Overcoming the Inhibitors of Myelin with a Novel Neurotrophin Strategy*

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Myelin inhibitors activate a p75NTR-dependent signaling cascade in neurons that not only inhibits axonal growth but also prevents neurotrophins (NT) from stimulating growth. Most strikingly, in addition to Trk receptors, neurotrophins also bind to p75NTR. We have designed a “mini-neurotrophin” called BAG to activate TrkB in the absence of p75NTR binding. We find that BAG is as effective as the natural TrkB ligands (brain-derived neurotrophic factor (BDNF) and NT-4) at promoting neurite outgrowth from cerebellar neurons. Furthermore, the neurite outgrowth responses stimulated by BDNF and BAG are inhibited by a common set of reagents, including the Trk receptor inhibitor K252a, as well as protein kinase A and phosphoinositide 3-kinase inhibitors. However, in contrast to BDNF, BAG promotes growth in the presence of a myelin inhibitor or when antibodies directly activate the p75NTR inhibitory pathway. On the basis of this observation, we postulated that the binding of BDNF to the p75NTR might compromise the ability of BDNF to stimulate neurite outgrowth in an inhibitory environment. To test this, we used NGF, and an NGF-derived peptide, to compete for the BDNF/p75NTR interaction; remarkably, in the presence of either agent, BDNF acquired the ability to promote neurite outgrowth in the presence of a myelin inhibitor. The data suggest that in an inhibitory environment, the BDNF/p75NTR interaction compromises regeneration. Agents that activate Trk receptors in the absence of p75NTR binding, or agents that inhibit neurotrophin/p75NTR binding, might therefore be better therapeutic candidates than neurotrophins.

The central nervous system shows very limited repair after injury, and this has been postulated to be due in part to the presence of inhibitory products associated with damaged myelin that prevent axonal regeneration (1). In support, biochemical studies on central myelin have identified two protein fractions that contain inhibitory activity (2), and monoclonal antibodies that bind to these fractions enhance the growth of axons from cultured sensory and sympathetic neurons into otherwise nonpermissive optic nerve explants (3). Further-
agent can also promote neurite outgrowth when the myelin inhibitory pathway is activated in neurons.

A second independent test of the hypothesis is that agents that interfere with the neurotrophin/p75NTR interaction should allow neurotrophins to promote neurite outgrowth in an inhibitory environment. NGF and BDNF are very effective at competing for binding to the p75NTR, but they do not compete effectively for each other’s binding their respective Trk receptor (23). We have taken advantage of the fact that cerebellar neurons do not express TrkA receptors (24), and we have used NGF to compete for BDNF binding to the p75NTR in these cells. We find that in the presence of NGF, BDNF acquires the ability to stimulate neurite outgrowth in an inhibitory environment. The same response was found when a monomeric peptide mimetic of a small loop that NGF uses to bind to the p75NTR was used instead of NGF. These experiments not only provide a plausible explanation as to why endogenous neurotrophins might not be effective at promoting regeneration in the damaged nervous system, they also point to at least two novel strategies for promoting brain repair. For example, agents that activate Trk receptors in the absence of p75NTR binding might be more effective at promoting regeneration in the damaged nervous system. NGF and BDNF are very effective at competing for binding to the p75NTR, but they do not compete effectively for each other’s binding their respective Trk receptor (23). We have taken advantage of the fact that cerebellar neurons do not express TrkA receptors (24), and we have used NGF to compete for BDNF binding to the p75NTR in these cells. We find that in the presence of NGF, BDNF acquires the ability to stimulate neurite outgrowth in an inhibitory environment. The same response was found when a monomeric peptide mimetic of a small loop that NGF uses to bind to the p75NTR was used instead of NGF. These experiments not only provide a plausible explanation as to why endogenous neurotrophins might not be effective at promoting regeneration in the damaged nervous system, they also point to at least two novel strategies for promoting brain repair. For example, agents that activate Trk receptors in the absence of p75NTR binding might be more effective than neurotrophins at promoting repair. Also, antagonists of the neurotrophin/p75NTR interaction might allow endogenous neurotrophins, which have long been known to be synthesized in response to injury (25, 26), to acquire the ability to promote regeneration.

