

# TAJ/TROY, an Orphan TNF Receptor Family Member, Binds Nogo-66 Receptor 1 and Regulates Axonal Regeneration

## Report

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### Summary

Myelin-associated inhibitory factors (MAIFs) are inhibitors of CNS axonal regeneration following injury. The Nogo receptor complex, composed of the Nogo-66 receptor 1 (NgR1), neurotrophin p75 receptor (p75), and LINGO-1, represses axon regeneration upon binding to these myelin components. The limited expression of p75 to certain types of neurons and its temporal expression during development prompted speculation that other receptors are involved in the NgR1 complex. Here, we show that an orphan receptor in the TNF family called TAJ, broadly expressed in postnatal and adult neurons, binds to NgR1 and can replace p75 in the p75/NgR1/LINGO-1 complex to activate RhoA in the presence of myelin inhibitors. In vitro exogenously added TAJ reversed neurite outgrowth caused by MAIFs. Neurons from *Taj*-deficient mice were more resistant to the suppressive action of the myelin inhibitors. Given the limited expression of p75, the discovery of TAJ function is an important step for understanding the regulation of axonal regeneration.

### Introduction

Following injury, axons in the peripheral nervous system (PNS) regenerate, whereas axons in the central nervous system (CNS) lack this ability. However, CNS axons can regenerate in a PNS environment (Richardson et al., 1980), indicating the presence of inhibitory signals unique to the local CNS environment. Three myelin-associated inhibitor factors (MAIFs) have been identified, namely myelin-associated glycoprotein (MAG), Nogo, and oligodendrocyte-myelin glycoprotein (OMgp), that can negatively affect growth cone extension and axon outgrowth through activation of the RhoA signaling pathway (Filbin, 2003). MAIF signaling is transduced by a three-component receptor complex of Nogo-66 receptor (NgR1), neurotrophin receptor (p75), and LINGO-1 (Fournier et al., 2001; Mi et al., 2004; Wang et al., 2002). NgR1 is a member of the leucine-rich repeat (LRR) superfamily of molecules (Fournier et al., 2001) consisting of eight LRRs with N- and C-terminal caps, a 120 amino acid stalk region, and a GPI anchor. Since it is GPI-

linked to the cell surface, NgR1 is incapable of signal transduction by itself. p75 is a member of the tumor necrosis factor receptor (TNFR) superfamily, originally identified as a low-affinity neurotrophin receptor that has since been suggested to play important roles in cell death signaling (Rabizadeh and Bredesen, 2003). LINGO-1 is also an LRR family member (Mi et al., 2004) that contains 12 LRRs with N and C-terminal caps, an Ig domain, a transmembrane domain, and a cytoplasmic tail that may have a signaling function. MAIFs and their receptor complex components represent potential therapeutic targets in neurodegenerative diseases and neurological injury such as traumatic spinal cord injury and stroke.

Although p75 participates in vitro in a functional MAIF receptor complex (Mi et al., 2004; Wang et al., 2002), the in vivo physiological relevance of p75 has been questioned because of its absence in certain MAIF-responsive neurons. In searching for other TNF family member receptors that have the potential to functionally replace p75, we focused on the orphan receptor TAJ (also referred to as TROY/TNFRSF19), because it is more widely expressed in the postnatal and adult brain than p75 and, among the orphan TNF receptors, it most closely resembled p75 (Eby et al., 2000; Hu et al., 1999; Kojima et al., 2000). We examined the role of TAJ in neuronal systems specifically in the context of the MAIF receptor complex. Here, we show that TAJ binds NgR1 and affects axon growth.

### Results

#### TAJ Binds to NgR1

To examine potential interactions between TAJ and the MAIF receptor, NgR1, p75, and LINGO-1 were expressed in CHO cells alone and in various combinations. AP-TAJ bound to the cells expressing NgR1, NgR1/LINGO-1, or NgR1/LINGO-1/p75 (Figures 1A and 1B) but not to vector-transfected cells or CHO cells expressing only p75 or LINGO-1. The apparent  $K_D$  for AP-TAJ binding to the *NgR1*-, *NgR1/LINGO-1*-, and *NgR1/LINGO-1/p75*-transfected cells ranged from 5 to 10 nM. The slight decreases in affinity for TAJ binding to the *NgR1/LINGO-1*- and *NgR1/LINGO-1/p75*-transfected cells may result from competition between TAJ and p75 and/or LINGO-1 for binding to NgR1.

