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Assessing spinal axon regeneration and sprouting in Nogo, MAG and OMgp deficient mice

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SUMMARY

A central hypothesis for the limited capacity for adult central nervous system (CNS) axons to regenerate is the presence of myelin-derived axon growth inhibitors, the role of which, however, remains poorly understood. We have conducted a comprehensive genetic analysis of the three major myelin inhibitors, Nogo, MAG and OMgp, in injury-induced axonal growth, including compensatory sprouting of uninjured axons and regeneration of injured axons. While deleting any one inhibitor in mice enhanced sprouting of corticospinal or raphespinal serotonergic axons, there was neither associated behavioral improvement nor a synergistic effect of deleting all three inhibitors. Furthermore, triple mutant mice failed to exhibit enhanced regeneration of either axonal tract after spinal cord injury. Our data indicate that while Nogo, MAG and OMgp may modulate axon sprouting, they do not play a central role in CNS axon regeneration failure.

Keywords

axon regeneration; axon sprouting; spinal cord injury; central nervous system (CNS) repair; myelin inhibition; corticospinal tract (CST); serotonergic axons

INTRODUCTION

A fundamental problem that impedes functional recovery from central nervous system (CNS) injuries is the failure of damaged adult CNS axons to regenerate. This limited ability for axon regeneration has been attributed to the growth inhibitory nature of CNS myelin (Gonzenbach and Schwab, 2008), the glial scar at the injury site (Silver and Miller, 2004), an insufficiency of growth-promoting/permissive factors and/or tissue bridges (Lu and Tuszynski, 2008), and a lack of neuron-intrinsic growth potential (Moore et al., 2009; Park et al., 2008). Despite considerable advances in our understanding of all four areas, it is yet not known what is primarily responsible for the lack of significant axon regeneration in the adult CNS.

Among the contributors to CNS regeneration failure, the molecular pathways underlying myelin inhibition of axon growth have been most extensively characterized (Filbin, 2003;

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Yiu and He, 2006). Three major myelin-derived inhibitors have been identified: Nogo (or Rtn4), MAG (myelin-associated glycoprotein) and OMgp (oligodendrocyte myelin glycoprotein, or Omg); each possesses potent inhibitory activity on neurite outgrowth *in vitro*. Extensive biochemical and cell culture studies have led to the current working model where the three inhibitors signal through multiple neuronal receptors including the shared ligand-binding receptors NgR1 and PirB as well as co-receptors such as $p75^{NTR}$, TROY and LINGO-1, with downstream effectors such as Rho and Rho-associated kinase (ROCK) (Atwal et al., 2008; Yiu and He, 2006). Despite these elegantly elucidated biochemical pathways, the role of Nogo, MAG and OMgp and that of myelin inhibitors in general in axon regeneration *in vivo* remain poorly understood.

Much of the initial evidence implicating Nogo in axon regeneration came from experiments showing that administration of the IN-1 antibody, which recognizes – but is not specific to – Nogo (Caroni and Schwab, 1988), promoted axon regeneration of the corticospinal tract (CST) after experimental spinal cord injury (Brosamle et al., 2000; Schnell and Schwab, 1990). More specific antibodies produced more limited effects (Liebscher et al., 2005). Furthermore, experiments with a function-blocking peptide that interferes with Nogo-NgR1 interaction generated mixed results (GrandPre et al., 2002; Li and Strittmatter, 2003; Steward et al., 2008a).

Genetic studies in mice have been applied to provide a more definitive assessment of the role of myelin inhibitors in axon regeneration (Zheng et al., 2006). However, no consistent and robust regeneration has been reported in mice lacking Nogo (Cafferty et al., 2007; Dimou et al., 2006; Kim et al., 2003; Lee et al., 2009b; Simonen et al., 2003; Steward et al., 2007; Zheng et al., 2003). Enhanced axon growth in an OMgp mutant has been reported, but it was not clear whether the injury models specifically assessed regeneration or sprouting (see below), and the CST did not regenerate in this mutant (Ji et al., 2008). For MAG, genetic studies have not implicated any significant role in injury-induced axonal growth (Bartsch et al., 1995). These observations, together with the ability of Nogo, MAG and OMgp to signal through common receptors, have led to the hypothesis that the three inhibitors play redundant roles in restricting axon regeneration (Filbin, 2003; Liu et al., 2006; Zheng et al., 2006).

