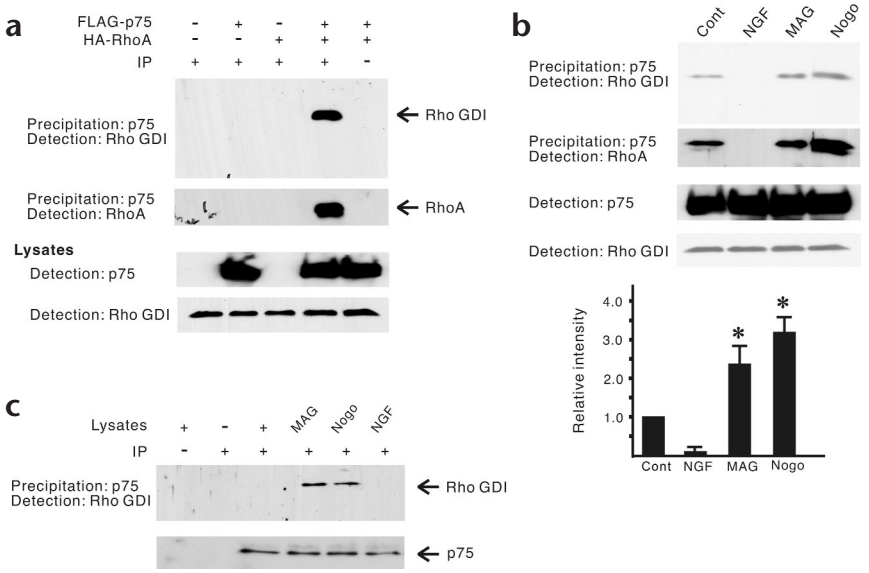

Fig. 1. Co-immunoprecipitation of p75^{NTR} with Rho-GDI. (**a**) Co-immunoprecipitation of p75^{NTR} with Rho-GDI or RhoA using lysates prepared from the transfected 293T cells. In the p75^{NTR} immunoprecipitates, the anti-Rho-GDI antibody revealed the presence of a protein corresponding to Rho-GDI. (**b**) The effects of MAG and Nogo on the interaction of p75^{NTR} with Rho-GDI or RhoA in the transfected N1E-115 cells. Data are mean ± s.e.m. (*$P < 0.01$; Student's *t*-test). (**c**) Co-immunoprecipitation of p75^{NTR} and Rho-GDI using lysates prepared from cerebellar neurons. Association was observed in MAG- and Nogo-treated cells.

the presence of a protein corresponding to Rho-GDI (**Fig. 1a**). As previously shown[2], RhoA was included in the complex. We next examined whether the interaction was strengthened by MAG or Nogo, which have been shown to activate RhoA through a p75^{NTR}-dependent mechanism. N1E-115 cells, which express the Nogo receptor endogenously (data not shown), were transfected with FLAG-tagged p75^{NTR}. The peptide corresponding to residues 31–55 of the extracellular fragment of Nogo (4 μM) (ref. 9) and soluble MAG-Fc (25 μg/ml) significantly enhanced the interaction of p75^{NTR} with Rho-GDI as well as RhoA (**Fig. 1b**). In contrast, NGF (100 ng/ml), which inactivates RhoA by p75^{NTR}, abolished the interaction of p75^{NTR} with Rho-GDI as well as RhoA. We previously noted that the interaction of endogenous p75^{NTR} with RhoA could not be observed in neurons[2]. Therefore, we examined the interaction of endogenous p75^{NTR} with Rho-GDI or RhoA using lysates prepared from cerebellar neurons from mice at postnatal day 9 (P9). An association of endogenous p75^{NTR} with RhoA and Rho-GDI was observed only after the stimulation with MAG or Nogo (**Fig. 1c**), suggesting that p75^{NTR} may not be a constitutive activator of RhoA in the cells expressing endogenous p75^{NTR}. These findings show that Rho-GDI in complex with RhoA interacts with p75^{NTR}, and that the interaction is strengthened by MAG and Nogo.

Direct interaction of p75^{NTR} with Rho-GDI

When RhoA was isolated as a p75^{NTR}-interacting protein by yeast two-hybrid screening, RhoA was suggested to bind directly to p75^{NTR} (ref. 2). However, the fact that endogenous Rho-GDI in yeast is active on mammalian Rho family members[10] leaves open an alternative possibility that RhoA in complex with yeast Rho-GDI may be associated with p75^{NTR} in the yeast. Therefore, we next examined the direct physical interaction of