To further characterize this binding and identify regions of NgR1 bound by TAJ, soluble, truncated forms of NgR1 were coated onto ELISA plates and used directly to measure the binding by AP-TAJ. Full-length NgR1 (sNgR1 27–431, LRR domain plus the entire 121 amino acid stalk region) bound AP-TAJ with an apparent  $K_D$  of 3 nM (Figure 1C). Truncated versions either containing the LRR domain plus 34 amino acids from the stalk region (sNgR1 27–344) or the LRR domain with no stalk region residues (sNgR1 27–310) bound to AP-TAJ with 3-fold and 10-fold lower affinities, respectively. In the same study, the binding of p75 to NgR1 was also evaluated (Figure 1D). Full-length NgR1 (sNgR1 27–431)

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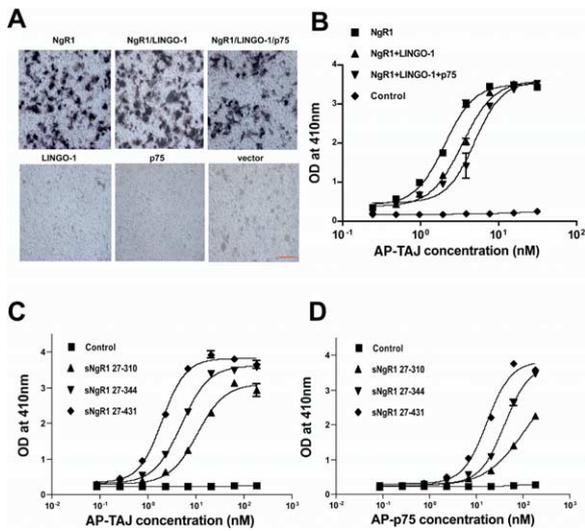


Figure 1. TAJ Binds NgR1

(A) AP-TAJ binding to CHO cells transfected with *NgR1*, *NgR1/LINGO-1*, *NgR1/LINGO-1/p75*, *LINGO-1*, *p75*, or vector alone (transfection efficiency 50%). Scale bar, 100  $\mu$ m. (B) Quantitation of AP-TAJ binding to CHO cells expressing *NgR1*, *NgR1/LINGO-1*, *NgR1/LINGO-1/p75*, or the vector-only control. ELISA of AP-TAJ binding (C) and of AP-p75 binding (D) to the various truncated forms of immobilized human *NgR1*. Data points in (B)–(D) are expressed as the mean  $\pm$  standard error of the mean.

bound AP-p75 with a 7-fold lower affinity than AP-TAJ, with an apparent  $K_D$  of 20 nM. As with AP-TAJ binding, shifts in titration curves for the binding of AP-p75 to sNgR1 27–344 and sNgR1 27–310 were observed; however, apparent  $K_D$  values could not be calculated, because maximal binding was not achieved at the highest concentration of AP-p75 tested. Finally, when compared to four other TNF family members (CD40, Fn14, TNF-R1, and TNF-R2) as Fc fusion proteins, only TAJ-Fc showed specific binding to *NgR1* (see Figure S3 in the Supplemental Data available with this article online).

The binding of *NgR1* by TAJ was further verified by immunoprecipitation studies. When lysates from COS-7 cells cotransfected with *Taj* and *NgR1* were subjected to immunoprecipitation with anti-*NgR1* antibodies, a TAJ-immunoreactive band was detected in Western blots probed with an anti-TAJ antibody (Figure 2A). No TAJ band was detected in control cells transfected with *NgR1/p75*, *NgR1/GFP*, *LINGO-1/Taj*, *LINGO-1/p75*, or *LINGO-1/GFP*. Similar experiments were performed with cells transfected with *Taj* and *LINGO-1*, in which immunoprecipitation was performed with anti-*LINGO-1* antibodies (Figure 2A). A strong immunoreactive band for bound TAJ protein was visualized in the *LINGO-1* immunoprecipitation that was not detected in cells transfected with *LINGO-1/p75*, *LINGO-1/GFP*, *NgR1/Taj*, *NgR1/p75*, or *NgR1/GFP*. The presence of TAJ in the *LINGO-1* immunoprecipitation indicates that, in addition to the TAJ/*NgR1* interactions, TAJ/*LINGO-1* interactions also occur. Since *LINGO-1* also binds *NgR1* (Mi et al., 2004), these data are consistent with a model wherein *NgR1*, *LINGO-1*, and TAJ interactions can lead