To assess the combined contribution of all three inhibitors, we generated a Nogo/MAG/OMgp triple null mutant and investigated its axon growth phenotype after injury. We applied four different injury models to examine the compensatory sprouting of uninjured axons and the regeneration of injured axons in two different axonal tracts. Results from these experiments illustrate that while modulating axon sprouting, the three major myelin-derived axon growth inhibitors do not play a central role in axon regeneration failure in the injured CNS.

RESULTS

Generation and baseline characterization of Nogo/MAG/OMgp triple null mutants

We have recently described the generation of a Nogo deletion mutant and an OMgp null mutant (Lee et al., 2009a; Lee et al., 2009b). The Nogo deletion mutant differs from all other Nogo mutants published in that it is the only Nogo null that is fully viable and lacks the expression of all known Nogo isoforms including Nogo-A,B,C, and thus would allow for unequivocal assessment of all Nogo isoforms (Lee et al., 2009b). Given the unusual location of the *OMgp* gene in the intron of the Neurofibromin 1 gene (*NF1*), we designed the OMgp mutation to minimize any effect on NF1 expression (Lee et al., 2009a). The Nogo and OMgp mutants were bred to a previously characterized MAG null mutant (Li et al., 1994) to obtain Nogo/MAG/OMgp triple null mutants in a mixed background with 129S7 and

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C57BL/6 (see supplemental Experimental Procedures), which proved viable, fertile and exhibited no gross morphological defects. Western blot analysis on total brain protein extracts confirmed the absence of Nogo-A, MAG and OMgp proteins and further indicated that the expression of their common receptors, NgR1 and PirB, was not altered in the triple mutants (Figure 1A).

In baseline behavioral assays, Nogo mutants exhibited subtle defects in open field locomotion as assessed by the Basso Mouse Scale (BMS) (Basso et al., 2006), while both MAG and OMgp single mutants exhibited defects in a rotarod test (Figures 1B and 1C). However, deleting all three proteins did not appear to cause an overt synergistic effect. In addition, none of the mutants showed any defect in a gridwalk or forepaw preference test (Figures 1D and 1E). In subsequent experiments, wherever appropriate, we used a behavioral assay that showed no or minimal baseline deficits as the primary behavioral measure following injury.

The effects of deleting one or all three inhibitors on neurite outgrowth *in vitro*

We first asked whether deleting all three myelin inhibitors would lead to an increased release of myelin inhibition of neurite outgrowth as compared with deleting Nogo alone. As expected, neurite outgrowth from postnatal day 7 cerebellar granule neurons was inhibited by wild type (WT) myelin (Figures 2A, 2B and 2E). Consistent with previous studies using Nogo-A,B or Nogo-A deficient myelin (Kim et al., 2003; Simonen et al., 2003; Zheng et al., 2003), this inhibition was partially released on Nogo null myelin (Figures 2C and 2E). However, this reduction in neurite inhibition was not enhanced by deleting all three inhibitors from the myelin (Figures 2D and 2E). MAG deficient myelin was as inhibitory as WT myelin, whereas OMgp deficient myelin displayed a trend for reduced inhibition that was not statistically significant (Figure 2E).