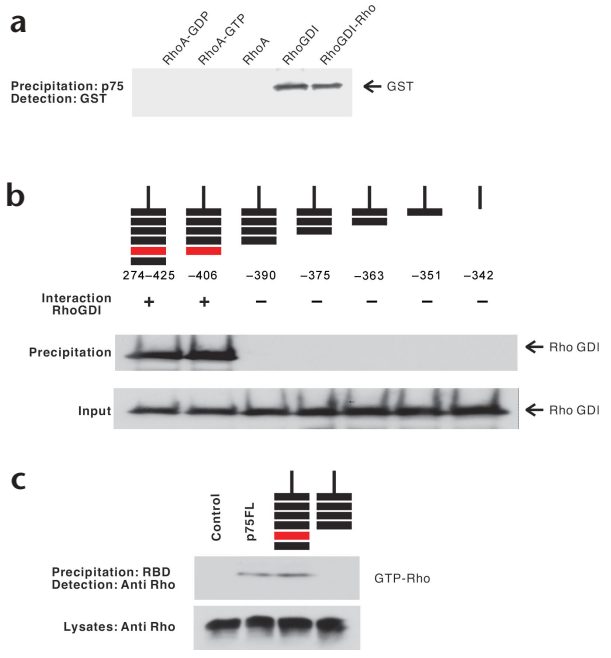

Fig. 2. p75^{NTR} directly associates with Rho-GDI. (**a**) Co-precipitation of p75^{NTR} with recombinant GST–Rho-GDI or GST–RhoA. Association was examined by western blot analysis of the precipitates produced with the purified p75^{NTR} and protein A sepharose. The anti-GST antibody revealed the presence of a Rho-GDI in the complex. (**b**) Co-precipitation of Rho-GDI with the deletion mutants of p75^{NTR}. A schematic representation of the constructs for the deleted mutants is shown. The indicated numbers correspond to residues of the mutants. (**c**) Affinity precipitation of RhoA in the transfected 293T cells. Overexpression of full-length p75^{NTR} or p75^{NTR} ICD elicits activation of RhoA, whereas the mutated p75^{NTR} that lacks the fifth helix fails to activate RhoA.

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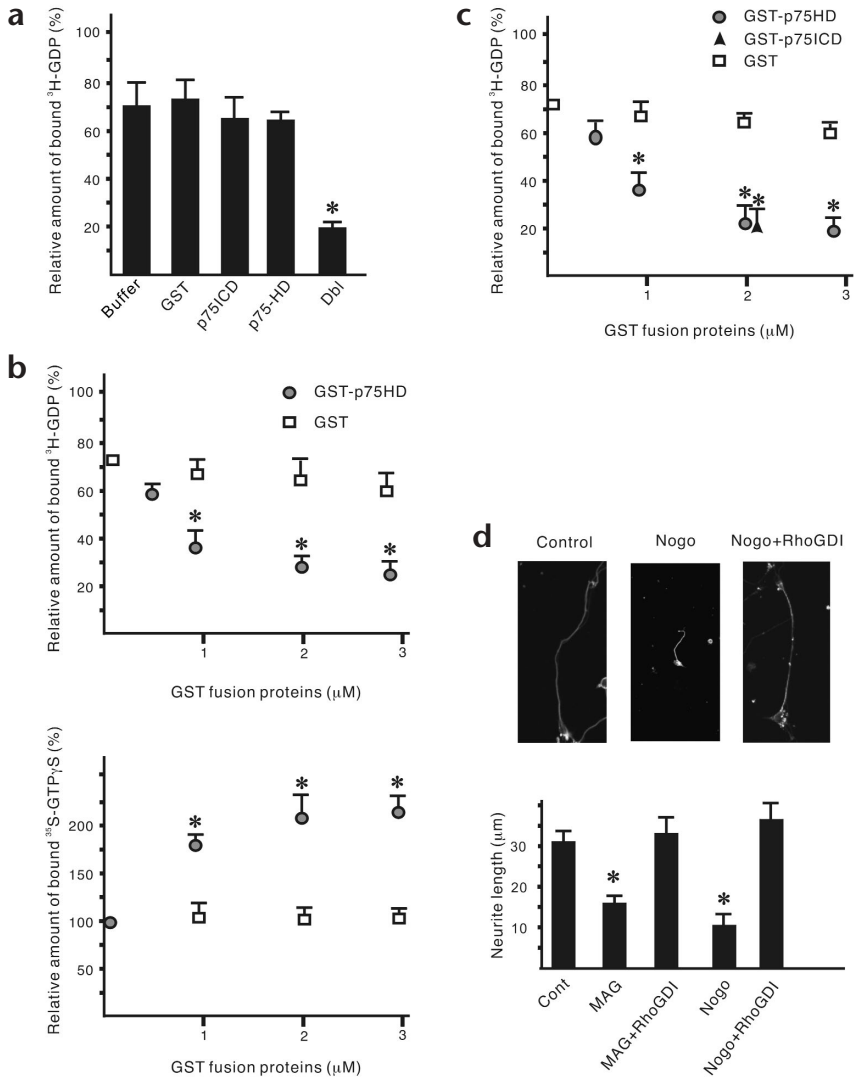