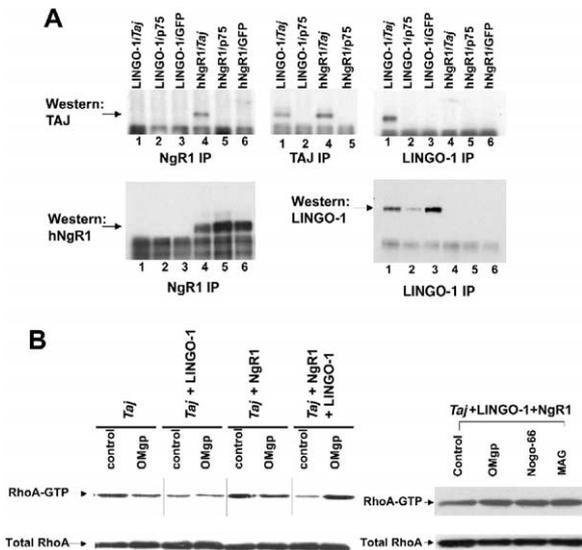


Figure 2. TAJ, *NgR1*, and *LINGO-1* Form a Functional Signaling Complex

(A) Coimmunoprecipitation of TAJ, *LINGO-1*, and *NgR1* by anti-*NgR1*, anti-*LINGO-1*, or anti-TAJ antibodies from COS-7 cells expressing combinations of *NgR1*, TAJ, and *LINGO-1* and detected by Western blotting.

(B) Effects of TAJ, *NgR1*, and *LINGO-1* expression on RhoA activation. (Left panel) RhoA activation data for COS-7 cells transfected with *Taj/NgR1*, *LINGO-1/Taj*, or *Taj/LINGO-1/NgR1* in the presence or absence of OMgp (10  $\mu$ g/ml) for 10 min. (Right panel) RhoA activation data for *Taj/LINGO-1/NgR1*-transfected COS-7 cells in the presence or absence of OMgp, Nogo-66, and MAG (all at 10  $\mu$ g/ml for 3 min).

to a structure analogous to the *NgR1/LINGO-1/p75* complex.

### The TAJ/*NgR1*/*LINGO-1* Complex Can Activate RhoA

Binding of the MAIF receptor by Nogo, OMgp, or MAG leads to the activation of RhoA, a step in the MAIF receptor signaling pathway that is critical for the inhibitory effects of myelin. To determine if the interactions between TAJ, *NgR1*, and *LINGO-1* are truly indicative of a functional MAIF receptor complex, we transfected COS cells with various combinations of *Taj*, *NgR1*, and *LINGO-1*, treated them with OMgp, Nogo-66, and MAG, and then measured the levels of activated RhoA in the cells. Only COS cells coexpressing *Taj*, *NgR1*, and *LINGO-1* showed increased RhoA activation when compared to control (Figure 2B). COS cells transfected with *Taj* alone, *Taj* and *LINGO-1*, or *Taj* and *NgR1* with OMgp showed no increase in RhoA GTP levels (Figure 2B, left panel). Similar data were obtained with MAG and Nogo-66 (data not shown). These findings indicate that the coexpression of TAJ, *LINGO-1*, and *NgR1* is sufficient to reconstitute a functional MAIF receptor, and therefore TAJ can substitute for p75 in the RhoA signaling pathway upon interaction with *NgR1* ligands.

### Soluble TAJ Reverses the Neurite Outgrowth Inhibition Caused by Myelin Inhibitors

We next tested whether the inhibitory response to MAIFs in normal CG neurons and DRG neurons can be

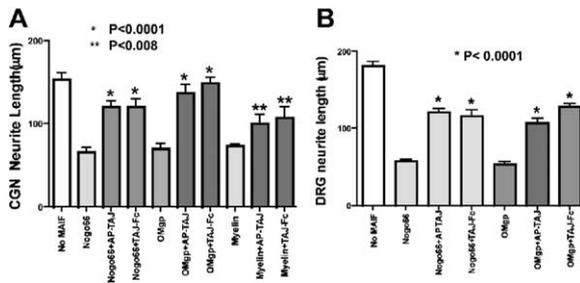


Figure 3. Effects of TAJ-Fc on Neurite Outgrowth

P7 mouse CG neurons or DRG neurons were grown on either no MAIF, OMgp, or Nogo-66 coated on poly-D-lysine slides, in combination with control-Fc, TAJ-Fc, or AP-TAJ as substrates. Statistical analysis by one-way ANOVA of neurite lengths from CG neurons (A) and DRG neurons (B) were shown, comparing TAJ- versus control-treated samples grown on MAIFs.

reversed with soluble TAJ presented as either TAJ-Fc or AP-TAJ. In theory, TAJ fusion protein will bind to NgR1 on neurons to produce a nonfunctional MAIF receptor, thereby promoting neurite outgrowth. Indeed, addition of AP-TAJ and TAJ-Fc led to longer CG neuron processes, suggesting that signaling by the MAIF receptor complex was compromised. Similar effects occurred when CG neurons were grown on myelin, Nogo-66, or OMgp (Figure 3A). Neurite outgrowth by DRG neurons was also restored when TAJ-Fc and AP-TAJ were added to the myelin inhibitor substrates (Figure 3B).