To determine whether this result can be extended to another neuronal type, we measured neurite outgrowth from dissociated adult dorsal root ganglion (DRG) neurons or DRG explants grown on spinal cord cryosections, a more physiologically relevant substrate than purified myelin. Similar to cerebellar neurons on myelin substrates, neurite outgrowth from DRG neurons was enhanced on triple null spinal cord sections to the same extent as on Nogo null sections in comparison to WT controls (Figures 2F–2K). MAG null sections were as inhibitory as WT controls whereas, again, OMgp null sections displayed a trend for reduced inhibitory activity that did not reach statistical significance (Figures 2H and 2K). Taken together, these *in vitro* assays indicate that, for the two neuronal types analyzed, deleting all three inhibitors, Nogo, MAG and OMgp, does not lead to significantly more neurite outgrowth than deleting Nogo alone.

The effects of deleting one or all three inhibitors on serotonergic axon sprouting

To ascertain whether deleting the three inhibitors enhances injury-induced axonal growth *in vivo*, we used four different injury models to assess axon regeneration or compensatory sprouting of two major descending pathways: the raphespinal serotonergic tract and the corticospinal tract (CST). These two axonal tracts are known to possess different growth capabilities after injury: the CST is very refractory to regeneration while the raphespinal serotonergic tract exhibits a high growth capacity following injury (Hollis et al., 2009; Saruhashi et al., 1996). Here we define *regeneration* as growth of injured axons beyond the injury site and *sprouting* as growth of uninjured axons for relatively shorter distances in response to an injury elsewhere in the CNS. We characterize both phenomena as *injury-induced axonal growth*.

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We first analyzed compensatory sprouting of raphespinal serotonergic axons across the midline following a lateral hemisection. These descending axons originate in the raphe nuclei in the brainstem, travel down the spinal cord and modulate locomotion (Schmidt and Jordan, 2000). Because serotonergic axons have a high growth capacity following injury, and because sprouting across the midline does not involve growth through a glial scar, another potent inhibitory barrier to regeneration (Silver and Miller, 2004), we reasoned that this model would be among the most likely to reveal enhanced injury-induced axonal growth in mice deficient in myelin inhibitors.

Mice were subjected to a right lateral hemisection (illustrated in Figure S1A) at the eighth thoracic (T8) vertebral level, leading to depletion of serotonergic input in the caudal ipsilateral spinal cord. Sprouting of uninjured serotonergic axons from the contralateral side, as assessed by 5-hydroxytryptamine (or 5-HT) immunoreactivity, was analyzed at the lumbar enlargement below the level of injury. Four weeks after injury, MAG and OMgp single mutants, but not Nogo mutants, exhibited an elevated level (~2 fold) of 5-HT immunoreactivity on the denervated side as compared with WT mice. However, this was not further enhanced in the triple mutants (Figures 3A–3C). This enhanced sprouting was not due to any gross developmental defects of the raphespinal serotonergic tract in the mutants (Figures S1B–S1D). Despite the increased serotonergic axon sprouting, MAG, OMgp and triple mutants did not perform significantly better than WT controls in the gridwalk (Figure 3D) or the BMS open field test (data not shown). Thus, deleting MAG or OMgp enhances compensatory sprouting of serotonergic axons while there is no additive or synergistic effect of deleting all three inhibitors.

The effects of deleting one or all three inhibitors on corticospinal axon sprouting

To determine whether an axonal tract that is more refractory to injury-induced growth displays increased sprouting in the mutants, we applied a pyramidotomy model to study compensatory sprouting of uninjured CST axons across the spinal cord midline. The CST originates from the sensorimotor cortex and crosses the midline at the medullary pyramids before the main cohort of CST axons course down the contralateral spinal cord through the ventral part of the dorsal column in rodents and innervate the contralateral gray matter. The CST was severed unilaterally at the (right) medullary pyramid above the pyramidal decussation (the point of CST axons crossing the midline), resulting in complete denervation of the contralateral (left) spinal cord from corticospinal neurons in the right sensorimotor cortex (Figures 4A and 4B). The neuronal tracer biotinylated dextran amine (BDA) was injected into the left sensorimotor cortex to trace uninjured CST axons (Figure 4C) corresponding to the right forelimb area. BDA labeling efficiency did not significantly differ between different genotypes (Figures S2A–S2C). To further control for variations in BDA labeling among individual animals, the axonal counts in the contralateral gray matter (as an indicator of axon sprouting) were normalized against the total number of BDA labeled CST axons in the medullary pyramid for each animal (see supplemental Experimental Procedures). The lesion was verified by the unilateral loss of PKCγ immunoreactivity in the spinal cord (Figures S2D–S2F), which marks the main CST.