Fig. 3. $p75^{NTR}$ reduces the Rho-GDI activity. (**a**) $p75^{NTR}$ is not a guanine nucleotide exchange factor for RhoA. The ability of the proteins to induce the dissociation of ^{3}H-labeled GDP from RhoA in 30 min was measured. GST protein or the incubation buffer was used as a control. The graph represents the relative amount of initial ^{3}H-GDP that remained bound (mean ± s.e.m.) in three separate experiments (*$P < 0.01$; Student's *t*-test). (**b**) $p75^{NTR}$ HD inhibits the Rho-GDI activity *in vitro*. The GDP/GTP exchange reaction of RhoA in complex with Rho-GDI was determined in the presence or absence of $p75^{NTR}$ HD. In the [^{3}H]GDP dissociation assay (top), the dissociation of [^{3}H]GDP from [^{3}H]GDP–RhoA complexed with Rho-GDI was assayed by measuring the radioactivity of [^{3}H]GDP bound to RhoA. In the [^{35}S] GTPγS binding assay (bottom), the binding of [^{35}S] GTPγS to GDP–RhoA complexed with Rho-GDI was assayed by measuring the activity of [^{35}S] GTPγS bound to RhoA (*$P < 0.01$; Student's *t*-test). (**c**) $p75^{NTR}$ inhibits the Rho-GDI activity. The GDP/GTP exchange reaction of RhoA stimulated with Dbl was determined. The [^{3}H]GDP–RhoA–Rho-GDI complex (50 nM) was incubated with 90 nM GST–Dbl and GST-fused proteins at the indicated concentrations (*$P < 0.01$; Student's *t*-test). (**d**) Overexpression of Rho-GDI abolishes the effect of MAG and Nogo. Top, images of representative cells transiently transfected with the control or Rho-GDI plasmid. MAG, MAG-Fc (25 μg/ml); Nogo, the Nogo peptide (4 μM); Rho-GDI, cells transfected with myc-tagged Rho-GDI. Bottom, the effect of Rho-GDI on the neurite outgrowth of dissociated cerebellar neurons. Data are mean ± s.e.m. (*$P < 0.01$; Student's *t*-test).

$p75^{NTR}$ with Rho-GDI or RhoA using purified recombinant proteins. Bacterially produced RhoA, in the GDP-bound, GTP-bound or the nucleotide-depleted state, was incubated with $p75^{NTR}$, which was precipitated from transfected 293T cells. However, we observed no interaction between them in any nucleotide state (**Fig. 2a**). Notably, recombinant Rho-GDI bound to $p75^{NTR}$. When prenylated RhoA was complexed with Rho-GDI, it associated with $p75^{NTR}$, suggesting that Rho-GDI, but not RhoA, directly complexes with $p75^{NTR}$.

We determined the structural basis of the interaction between Rho-GDI and $p75^{NTR}$. The fifth of the six α helices of the intracellular domain (ICD) of $p75^{NTR}$ shows significant similarity with the 14-base peptide mastoparan[11]. Mastoparan is an amphiphilic component of wasp venom known to activate RhoA[12]. Experiments with the deletion mutant of $p75^{NTR}$ ICD showed that the fifth helix is necessary for the interaction of $p75^{NTR}$ with Rho-GDI (**Fig. 2b**). These results suggest that the activation of RhoA by MAG and Nogo may be dependent on the interaction of Rho-GDI with the fifth helix of $p75^{NTR}$ ICD. To test this hypothesis more directly, we used 293T cells, which do not express $p75^{NTR}$ endogenously. Affinity precipitation of the GTP-bound form of RhoA revealed that RhoA was activated by