#### Analysis of Neurite Outgrowth Using Neurons from *Taj*-Deficient Mice

To further confirm the biological role of TAJ on axon regeneration, CG and DRG neurons from *Taj* knockout mice were tested in vitro using the neurite outgrowth assay. The *Taj* knockout mouse was generated as described in Figure S1A, and loss of *Taj* expression was confirmed using RT-PCR (Figure S1B). *Taj* knockout mice exhibited no obvious physical abnormalities or alterations in behavior, locomotion, or fecundity. To analyze the effects of *Taj* deletion on the response of neurons to MAIFs, postnatal day 7 (P7) CG and DRG neurons from *Taj* knockout and wild-type mice were plated onto myelin inhibitor-coated slides, and neurite outgrowth was measured 1 day later. Quantitation revealed that MAIF-treated neurons from *Taj*-deficient mice had longer processes than the corresponding wild-type neurons (Figures 4A–4D), with the effect in the DRG neuron cultures being particularly dramatic (Figures 4C and 4D). By varying the concentration of myelin, Nogo-66, or OMgp, it was shown that the *Taj* deficiency overcame the ability of 500 ng and 1 µg, but not 2 µg, of myelin inhibitors to block neurite outgrowth.

#### Expression of *Taj* in Rat Brain

Previous analyses have revealed that *Taj* is highly expressed in both mouse and human brain (Eby et al., 2000; Kojima et al., 2000; Pispas et al., 2003). Northern blot analysis of various regions of the human brain using a commercial blot showed that *Taj* was expressed in most regions of the brain (Figure S2A). Semi-quantitative RT-PCR revealed that purified cultures of

CG neurons, oligodendrocytes, astrocytes, and DRG neurons all expressed *Taj* (Figure S2B). The expression of *Taj* in brain was further localized by in situ hybridization. As shown in Figure 5A, a *Taj* antisense probe detected its expression in DRG neurons, cerebellar neurons, cortical neurons, and spinal cord neurons as evident from costaining with an anti-MAP-2 antibody that was used as a neuronal cell marker. No staining was detected using a *Taj* sense probe. To gain further insight into the potential composition of the MAIF receptor complex in the developing and adult brain, quantitative real-time RT-PCR was performed for *Taj*, *p75*, *NgR1*, and *LINGO-1* on whole rat brain homogenates taken over a developmental time course including days E14, E18, P0, P4, P8, and P23 and adult stage. *Taj* and *p75* showed different temporal expression patterns, with *p75* peaking at E14 and E18 but dropping in adulthood, and *Taj* expressing maximally at P0–P8 (Figure 5B). Substantially higher levels of *Taj* were present in P23 and adult samples when compared to *p75*. mRNA levels of *LINGO-1* and *NgR1* resembled those of *Taj*, with the highest at P4 and declining slowly into adulthood. An identical developmental expression pattern for *p75* and *Taj* was observed when CG neurons were subjected to the same quantitative real-time RT-PCR analysis. The relative abundance of the *Taj* and *p75* mRNAs on average were 5-fold higher in the CG neuron samples than in the whole-brain extracts (data not shown). The higher expression of *Taj* than *p75* in brain, particularly after birth, suggests an important biological role for the molecule in CNS function.

#### Discussion

The identification of NgR1 was a critical step in understanding the signaling pathway by which components of myelin, namely Nogo-66, OMgp, and MAG, inhibited neurite outgrowth (Fournier et al., 2001). However, the lack of a NgR1 intracellular signaling domain made it immediately evident that other molecules must be involved in MAIF inhibition. Two other molecules, *p75* and *LINGO-1*, have been identified that participate in the MAIF receptor complex in vitro (Mi et al., 2004; Wang et al., 2002). However, the physiological role of *p75* has been controversial because of its lack of expression in a number of MAIF-responsive neuronal populations (Coprav et al., 2004; Giehl et al., 2001; Mi et al., 2004; Song et al., 2004). For this reason, we were intrigued by TAJ, an orphan member of the TNF receptor family that is widely expressed in postnatal and adult neurons. The data presented here show that TAJ can bind NgR1 and substitute for *p75* in the Nogo receptor-dependent signaling pathway for neurite outgrowth. *p75* and TAJ appear to share similar domain interactions with NgR1, as truncated NgR1 containing stalk deletions showed severely reduced binding to both proteins. NgR1/LINGO-1/TAJ complexes responded to MAIFs to activate RhoA, showing that, as with *p75*, a functional complex is obtained with TAJ. Lastly, exogenously added TAJ-Fc, like *p75*-Fc (Wang et al., 2002), can reverse the MAIF inhibition of neurite outgrowth. We postulate that this is due to the binding of soluble TAJ to NgR1 expressed on the surface of the neurons, resulting in the formation of nonfunctional MAIF receptor complexes.