EXPERIMENTAL PROCEDURES

Neurite Outgrowth Assays—Cerebellar neurons isolated from postnatal day 2/3 rat pups were cultured over monolayers of parental 3T3 cells or N-cadherin-expressing 3T3 cells (the LK8 cell line) (27), essentially as described previously (28). Monolayers were established by seeding ~80,000 cells into individual chambers of an eight-chamber tissue culture slide coated with poly-L-lysine and fibronectin. The cell lines, and monolayers, were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. When looking for trophic factor or peptide stimulation of neurite outgrowth, the monolayers were set up 4 h before the addition of the neurons, and the co-cultures were maintained for ~16 h before fixation. The same procedure was followed when looking at the MAG-induced inhibition of neurite outgrowth over N-cadherin-expressing 3T3 cells. However, when looking at MAG inhibition of growth over parental 3T3 cells, the monolayers were established for 24 h. Monolayers were established by seeding ~80,000 cells into individual chambers of an eight-chamber tissue culture slide coated with poly-L-lysine and fibronectin. The cell lines, and monolayers, were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. When looking for trophic factor or peptide stimulation of neurite outgrowth, the monolayers were set up 4 h before the addition of the neurons, and the co-cultures were maintained for ~16 h before fixation. The same procedure was followed when looking at the MAG-induced inhibition of neurite outgrowth over N-cadherin-expressing 3T3 cells. However, when looking at MAG inhibition of growth over parental 3T3 cells, the monolayers were established for 24 h. Monolayers were established by seeding ~80,000 cells into individual chambers of an eight-chamber tissue culture slide coated with poly-L-lysine and fibronectin.

Neuronal survival and neurite outgrowth were assessed after 23 h. This increases the basal neurite outgrowth and gives a better window for looking at inhibition of neurite outgrowth by MAG. Following careful fixation with 4% paraformaldehyde, the neurons were retained for 24 h prior to addition of the neurons, and the cultures were maintained for 23 h. This increases the basal neurite outgrowth and gives a better window for looking at inhibition of neurite outgrowth by MAG. Following careful fixation with 4% paraformaldehyde, the neurons were retained for 24 h prior to addition of the neurons, and the cultures were maintained for 23 h. This increases the basal neurite outgrowth and gives a better window for looking at inhibition of neurite outgrowth by MAG.
over monolayers of control 3T3 fibroblasts in control media or media supplemented with NT-4 (5 ng/ml) as indicated in the presence and absence of a constrained peptide mimetic of the NT-4 SRRGE motif (N-Ac-CSRRGEC-NH₂) at 125 μM (hatched bars), or 125 μM of a linear version of this motif (N-Ac-SRRGELA-NH₂) (black bars). B–D, the cultures were maintained for 16 h before being fixed and stained for GAP-43. The mean length of the longest neurite was then determined from 100 to 120 neurons under each culture condition. The results show absolute mean neurite lengths pooled from at least three independent experiments. The bar(s) show S.E.

The SRRGE motif at the amino terminus of NT-4 is highlighted in gray. The NT-4 dimer is denoted as chains a₁ (yellow) and a₂ (green), with the two Ig domains from the TrkB receptor (denoted as chains b₁ and b₂) shown in grey. The SRRGE motif at the amino terminus of NT-4 is highlighted in red, and can be seen to make the dominant contact at the a₁/b₁ and reciprocal ligand/receptor interfaces.

Overcoming Myelin Inhibition

Design and testing of a TrkB antagonist peptide. A, the NT-4/TrkB Ig domain 5 crystal structure (Protein Data Bank code 1HCF) as solved by Banfield et al. (29) is shown as a ribbon diagram. The NT-4 dimer is denoted as chains a₁ (yellow) and a₂ (green), with the two Ig domains from the TrkB receptor (denoted as chains b₁ and b₂) shown in grey. The SRRGE motif at the amino terminus of NT-4 is highlighted in red, and can be seen to make the dominant contact at the a₁/b₁ and reciprocal ligand/receptor interfaces. B, dissociated cerebellar neurons were cultured over monolayers of control 3T3 fibroblasts in control media or media supplemented with NT-4 (5 ng/ml) as indicated in the presence and absence of a constrained peptide mimetic of the NT-4 SRRGE motif (N-Ac-CSRRGEC-NH₂) or the equivalent motifs in NGF (N-Ac-CFHRGEC-NH₂) and NT-3 (N-Ac-CSHRGEC) as indicated. C, the same as B except BDNF (5 ng/ml) was used instead of NT-4. D, cerebellar neurons were cultured over monolayers of 3T3 cells in control media or media supplemented with NT-4, BDNF, and FGF2 (all at 5 ng/ml) as indicated. In addition, neurons were grown in control media over monolayers of 3T3 cells that express transfected N-cadherin on their cell surface (NCAD). The experiments were done in control media (open bars), in the presence of the constrained peptide mimetic of the NT-4 SRRGE motif (N-Ac-CSRRGEC-NH₂) at 125 μM (hatched bars), or 125 μM of a linear version of this motif (N-Ac-SRRGELA-NH₂) (black bars). B–D, the cultures were maintained for 16 h before being fixed and stained for GAP-43. The mean length of the longest neurite was then determined from 100 to 120 neurons under each culture condition. The results show absolute mean neurite lengths pooled from at least three independent experiments. The bar(s) show S.E.