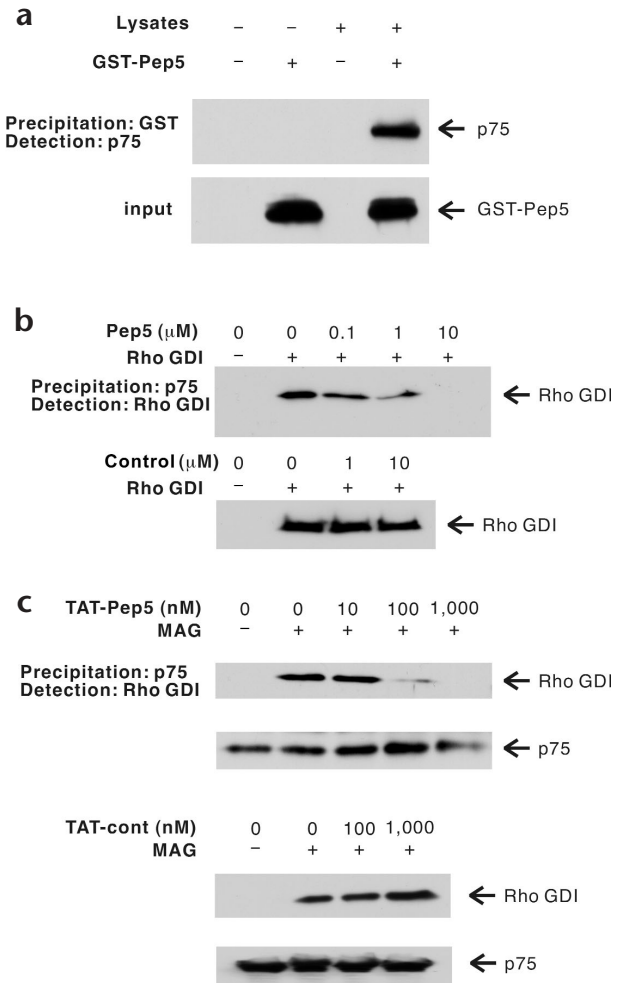

Fig. 4. Pep5 inhibits interaction of Rho-GDI with $p75^{NTR}$. (**a**) Co-precipitation of $p75^{NTR}$ with recombinant GST–Pep5. (**b**) Pep5 inhibits the binding of $p75^{NTR}$ with Rho-GDI in a dose-dependent manner. (**c**) Co-immunoprecipitation of $p75^{NTR}$ and Rho-GDI using lysates prepared from cerebellar neurons. The interaction was diminished by TAT–Pep5.

the overexpression of full-length $p75^{NTR}$ or $p75^{NTR}$ ICD, as shown previously[2]. As expected, the deletion mutant that lacks the fifth helix failed to activate RhoA (**Fig. 2c**), indicating that the fifth helix is necessary for the activation of RhoA by $p75^{NTR}$.

$p75^{NTR}$ releases RhoA from Rho-GDI

In vitro assays with bacterially expressed $p75^{NTR}$ did not indicate GDP/GTP exchange activity on recombinant RhoA (**Fig. 3a**). These results, in combination with the fact that RhoA does not directly associate with $p75^{NTR}$, raise the possibility that $p75^{NTR}$reduces the activity of Rho-GDI, thus facilitating the release of RhoA from Rho-GDI. This step allows for the activation by guanine nucleotide exchange factors and membrane association of the GTP-bound form of Rho proteins[8]. We first examined the effect of the interaction of Rho-GDI with the helical domain (HD) of $p75^{NTR}$ on its ability to inhibit the GDP/GTP exchange reaction of RhoA at low Mg^{2+} concentrations, as the inhibitory effect of Rho-GDI is more obvious at low Mg^{2+} concentrations[13]. This reaction was estimated by measuring the dissociation of [^{3}H]GDP from [^{3}H]GDP-RhoA complexed with Rho-GDI and the binding of [^{35}S]GTPγS to GDP-RhoA complexed with Rho-GDI. $p75^{NTR}$ HD reduced this Rho-GDI activity in a dose-dependent manner (**Fig. 3b**). Under comparable conditions, glutathione S-transferase (GST) did not affect the Rho-GDI activity (**Fig. 3b**). These results show that the $p75^{NTR}$ HD has a potency to directly interact with Rho-GDI and reduce its ability to inhibit the GDP/GTP exchange reactions of RhoA. We next examined the effect of $p75^{NTR}$ HD on the ability of Rho-GDI to inhibit the GDP/GTP exchange reaction of RhoA at high Mg^{2+} concentrations. Rho guanine nucleotide exchange factors (Rho GEFs), such as Dbl, stimulate the GDP/GTP exchange reaction of GDP-RhoA in the absence of Rho-GDI, but not when GDP-RhoA is complexed with Rho-GDI at high Mg^{2+} concentrations[14]. Dbl stimulated the dissociation of GDP from GDP-RhoA (**Fig. 3a**), but the dissociation of GDP from GDP-RhoA complexed with Rho-GDI was markedly reduced (**Fig. 3c**). However, the dissociation of GDP was restored by $p75^{NTR}$ HD. This inhibitory effect of p75 HD on the Rho-GDI activity was dose-dependent. $p75^{NTR}$ ICD showed the inhibitory effect to the same extent as $p75^{NTR}$ HD (**Fig. 3c**). These results demonstrate that the interaction of Rho-GDI with p75 HD increases its activity in both the RhoGEF-independent and RhoGEF-dependent GDP/GTP exchange reactions of RhoA.

As $p75^{NTR}$ has an ability to release RhoA from Rho-GDI *in vitro*, the activation of RhoA by MAG and Nogo through $p75^{NTR}$ may be attributable to the activity that releases Rho from Rho-GDI. Although MAG, as well as the Nogo peptide, significantly inhibited the neurite outgrowth from postnatal cerebellar neurons, overexpression of Rho-GDI abolished these inhibitory effects (**Fig. 3d**). These results are consistent with our suggestion that $p75^{NTR}$ acts as a Rho-GDI displacement factor.