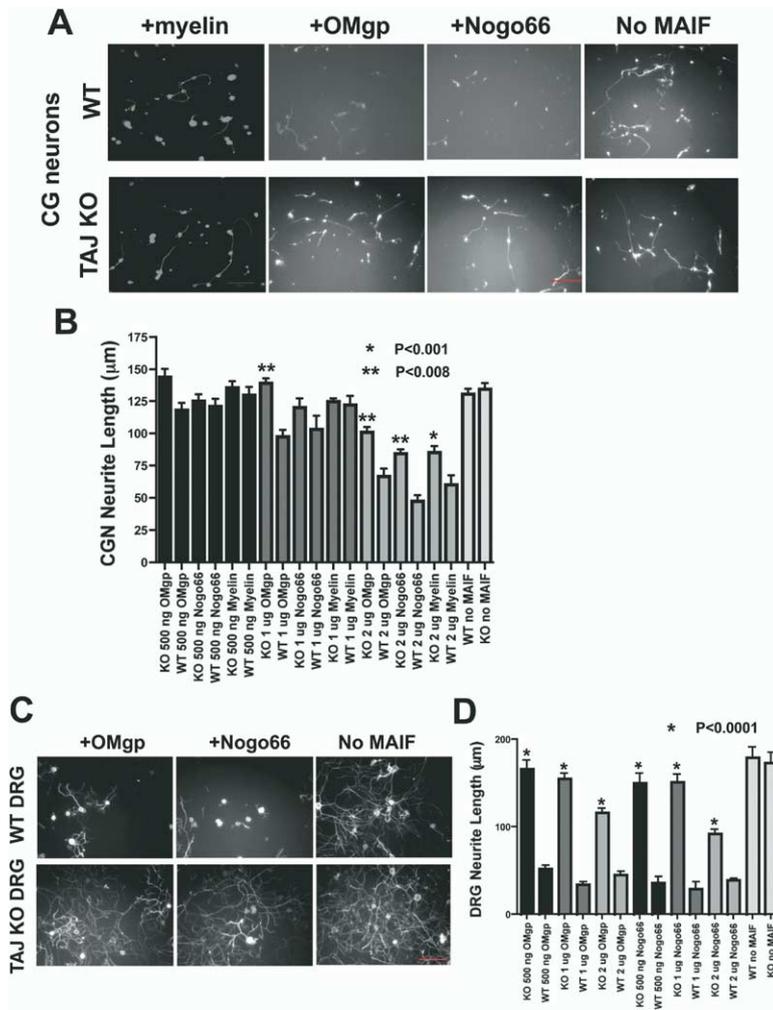


Figure 4. Neurite Outgrowth Using *Taj* Knockout Neurons

P7 CG neurons (CGN) (A and B) and DRG neurons (C and D) from wild-type or *Taj* knockout mice were grown on either myelin-, Nogo66-, or OMgp-coated slides (data from 1 µg MAIF treatments are shown). Scale bar, 50 µm. (B and D) Statistical analysis of neurite lengths in (A) and (C) by one-way ANOVA, comparing each treatment for the corresponding wt and KO samples.

The physiologic role of TAJ in MAIF signaling was verified by the altered response of cultured CG and DRG neurons from *Taj* knockout mice. When cultured on inhibitory myelin substrates, CG and DRG neurons from *Taj* knockout mice showed longer neurite outgrowth, i.e., decreased inhibition by MAIFs, as compared to the corresponding wild-type neurons. However, at the highest concentration of MAIFs tested, neurite outgrowth inhibition remained in the knockout samples, suggesting that other molecules such as p75 can substitute for TAJ. The functional similarities between p75 and TAJ in vitro may translate into redundancies in vivo that account for the lack of CNS defects in *p75* (Song et al., 2004) and *Taj* knockout mice (this paper). There is also the possibility, since LINGO-1 and NgR1 are members of paralog gene families (Mi et al., 2004; Pignot et al., 2003), that additional diversity in subunit composition may exist in MAIF receptor complexes.