dependent manner with a maximal response seen at 10 μg/ml (~6 μM) (Fig. 2B). Bₐg is as effective as maximally active concentrations of NT-4, BDNF, and FGF2 in promoting neurite outgrowth from cerebellar neurons (Fig. 2C). Like the response to BDNF (Fig. 1B), the response to Bₐg was fully inhibited by the BₐNT peptide (Fig. 3). Likewise, the response to Bₐg and BDNF is fully inhibited by K252a, a drug that inhibits the tyrosine kinase activity of the Trk receptor (33). Furthermore, PKA and PI3K activity couple the activated TrkB receptor to neurite outgrowth (34, 35), and inhibitors of both pathways (KT5720 or H-89 for PKA and wortmannin or LY294002 for PI3K) substantially inhibit the response to BDNF and the Bₐg (Fig. 3). All of the above inhibitors have little or no effect on the neurite outgrowth over 3T3 cells or neurite outgrowth stimulated by FGF2 and other agents (Fig. 1B and Fig. 3). These data support the view that BDNF and Bₐg stimulate neurite outgrowth by activating a common TrkB-dependent signaling cascade in the cerebellar neurons.

Bₐg, but Not BDNF, Promotes Neurite Outgrowth in an Inhibitory Environment—MAG was the first inhibitory component of myelin to be identified (see Introduction), and it can inhibit neurite outgrowth when presented to neurons as a soluble Fc chimera (36). To look at MAG inhibition, we cultured cerebellar neurons over monolayers of N-cadherin-expressing fibroblasts, as this gives an enhanced basal outgrowth response over an ~16-h culture period when compared with parental 3T3 cells (Fig. 1D) (28). Under these conditions, Bₐg and BDNF do not promote additional growth, suggesting that the rate of growth is optimal (Fig. 4). The MAG-Fc inhibited neurite outgrowth at 5 μg/ml, with a more robust inhibition seen at 25 μg/ml (Fig. 4). As reported previously (see Introduction), MAG does not inhibit growth when the Rho kinase inhibitor Y27632 (10 μM) is present (Fig. 4). Also in agreement with Cai et al. (20) we found that BDNF was unable to promote neurite outgrowth in the presence of the MAG-Fc (Fig. 4). However, the Bₐg peptide was able to stimulate neurite outgrowth in this inhibitory environment to the extent that it completely circumvented the inhibitory activity of MAG (Fig. 4). In order to ascertain the robustness of these observations, we repeated the experiments with neurons cultured for a longer period of time (~23 h) over established monolayers of 3T3 fibroblasts. Again, the MAG-Fc inhibits neurite outgrowth in control media and media containing BDNF; however, this inhibition was not seen when the Bₐg peptide was present (Fig. 5).

To date, all of the inhibitory molecules in myelin have been reported to signal via p75NTR. It follows that direct activation of...
this complex in neurons should also inhibit neurite outgrowth. In this context, direct clustering of p75NTR with an antibody might be expected to inhibit neurite outgrowth. Indeed, we observed that pretreatment of cerebellar neurons with a p75NTR polyclonal antibody for 60 min was sufficient to inhibit the subsequent growth of the neurons over the N-cadherin monolayers (Fig. 6). The response to the p75NTR antibody was not seen when Rho kinase was inhibited after the pretreatment.
overcome by BAG, but not BDNF. Cerebellar neurons were cultured over monolayers of N-cadherin-expressing 3T3 cells in control media (open bars) or media supplemented with the MAG-Fc at 25 μg/ml (hatched bars). These experiments were done with no other agents added to the media (control), or in the presence of the BAG peptide at 10 μg/ml or BDNF at 5 ng/ml. The cultures were maintained for 23 h before being fixed and stained for GAP-43. The mean length of the longest neurite was then determined from 100 to 120 neurons under each culture condition. The results are pooled from three independent experiments, and the bars show the S.E.