Pep5 inhibits the interaction of $p75^{NTR}$ with Rho-GDI

As all the myelin-derived inhibitors of axonal regeneration identified so far act on neurons through $p75^{NTR}$, intervening with $p75^{NTR}$ signaling after injury to the central nervous system may alleviate myelin-dependent inhibition of axonal regeneration. Pinpointing the region of Rho-GDI association allowed us to develop a strategy to specifically inhibit the function of $p75^{NTR}$. The specific peptide ligand to the $p75^{NTR}$ HD is obtained from a combinatorial library[15]. This ligand is a 15-amino acid residue peptide (Pep5; CFFRGGFFNHNPRYC), and the binding site is mapped by nuclear magnetic resonance spectroscopy onto a hydrophobic patch framed by helices 5 and 6. Although the sequence of the peptide did not immediately suggest a protein that exists in mammals, we were interested in the possibility that it could act as a silencer that disrupts the recruitment of Rho-GDI to $p75^{NTR}$ HD. We first determined whether $p75^{NTR}$ associates with Pep5. Glutathione S-transferase fusion protein containing Pep5 (GST-Pep5) was incubated with lysates prepared from postnatal cerebellum that abundantly express $p75^{NTR}$. In the GST-Pep5 precipitates, the anti-$p75^{NTR}$ antibody revealed the presence of a protein corresponding to $p75^{NTR}$ (**Fig. 4a**). Then, binding affinity was compared between Pep5 and Rho-GDI. $p75^{NTR}$, immunoprecipitated and purified from the lysates of the transfected 293T cells, was incubated with 1 μM GST–Rho–GDI and Pep5 at the indicated concentrations (**Fig. 4b**). Pep5, but not the control peptide, inhibited the association of $p75^{NTR}$ with Rho-GDI in a dose-dependent manner. Therefore, Pep5 has a potential to disrupt the signal mediated by $p75^{NTR}$ *in vitro*. As the peptide ligand must gain entry into the cell if it is to act directly on the $p75^{NTR}$ HD *in vivo*, we generated

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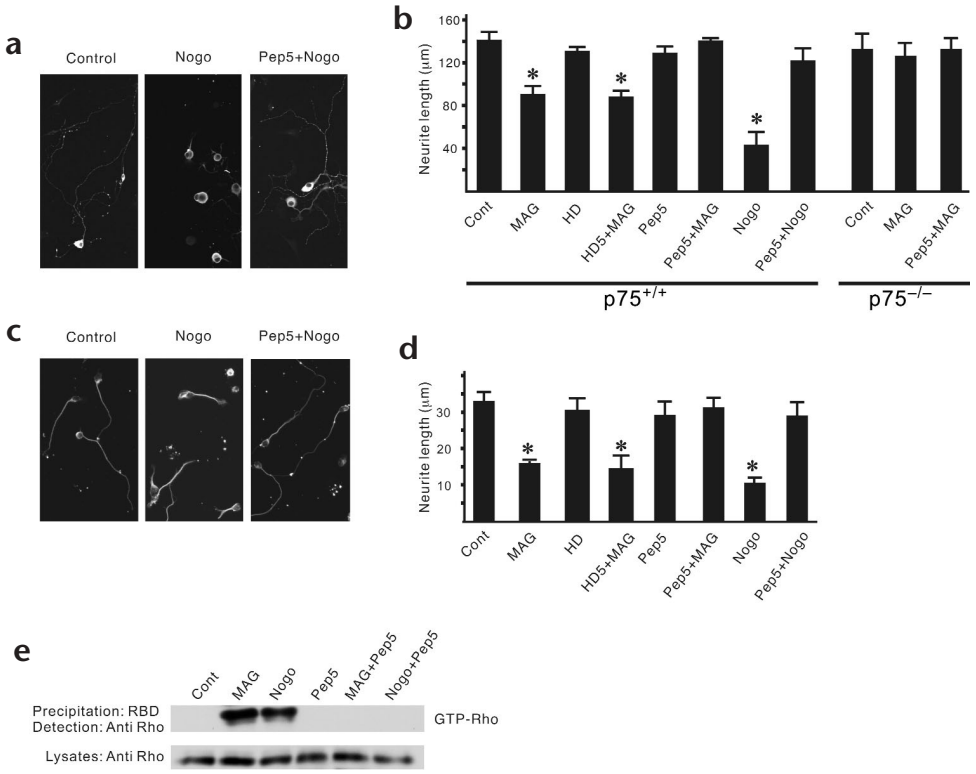

Fig. 5. Pep5 silences the inhibitory action of $p75^{NTR}$. (**a**) Dissociated DRG neurons were incubated for 24 h with or without the Nogo peptide and were then immunostained with monoclonal antibody (TuJ1) recognizing the neuron-specific β tubulin III protein. Nogo, the Nogo peptide; Pep5, TAT–Pep5. (**b**) Neurite outgrowth of DRG neurons. MAG, MAG-Fc; HD, the peptide corresponding to the $p75^{NTR}$ HD (residues 368–381); $p75^{+/+}$, wild type; $p75^{-/-}$, mice carrying a mutation in the $p75^{NTR}$ gene. Data are mean ± s.e.m. (*$P < 0.01$, Student's *t*-test). (**c**) Dissociated cerebellar neurons were incubated for 24 h with or without the Nogo peptide. (**d**) Neurite outgrowth of cerebellar neurons. Data are mean ± s.e.m. (*$P < 0.01$, Student's *t*-test). (**e**) Affinity precipitation of RhoA in cerebellar neurons. The Nogo peptide (4 μM) and MAG-Fc (25 μg/ml) elicit activation of RhoA, whereas TAT–Pep5 (1 μM) completely abolishes these effects.