Pyramidotomy in WT mice induced spontaneous sprouting of CST axons across the midline from the intact to the contralateral side (Figures 4D, 4E, and 4J). Consistent with a previous study using a Nogo-A,B mutant line (Cafferty and Strittmatter, 2006), Nogo null mutants exhibited enhanced CST sprouting, albeit at levels lower than expected based on the previous report (Figures 4F and 4K). Whereas OMgp mutants did not differ significantly from WT controls, MAG mutants unexpectedly displayed reduced CST sprouting (Figures 4G, 4H and 4K). In line with this observation, the degree of CST sprouting in the triple mutants was similar to that of WT mice (i.e. intermediate between Nogo and MAG mutants), suggesting antagonistic effects of deleting Nogo and MAG (Figures 4I, 4J and

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4K). Despite the spectrum of CST sprouting phenotypes in various mutants, recovery of forepaw preference during rearing behavior after pyramidotomy (Starkey et al., 2005) did not differ significantly among genotypes (Figure 4L).

Because the reduced CST sprouting in MAG mutants was unexpected, we sought to confirm this finding with a different injury model. In the dorsal hemisection model used to assess CST axon regeneration (illustrated in Figure S4A, described below), CST axons rostral to the injury site often sprouted into the contralateral gray matter in WT mice (Figures S3A and S3B). Consistent with reduced CST sprouting in the pyramidotomy model, CST axons rostral to the injury site in MAG mutants also displayed reduced sprouting into the contralateral gray matter after dorsal hemisection (Figures S3C–S3E). This was not due to a difference in BDA labeling efficiency, because 1) the average total number of CST axons labeled in the medullary pyramids did not differ between the two genotypes (Figure S3F), and 2) inter-animal variability in labeling efficiency was further controlled by normalizing the axonal density on transverse spinal cord sections against the total number of BDA labeled CST axons in the medullary pyramid. Taken together, genetic deletion of Nogo increases sprouting of CST axons while deleting MAG decreases CST sprouting, and there is no synergistic effect of deleting all three proteins.

Lack of enhanced corticospinal or raphespinal serotonergic axon regeneration in Nogo/MAG/OMgp triple mutants

Axon regeneration has been pursued as a major goal when targeting myelin-derived axon growth inhibitors (Gonzenbach and Schwab, 2008), but genetically deleting Nogo, MAG or OMgp in mice leads to no or little enhancement of axon regeneration (Bartsch et al., 1995; Ji et al., 2008; Kim et al., 2003; Lee et al., 2009b; Simonen et al., 2003; Steward et al., 2007; Zheng et al., 2003). To determine whether deleting all three inhibitors would promote axon regeneration after spinal cord injury, we analyzed CST and serotonergic axon regeneration in the triple mutants following a dorsal hemisection and a complete transection of the spinal cord respectively. Following a T8 dorsal hemisection injury (illustrated in Figure S4A), unilateral BDA tracing was performed to assess the regeneration of CST axons corresponding to the hindlimb area. In both WT controls and triple mutants, injured CST axons exhibited typical retraction from the injury site and were rarely detected at the injury site or more caudal regions (Figures 5A and 5B), which was confirmed with quantification of the axon density along the rostral – caudal axis (Figure 5C). Consistent with the lack of enhanced regeneration, behavioral analyses did not reveal any significant differences between the two genotypes in the gridwalk (Figure 5D), open field locomotion or rotarod tests (Figures S4C and S4D) throughout the 6-week survival period. To address the possibility that deleting MAG could counteract any detectable regeneration-promoting effect of deleting Nogo and OMgp, we also analyzed the three single mutant lines individually. We found no evidence of significant CST regeneration after dorsal hemisection in Nogo, MAG or OMgp single mutants (Figure S5), confirming previous studies (Bartsch et al., 1995; Ji et al., 2008; Lee et al., 2009b). Thus, deleting any one inhibitor (Nogo, MAG or OMgp) or the three inhibitors altogether does not promote CST axon regeneration.