NGF and an NGF Loop 1 Peptide Allow BDNF to Circumvent MAG Inhibition—We wanted to test if agents that interfere with the BDNF/p75NTR interaction can influence neurite outgrowth in an inhibitory environment. BDNF and NGF can effectively compete with each other for binding to the p75NTR, but they are not effective at competing for binding at their respective Trk receptors (23). When neurons are grown over established 3T3 monolayers for ~23 h in media containing 25 μg/ml of the MAG-Fc, neither NGF nor BDNF inhibited the MAG response (Fig. 7); we can therefore conclude that the neurotrophin-binding site on p75NTR is not required for the formation and/or function of the p75NTR inhibitory signaling complex that is activated by the MAG-Fc. Based on this observation, and the fact that p75NTR is the only NGF receptor expressed by cerebellar neurons, we conclude that NGF is a reasonable agent to use to compete out BDNF binding to p75NTR in the cerebellar neurons.

Effective competition between NGF and BDNF at the p75NTR receptor requires an ~100-fold excess of one neurotrophin to the other (23), and we therefore used BDNF at 1 ng/ml and NGF at 100 ng/ml. In contrast to the results seen when these agents are added in the presence of the MAG-Fc on their own, a significant neurite outgrowth response is seen when BDNF (1 ng/ml) and NGF (100 ng/ml) are added together (Fig. 7). Increasing the BDNF concentration to 5 and then 10 ng/ml diminished the molar excess of NGF to BDNF and resulted in a considerably smaller neurite outgrowth response, demonstrating the competitive nature of the interaction between the two factors (Fig. 7). NGF contains four main exposed loops (47) with a considerable body of evidence showing that the TDIKGKE motif that constitutes the first β-hairpin loop plays a crucial role in binding to p75NTR (22, 37). Constrained peptide mimetics of this motif interact with p75NTR and would be expected to compete for neurotrophin binding to the receptor (38). In the present study we tested a constrained monomeric version of the motif (N-Ac-CTDIKGGKEC-NH$_2$) for its ability to mimic the effect that NGF has on the BDNF response. When
We wanted to test if, in the presence of the MAG-Fc, the three myelin inhibitors. Neurotrophin activation of p75NTR can increase neurite outgrowth in the absence of the BAG peptide (but in the presence of the MAG-Fc) (data not shown).

When neurons are grown for 23 h over 3T3 cell monolayers or for 16 h over N-cadherin-expressing 3T3 cell monolayers, or for 23 h over 3T3 cell monolayers, or for 16 h over N-cadherin-expressing 3T3 cell monolayers, a robust neurite outgrowth response is measured. In both instances, soluble MAG-Fc inhibited growth by around 40–50%. A similar inhibition of growth was seen when neurons were treated with a p75NTR binding. In both cases, Rho kinase activity seems to be central to this suppression of growth as the responses could be fully inhibited by the Rho kinase inhibitor, Y27632. As reported previously (see Introduction), BDNF was unable to promote neurite outgrowth in the presence of the MAG-Fc or after the direct activation of p75NTR with a p75NTR agonist in an inhibitory environment. Another way to test this would be to see if BANT acquires the ability to promote growth in an inhibitory environment when its ability to interact with the p75NTR is compromised.
antibody. However, the BAG peptide was able to stimulate growth in both inhibitory environments to the extent that inhibition by both the MAG-Fc and the p75NTR antibody was fully circumvented. An antibody to GT1b also inhibits neurite outgrowth by activating the MAG receptor complex in neurons (46); we have confirmed this observation and also find that this inhibitory response can also be circumvented by the BAG peptide but not BDNF (not shown).

BAG was rationally designed to be a TrkB agonist, and the possibility that BAG might be stimulating growth via a non-Trk pathway is extremely unlikely as several lines of evidence suggest that this peptide is functioning as a TrkB agonist. First, the response to the peptide can be fully inhibited by a monoclonal version of the same binding motif that has been shown to function as a TrkB antagonist. Second, the response to BAG is fully inhibited by K252a, an established and relatively specific inhibitor of the tyrosine kinase activity of the Trk receptor family (33). In addition, PKA and PI3K antagonists inhibited both BAG and BDNF responses in the absence of any substantial effect on basal neurite outgrowth or neurite outgrowth stimulated by FGF2. Finally, in the presence of the MAG-Fc, BDNF (but not NGF) can compete out the BAG response, suggesting that they interact with a common receptor. Thus, by several independent criteria it would appear that BAG-stimulated neurite outgrowth requires the function of the TrkB receptor and its associated signaling cascade. Nonetheless, peptides of this size and affinity are rarely good tools for binding and biochemical studies, even though they work well in biological assays. Indeed, we have been unable to obtain direct evidence for BAG binding to TrkB. For this reason, it was essential to use an independent approach to test further the hypothesis.