Pep5 fused with the amino (N)-terminal protein transduction domain (11 amino acids) from the human immunodeficiency virus protein TAT (TAT–Pep5) (ref. 16). The interaction of $p75^{NTR}$ with Rho-GDI induced by MAG-Fc in the dissociated cerebellar neurons was significantly inhibited by TAT–Pep5 in a competitive fashion, but not by TAT-fused control peptide (**Fig. 4c**). Thus, Pep5 may be used as an inhibitor of Rho-GDI association with $p75^{NTR}$.

Pep5 silences the myelin signal

We next asked whether Pep5 inhibits the effect of MAG or Nogo. We used the neurite growth assay to measure the effect of MAG or Nogo. We used another control peptide derived from rat $p75^{NTR}$ corresponding to residues 368–381. This peptide, at the concentration of 100 nM (**Fig. 5b**) or 10 μM (data not shown), had no effect on neurite outgrowth of dorsal root ganglion (DRG) neurons, and it did not influence the action of MAG-Fc (**Fig. 5b**) or the Nogo peptide (data not shown). However, TAT–Pep5, added exogenously to cultured neurons at the concentration of 100 nM, abolished their responsiveness to both MAG (25 μg/ml) and the Nogo peptide (4 μM) (**Fig. 5a** and **b**). Postnatal cerebellar neurons were used to examine the effects of Pep5. As observed in DRG neurons, TAT–Pep5 efficiently silenced the inhibitory effect of MAG (25 μg/ml) and the Nogo peptide (4 μM) (**Fig. 5c** and **d**). Finally, to show more clearly that the peptide acts as a silencer of $p75^{NTR}$ signaling, we measured Rho activity by affinity precipitation. As expected, although RhoA was activated 30 min after the addition of MAG-Fc or the Nogo peptide to the postnatal cerebellar neurons, TAT–Pep5 inhibited the activation of RhoA induced by MAG-Fc or the Nogo peptide on these cells (**Fig. 5e**). These findings strongly suggest that Pep5 inhibits the activation of RhoA through $p75^{NTR}$ by inhibiting the association of Rho-GDI with $p75^{NTR}$.

Discussion

Our data show that the association of $p75^{NTR}$ with Rho-GDI was enhanced by MAG and Nogo. As $p75^{NTR}$ has an ability to release RhoA from Rho-GDI *in vitro*, activation of RhoA by MAG and Nogo through $p75^{NTR}$ may be attributable, at least partly, to Rho-GDI displacement. The release of Rho from Rho-GDI is an important step allowing the GDP-bound form of Rho to be activated by guanine nucleotide exchange factors and to become associated with the membrane. As $p75^{NTR}$ itself may not mediate the process of guanine nucleotide exchange, some Rho guanine nucleotide exchange factors might cooperate with $p75^{NTR}$, which is an issue to be addressed in the future. It is noted that another Rho-GDI displacement factor, ezrin/radixin/moesin, also induces activation of RhoA in Swiss 3T3 cells[13], which is similar to our findings that $p75^{NTR}$ activates RhoA.

There is growing evidence that $p75^{NTR}$ has a key role in axon guidance or growth during the developmental stage[1]. Axon outgrowth from spinal motoneurons or forelimb motor neurons in

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mice carrying a mutation in $p75^{NTR}$ is significantly retarded *in vivo*[2,17]. This phenotype may be attributable to ligand binding to $p75^{NTR}$, as the chick ciliary neurons, which express $p75^{NTR}$ but not TrkA, extend neurites in response to NGF. Contrary to these observations, aberrant axonal elongation is observed in myelin-rich areas where these axons would normally not grow in mice carrying a mutation in $p75^{NTR}$ (ref. 18). In line with this finding, all of the myelin-derived inhibitors of neurite outgrowth identified so far inhibit the growth that is dependent on $p75^{NTR}$ (refs. 5–7). Our findings suggest that these effects may result from the Rho-GDI displacement activity of $p75^{NTR}$. In addition, axon pathfinding errors of $p75^{NTR}$-expressing neurons are prominent among the phenotypes observed in mice carrying a mutation in $p75^{NTR}$, including mistargeting of sympathetic and cortical subplate axons[19,20]. As Rho seems to be involved in the regulation of axon pathfinding in the developmental stages, it is possible that the mistargeting in the absence of $p75^{NTR}$ may be attributable to the failure of appropriate regulation of Rho activity. Interestingly, a recent report suggests a role of Rho-GDI in spatial and temporal activation of the downstream pathway of Rac1 (ref. 21). Although Rho-GDI associates with Rac1 and blocks effector binding, release of Rac1 from Rho-GDI at specific regions where integrin localizes allows Rac1 to bind its effectors. Thus, Rho-GDI may spatially restrict the interactions between Rho GTPases and their effectors. In future studies, it will be interesting to test whether spatial control of Rho signaling, as regulated by Rho-GDI, has an impact on axon pathfinding.