p75 and TAJ also exhibit significant differences. Since p75 is a receptor for neurotrophins, we tested and failed to detect TAJ binding to NGF by BiaCore analysis (data not shown), although the entire spectrum of neurotrophins requires further examination. Importantly, while p75 and TAJ are both expressed in the em-

bryonic brain, where they are likely to play a role in neurogenesis and differentiation, only *Taj* mRNA levels are maintained at significant levels in the adult brain (this paper and Kojima et al., 2000), which interestingly parallels both *NgR1* and *LINGO-1* expression. The observed differences in the gene expression patterns of TAJ and p75, specifically the limited expression of p75 after birth, suggest that TAJ may play an even more important role than p75 in CNS axon regeneration in adulthood. In addition to its putative participation in neurite outgrowth inhibition, p75, as a neurotrophin receptor, is involved in neuronal proliferation, survival, and death (Chao et al., 1995). It remains to be determined if TAJ shares similar functions. TAJ has been associated with migrating/proliferating embryonic neuron populations (e.g., hippocampus, thalamus, cortex) (Hisaoka et al., 2003), and its overexpression can trigger caspase-independent cell death (Wang et al., 2004).

In summary, we have identified TAJ as a critical component of the NgR1 receptor complex. TAJ is broadly expressed in postnatal and adult neurons and can substitute for p75 in the p75/LINGO-1/NgR1 complex to activate the RhoA signaling pathway regulating neurite outgrowth. The subunit complexity of the MAIF receptors may indicate that receptor action is tailored to dif-

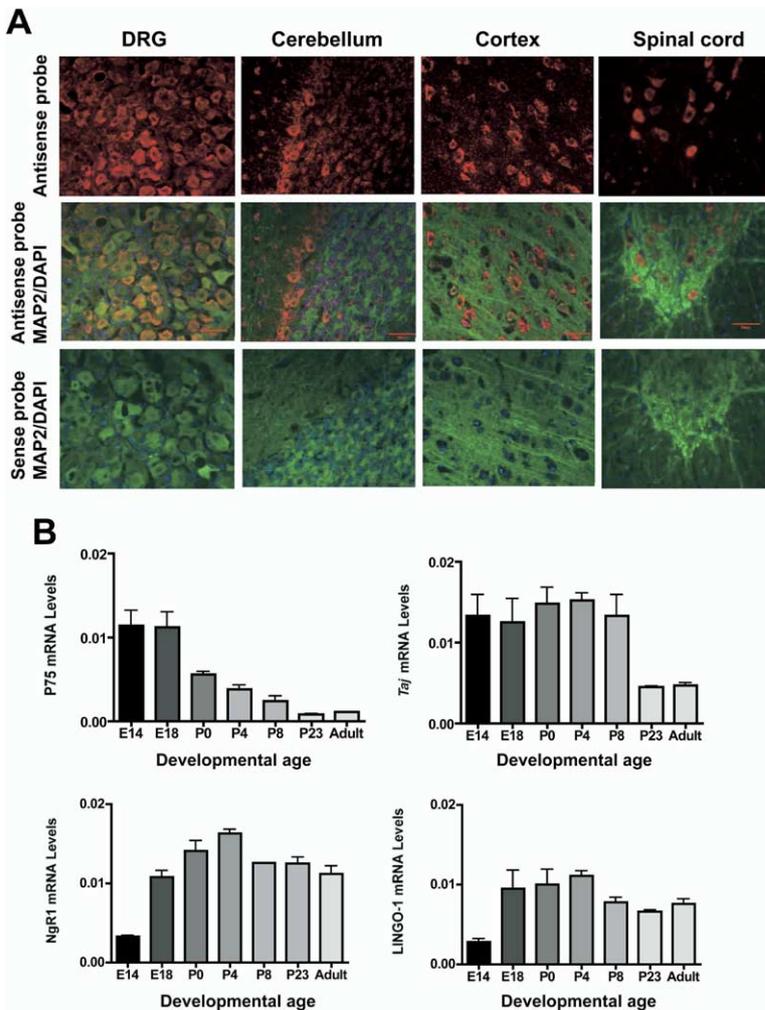


Figure 5. Expression of *Taj* in the CNS

(A) In situ hybridizations showing *Taj* expression in rat brain and spinal cord sections. Red, probed with *Taj* antisense or sense mRNA; green, stained with anti-MAP2 antibody; blue, DAPI staining. Scale bars, 50  $\mu$ m. (B) Taqman RT-PCR quantitation of *p75*, *Taj*, *LINGO-1*, and *NgR1* mRNA levels in mouse brain at different developmental stages. Data were normalized with respect to *GAPDH* mRNA levels.

ferent settings by variations in its molecular composition. Resolution of the actual biochemical picture and TAJ's involvement in MAIF receptor signaling should contribute considerably to solving this puzzle.