The hypothesis is that BDNF is unable to promote growth in an inhibitory environment because it can bind to both TrkB and p75NTR; this can be independently tested by determining whether agents that compete for the BDNF/p75NTR interaction allow BDNF to stimulate growth in the inhibitory environment. As NGF will compete effectively for BDNF binding to the p75NTR, but not TrkB, the addition of NGF into the cultures can clearly give an insight into this question. In this context, p75NTR is the only NGF receptor in cerebellar neurons, and it is the only known target for which NGF and BDNF can effectively compete for binding. The results clearly show that when NGF is added to the cultures at an ~100-fold molar excess, BDNF acquires the ability to stimulate considerable neurite outgrowth in the presence of the MAG-Fc. The BDNF response (~40% increase over control) was not as good as that seen with the BAG peptide under the same conditions (~60% increase), and this probably reflects the fact that 1 ng/ml BDNF is a suboptimal concentration for axonal growth. It is self-evident that as the BDNF concentration increases, NGF will become less effective at competing out its binding to the p75NTR. When the BDNF concentration is increased in the presence of NGF (held at 100 ng/ml) and the MAG-Fc, the neurite outgrowth response decreases in a dose-dependent manner. These results establish the competitive nature of an NGF/BDNF interaction in the inhibitory environment, and clearly support the hypothesis that BDNF acquires the ability to promote growth because NGF prevents it from binding to the p75NTR.

The above observation with NGF and BDNF might allow some insights into why a BDNF/p75NTR interaction prevents growth. In a purely “signaling” model, BDNF binding to the p75NTR might generate a signal that compromises TrkB signaling, and other neurotrophins might also be expected to be able to deliver this signal. The fact that NGF does not inhibit the response to the TrkB agonists would tend to argue against this. However, signaling by NGF and BDNF via the p75NTR might not be equivalent in terms of modulating TrkB function. Nonetheless, another possibility is a “scaffold” model where the ability of BDNF to bind to both p75NTR and TrkB, perhaps simultaneously, would play a role in recruiting TrkB into a p75NTR inhibitory signaling complex. In turn, this might facilitate cross-talk between the receptors in a manner that allows the inhibitory signal to dominate. Indeed, recent crystal structures suggest that a neurotrophin dimer might be able to bind simultaneously a Trk and p75NTR monomer (22). The observation that NGF allows BDNF to promote growth in an inhibitory environment, most probably by competing for BDNF/p75NTR binding, would fit with such a model. In support, NGF contains four exposed β-hairpin loops (47), with a considerable body of evidence showing that the TD1GKKGE motif that constitutes loop 1 plays a crucial role in binding to p75NTR (22, 37). A constrained monomeric version of the motif (N-Ac-CCTDIKKGEC-NH2) behaved in the same manner as NGF and allowed BDNF to promote neurite outgrowth in the presence of the MAG-Fc. Thus, this novel activity of NGF can be localized to a β-hairpin loop that is required for p75NTR binding.

In summary, within an inhibitory environment, p75NTR is the signaling component of an inhibitory receptor complex. The experiments in this study have been designed to test the hypothesis that BDNF fails to promote neurite outgrowth in such an inhibitory environment because it binds to both the TrkB receptor and p75NTR and perhaps thereby recruits the TrkB receptor to the inhibitory complex. The ability of the BAG peptide to promote growth in this environment would be accounted for by its inability to bind to p75NTR, with the ability of BDNF to promote growth when NGF (or the NGF loop 1 peptide) is added accounted for by competition for binding to p75NTR. The TrkB receptor has been validated in a number of studies as a therapeutic target for brain repair (48–50). However, as with other “large molecule” strategies, continuous delivery of effective levels of BDNF and/or NT-4 to the vulnerable neurons presents a considerable therapeutic challenge. Our studies now suggest that the use of these natural ligands of the receptor in a therapeutic context might also be compromised by the fact that they can engage with p75NTR in neurons. It follows that small molecule agonists of the TrkB receptor that do not bind to p75NTR might have therapeutic potential in a wide range of applications. Likewise, as neurotrophins are often present at injury sites (25, 26), their ability to promote regeneration might be enhanced by agents that inhibit their binding to p75NTR. Both types of agent might be useful for combating neurodegenerative conditions such as Alzheimer’s and Parkinson’s disease and/or promoting brain repair following injury associated with events like stroke or spinal cord hemi-section (51, 52). The peptide mimetics used in the present study were designed to test an hypothesis and not as drugs for animal studies. Nonetheless, rational approaches can be adopted for the development of peptides into compounds suitable for such studies.

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