A short isoform of $p75^{NTR}$, which lacks three of the four cystein-rich repeats in the extracellular ligand-binding domain but has the intact intracellular domain, has been found[22]. The cells from mice bearing a targeted disruption of the third exon of the $p75^{NTR}$ gene[23] express this short isoform of $p75^{NTR}$, but are insensitive to the inhibitory molecules[5–7]. Our data show that Pep5 did not affect the neurite outgrowth of neurons that express the short isoform but not the full-length $p75^{NTR}$ (**Fig. 5b**), indicating that the short isoform might not act as a regulator of the neurite outgrowth.

It is now well established that axons of the adult central nervous system are capable of only a limited amount of regrowth after injury, and that an unfavorable environment is tightly linked to this lack of regeneration. Much of axon growth inhibition is associated with myelin. Identification of the myelin-derived inhibitors has led to an understanding of the molecular mechanisms underlying this limitation in growth, and this knowledge will be useful for developing methods to overcome the inhibitory signals. We note that Pep5 seems to specifically inhibit the action mediated by myelin-derived inhibitors, as Pep5 did not inhibit the NGF-induced promotion of the neurite outgrowth from the hippocampal neurons (see **Supplementary Fig. 1a** online) or the cell death of superior cervical ganglion neurons treated with 100 ng/ml BDNF (**Supplementary Fig. 1b**). Specific inhibition of myelin-associated inhibitors may provide a practical therapeutic approach to treat injuries to the central nervous system.

Methods

Animals. We used a strain of mice bearing a targeted disruption of the third exon of the $p75^{NTR}$ gene[23]. They were originally obtained from the Jackson Laboratory (Bar Harbor, Maine) on a C57BL/6J background. All experimental procedures were approved by the institutional committee at Osaka University.

Co-immunoprecipitation. Amino-terminally FLAG-tagged human $p75^{NTR}$ and/or HA-tagged RhoA[2] were transfected with 293T cells or N1E-115 cells by lipofection using Lipofectamine 2000 (Invitrogen, Carlsbad, California). Cells were lysed on ice for 20 min with lysis buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.2% NP-40, 25 μg/ml leupeptin and 25 μg/ml aprotinin). The lysates were centrifuged at 13,000*g* for 20 min, and then the supernatants were collected. They were then incubated with the anti-FLAG antibody (for transfected FLAG-$p75^{NTR}$) or anti-p75 antibody (Chemicon, Temecula, California) (for cerebellar neurons) for 3 h. The immunocomplex was collected with protein A sepharose (Amersham Biosciences, Buckinghamshire, UK). The suspension was centrifuged at 1,000*g* for 5 min. The pellets were washed four times with lysis buffer and subjected to SDS-PAGE, followed by immunoblot analysis using anti-Rho-GDIα antibody (Sigma, St. Louis, Missouri) or anti-RhoA antibody (Santa Cruz Biotechnology, Santa Cruz, California). Where indicated, recombinant rat MAG-Fc chimera (25 μg/ml, R&D Systems, Minneapolis, Minnesota), the Nogo peptide (4 μM, Alpha Diagnostic, San Antonio, Texas), TAT-fused Pep5 or TAT-fused control peptide (TAT–GGWKWWPGIF) was used. The peptides were chemically synthesized, and the composition was verified by amino acid analysis and mass spectrometry (Sigma-Genosys). Amino-terminally FLAG-tagged human $p75^{NTR}$ was cloned into pcDNA3.1 expression plasmid (Invitrogen).

Co-precipitation of $p75^{NTR}$ and Rho-GDI. $p75^{NTR}$, precipitated from the transfected 293T cells using anti-FLAG antibody and protein A sepharose, was incubated with recombinant human GST–Rho-GDI or GST–RhoA (both from Cytoskeleton, Denver, Colorado) in 200 μl buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM EDTA, 0.025% Tween20) for 2 h and then washed. The resultant precipitates were electrophoretically transferred to polyvinylidene difluoride membranes after SDS-PAGE and were immunoblotted with the anti-GST antibody (Sigma). To examine the nucleotide dependency, GST-RhoA was preloaded with the appropriate nucleotide, and EDTA was replaced with 10 mM $MgCl_2$. Where indicated, Pep5 or the control peptide (GGWKWWPGIF) was used.