#### Experimental Procedures

##### Reagents

TAJ-Fc (residues 1–168 of mouse TAJ fused to the hinge and Fc region of human IgG1) was expressed in CHO cells and purified on Protein A Sepharose as described (Browning et al., 1995). AP-TAJ (human placental AP with an N-terminal six-histidine tag and fused at its C terminus to human TAJ residues 26–167) was expressed in CHO cells. AP-TAJ was purified by sequential chromatography steps on TMAE-Fractogel (EM Industries) and Ni-NTA Agarose (Qiagen). Similar strategies were used for the construction, expression, and purification of AP-OMgp (human placental AP with an N-terminal six-histidine tag and fused at its C terminus to human OMgp residues 18–392) and AP-75 (human placental AP with an N-terminal six-histidine tag and fused at its C terminus to rat p75 residues 31–243) proteins. AP-Nogo-66 was expressed in 293 cells. Human NgR1 (amino acid residues 27–431) was expressed in CHO cells and purified on TMAE-Fractogel and Ni-NTA Agarose. Human NgR1 (27–310) and NgR1 (27–344) were expressed as histidine-tagged proteins in baculovirus and purified on Ni-NTA Agarose.

##### AP Binding Assay

Ninety percent confluent CHO cells were transfected with various combinations of human *NgR1*, rat *p75*, and human *LINGO-1* ex-

pression plasmids using Fugene 6 reagents (Roche). After 48 hr, the transfected cells were washed with Hank's balanced salt solution, 1 mg/ml BSA, 20 mM HEPES (pH 7.0) (HBH) and incubated for 1.5 hr at 23°C with 4  $\mu$ g/ml (25 nM) of AP-TAJ or other AP fusion proteins in HBH. Bound AP-TAJ was detected directly using BCIP/NCT (Pierce). In other studies, cells were treated with serial dilutions of AP-TAJ or control AP only, and following detergent lysis of the cells, enzymatic activity was quantified with 4-nitrophenylphosphate (4-NPP) (Flanagan et al., 2000) (Mi et al., 2004). Each sample was analyzed in triplicate, and dose-response curves were generated using GraphPad Prism software.

##### ELISA

ELISA plates (Costar) were coated overnight at 4°C with 10  $\mu$ g/ml soluble NgR1 (human NgR1 residues 27–431, 27–344, or 27–310) in 50 mM sodium bicarbonate (pH 9.2), blocked with 1% BSA, 0.1% ovalbumin, and 0.1% nonfat dry milk in Hank's balanced salt buffer plus 25 mM HEPES (pH 7.0) for 2 hr at 37°C, and incubated at 4°C overnight with serial dilutions of AP-TAJ, AP-p75, or control-AP in blocking buffer with 0.1% BSA. The plates were washed with PBS plus 0.05% Tween-20 after each step. Bound AP-TAJ was quantified with 4-NPP. Each sample was analyzed in triplicate, and dose-response curves were generated using GraphPad Prism software.

##### Immunoprecipitations and Western Blots

COS-7 cells (100 mm dishes) were transfected with combinations of full-length human *Taj* (human TAJ residues 27–417 were inserted into pV90 HA tag expression vector), human *NgR1*, human *LINGO-1*, and rat *p75*. The cells were harvested after 48 hr and lysed in 1 ml

lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1% Triton X-100, and 10% glycerol) for 30 min at 4°C. After centrifugation at 14,000 × g for 15 min, the supernatants were incubated with Protein A/G-Sepharose beads (Santa Cruz Biotechnology, Inc.) at 4°C for 1 hr and then incubated at 4°C for 1 hr with either anti-Ngr1 antibody (6F8; Biogen Idec) or anti-TAJ antibody (A13.F1.8; Biogen Idec) plus Protein A/G-Sepharose beads, or myc-tagged anti-LINGO-1 antibody (1968; Biogen Idec) plus anti-myc (9E10) agarose beads. The beads were washed three times with lysis buffer and boiled in Laemmli sample buffer. Samples were subjected to 4%–20% SDS-PAGE and analyzed by Western blotting with anti-Ngr1 (7E11; Biogen Idec), anti-LINGO-1 (201; Biogen Idec), or anti-TAJ/TROY (Alexis Biochemical) antibodies.

#### Taj mRNA Quantitation

Taj mRNA from purified cultures of P6–7 CG neurons, P2 cerebral cortex oligodendrocytes, P4 cerebral cortex astrocytes, and P7 DRG neurons were prepared as described (Mi et al., 2004) using Trizol reagent following the manufacturer's protocol (Invitrogen). Purified RNAs were used to generate cell type-specific cDNAs (GeneAmp kit; Applied Biosystems) that served as PCR templates for Taj evaluation. The cDNAs were analyzed by semiquantitative PCR using forward primer 5'-tatggggaggatgcacagtggtg and reverse primer 5'-agaccagctgggtttcttctccat. Amplification of GAPDH was carried out in parallel for normalization purposes.