Production of recombinant proteins. The $p75^{NTR}$ ICD coding sequence, with or without the deletion, was cloned into the pGEX-5X bacterial expression vectors (Amersham Biosciences) to generate GST-fused proteins from *Escherichia coli*. pGEX–GST–Rho-GDI was provided by Y. Takai. After cell growth to an OD_{600} of 1.0, 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to induce protein synthesis, and then cells were grown for another 16 h at 25 °C. Fusion proteins were purified using glutathione-Sepharose 4B (Amersham Biosciences), and the GST moiety was removed to produce recombinant Rho-GDI. Purity of the proteins was determined by SDS-PAGE, and the concentration was measured. The deletion mutants of rat $p75^{NTR}$ ICD are from residue 274 to residue 342, 351, 363, 375, 390, 406 and 425 (ref. 24). Complex formation of GST–$p75^{NTR}$ mutants with Rho-GDI was assessed by precipitating the GST–$p75^{NTR}$ mutants.

Affinity precipitation of GTP–RhoA. Amino-terminally FLAG-tagged human $p75^{NTR}$ or the deletion mutants of $p75^{NTR}$ ICD were cloned into a pcDNA3.1 expression plasmid and were transfected with 293T cells. Cells were lysed in 50 mM Tris (pH7.5) containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$ and 10 μg/ml each of leupeptin and aprotinin[25]. Cell lysates were clarified by centrifugation at 13,000*g* at 4 °C for 10 min, and then the supernatants were incubated with the 20 μg of GST–Rho binding domain of Rhotekin beads (Upstate Biotech., Charlottesville, Virginia) at 4 °C for 45 min. The beads were washed four times with washing buffer (50 mM Tris, pH 7.5, containing 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml each of leupeptin and aprotinin). Bound Rho proteins were detected by western blotting using a monoclonal antibody against RhoA (Santa Cruz Biotechnology.).

***In vitro* nucleotide exchange assay.** Lipid-modified RhoA was purified from yeast membranes as described[26]. [^{3}H]GDP– or GDP–RhoA complexed with Rho-GDI was obtained by first incubating GDP–RhoA with or without [^{3}H]GDP, followed by incubation with Rho-GDI for 30 min, as described previously[13]. The sample, subjected to gel filtration, was equilibrated with 20 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$,

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1 mM dithiothreitol and 0.1% CHAPS. The GDP dissociation and GTP binding assays were carried out by the filter binding method as described previously[27]. In the [^{3}H]GDP dissociation assay, 50 nM of the complex was incubated for 20 min with various concentrations of GST-fused proteins in a reaction mixture (50 μl) containing 30 mM Tris-HCl (pH 7.5), 5 mM or 0.5 μM $MgCl_2$, 1 (for low Mg) or 10 (for high Mg) mM EDTA, 0.1 mM GTP, 1 mM dithiothreitol, 0.12% CHAPS and 0.2 mg/ml bovine serum albumin. In the [^{35}S] GTPγS binding assay, the complex was incubated as described above except that 1 μM [^{35}S] GTPγS was used instead of 0.1mM GTP. At the indicated time, an aliquot of the reaction sample was removed and passed through nitrocellulose filters (IPVH 000, Millipore). The filters were washed and used for scintillation counting. GST protein or the buffer was used as a control. His-tagged catalytic domain of Dbl was used at a concentration of 90 nM.

Neurite outgrowth assay. Dorsal root ganglia were removed from adult mice and dissociated into single cells by incubation with 0.025% trypsin and 0.15% collagenase type 1 (Sigma) for 30 min at 37 °C. For cerebellar neurons, the cerebella from two animals were combined in 5 ml of 0.025% trypsin, triturated and incubated for 10 min at 37 °C. DMEM containing 10% FCS was added, and then the cells were centrifuged at 800 r.p.m. Neurons were plated in Sato media[28] on poly-L-lysine coated chamber slides. For outgrowth assays, plated cells were incubated for 24 h and were fixed in 4% (wt/vol) paraformaldehyde, and they were immunostained with a monoclonal antibody (TuJ1) recognizing the neuron-specific β tubulin III protein. The length of the longest neurite or the total process outgrowth for each β tubulin III-positive neuron was determined. Where indicated, MAG-Fc (25 μg/ml) or the Nogo peptide (4 μM) was added to the medium after plating. pEF–BOS–myc–Rho-GDI plasmid or pEGFP plasmid, as a control, was used for the transfection. Twenty four hours after transfection by lipofection, the cells were re-plated and incubated for 24 h. To determine the transfected cells, cells were permeabilized and immunostained with the anti-myc antibody (1:1,000, Sigma).

Note: Supplementary information is available on the Nature Neuroscience website.

Acknowledgments

We thank Y. Takai for gifts of pGEX–GST–Rho-GDI, pEF–BOS–myc–Rho-GDI and pRSET-Dbl, R. Beliveau for yeast expressing GST-RhoA, Y. Nagai for the control TAT-fused peptide and Y. Hara for technical assistance. This work was supported by a Grant-in-Aid from the Japan Brain Foundation.

Competing interests statement

The authors declare that they have no competing financial interests.

RECEIVED 2 JANUARY; ACCEPTED 24 MARCH 2003

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