Quantitative real-time PCR was performed for Taj, p75, NgR1, and LINGO-1 on whole mouse brain homogenates taken over a developmental time course. Mouse mRNA was extracted from embryonic day 14 (E14), E18, P0, P4, P8, P23, and adult brains using Trizol reagent (Invitrogen). These RNAs were subjected to Taqman RT-PCR to quantify Taj, p75, LINGO-1, and NgR1 mRNA levels, using the following forward primers, reverse primers, and FAM-labeled probes. p75 forward primer is 5'-CCATCTTGGCTGCTG TGGTT, reverse primer is 5'-GCTGTTCCATCTCTTAAAGCAA, and probe is 5'-TGGGCCTTGTGGCTT. NgR1 forward primer is 5'-AAG TGCTGCCAGCCAGATG, reverse primer is 5'-GCCTCCCGGTTT CAGTA, and probe is 5'-TGCAGACAAAGCCTC. Taj forward primer is 5'-CCCTCAATCCCGAAAACGA, reverse primer is 5'-TGGCCGC CACTGGAAT, and probe is 5'-AGCGCAGCATCTC. LINGO-1 forward primer is 5'-CTTCCCCTTCGACATCAAGAC, reverse primer is 5'-CAGCAGCACCCAGGCAGAA, and probe is 5'-ATCGCCACCA CCATGGGCTTCAT. The primers and FAM-labeled probes were designed using Primer Express v1.0 (Applied Biosystems).

#### Assay of Rho Activation

COS cells were transfected with various combinations of Taj, NgR1, and LINGO-1. After 48 hr, the transfected cells were treated with AP-OMgp, GST-Nogo-66, MAG-Fc, or control (all at 10 µg/ml). Cells were then lysed in 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl<sub>2</sub>, plus protease inhibitors. GTP bound and total RhoA proteins were detected by Western blotting using anti-RhoA mAb (Santa Cruz) as described (Mi et al., 2004). Each experiment was repeated three times. Data shown are from a representative study.

#### Taj Knockout Mice

Taj knockout mice were created by deleting the first two coding exons for Taj using homologous recombination technique with a human CD2-neomycin fusion construct (Figure S1A). The absence of Taj mRNA expression was verified by RT-PCR of Taj knockout mouse brain RNA. Forward primer is 5'-ACTCAAGGTCTACCT CTACACA, and reverse primer is 5'-GAAGCCACATTCCTTGGGA CAA. Taj knockout mice were created on a mixed Sv129/C57Bl6 background and backcrossed to C57Bl6.

#### Preparation and Analysis of Primary CGN and DRG Neuron Cultures

Primary CG neuron or DRG neuron cultures were prepared using 4-well Labtek culture slides coated with 0.1 mg/ml poly-D-lysine (Sigma) before spotting with 1 µg/3 µl AP-Nogo-66, or 1 µg/3 µl AP-OMgp, or 1 µg/3 µl bovine myelin alone or in combination with 1 µg/3 µl TAJ-Fc or 1 µg/3 µl AP-TAJ or control-Fc (human IgG1).

The slides were dried at room temperature for 2 hr prior to plating of cells. CG neurons (1.5 × 10<sup>5</sup> cells per well) or 5000 DRG neurons per well from P7 mice were dissociated, seeded onto the slides, and incubated at 37°C in 5% CO<sub>2</sub> for 16 hr. The slides were fixed in 4% paraformaldehyde/20% sucrose and stained with anti-βIII-tubulin (Covance TUJ1). Average neurite length was quantified by measuring lengths of individual neurites from 12 microscope fields (300 to 500 cells per sample) and dividing by the total number of cells counted. Data are presented as mean ± standard error of the mean. Counting/measuring was done using OpenLab software (Improvision, Inc., Lexington, MA).

#### In Situ Hybridization

Rat brain, spinal cord, and dorsal root ganglia frozen sections were prepared and processed as described (Mi et al., 2004) and were probed with digoxigenin-labeled Taj antisense and sense RNA. Sections were stained using the TSA plus fluorescence anti-digoxigenin-conjugated antibodies kit (Perkin Elmer) following the manufacturer's instructions.

#### Supplemental Data

Supplemental Data include three figures and can be found with this article online at <http://www.neuron.org/cgi/content/full/45/3/353/DC1/>.

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