To study raphespinal serotonergic axon regeneration, we subjected the triple mutants to a complete transection spinal cord injury (illustrated in Figure S4B). Because serotonergic axons descend throughout the dorsal and ventral spinal cord, this model is required to completely eliminate these axons en route to caudal segments. Lesion effectiveness was verified by a GFAP-negative area at the lesion epicenter in serial sections throughout the entire width of the spinal cord (Figures 5E, 5F and data not shown). Serotonergic axons were observed approaching the injury site and exhibited the highest density immediately rostral to the injury site in both WT and triple mutant mice. Quantification revealed a significantly higher level of 5-HT immunoreactivity just rostral to injury in the triple

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mutants (Figure 5G), indicative of enhanced serotonergic axon sprouting consistent with data from the lateral hemisection model (Figures 3A–3C). However, serotonergic axons did not traverse the injury site to any significant extent in either WT controls or triple mutants (Figures 5E–5G). In addition, mice of both genotypes displayed similarly minimum open field locomotor recovery as assessed by the BMS score (Figure 5H). Thus, deleting all three inhibitors does not promote raphespinal serotonergic axon regeneration.

DISCUSSION

The failure of axon regeneration in the injured adult CNS is a fundamental problem in neuroscience. Myelin inhibition of axon growth has been proposed as a major mechanism of CNS axon regeneration failure. However, considerable controversies exist regarding the role of myelin inhibitors in regeneration *in vivo* (Bartsch et al., 1995; Cafferty et al., 2007; Kim et al., 2003; Simonen et al., 2003; Steward et al., 2007; Zheng et al., 2003). Here we presented data from rigorous and comprehensive genetic analyses of Nogo, MAG and OMgp, the three major myelin inhibitors, in injury-induced axonal growth. By applying different injury models, we investigated two distinct forms of axonal growth: compensatory sprouting of uninjured axons and regeneration of injured axons. By comparing the phenotypes of mice lacking one or all three inhibitors, we addressed the question of functional redundancy among the three inhibitors. In addition, our analysis encompassed two axonal tracts with different growth capabilities. Results from these experiments provided important new insights on the role of Nogo, MAG and OMgp in injury-induced axonal growth and have implications on therapeutic development for spinal cord injury by targeting these molecules.

First and foremost, our data demonstrate that, contrary to what has been proposed, Nogo, MAG and OMgp are not primarily responsible for the limited axon regeneration in the adult CNS. Axon regeneration is defined here as the growth of injured axons beyond the injury site. Using a dorsal hemisection and a complete transection injury model respectively, we found that deleting Nogo, MAG and OMgp did not promote CST or serotonergic axon regeneration. Furthermore, no improved behavioral recovery was observed in Nogo/MAG/OMgp triple mutants after injury. In the complete transection model, the lack of enhanced serotonergic axon regeneration can be potentially explained by the inability of any regenerating axons to penetrate the glial scar, the complex role of which is highlighted by the recent discovery of the opposing effects of macrophages and NG2+ cells on axonal dieback in addition to the more extensively characterized roles of reactive astrocytes (Busch et al., 2010). In the case of dorsal hemisection, however, the spared ventral spinal cord could serve as a tissue bridge through which CST axons may regenerate (Steward et al., 2008b). Thus, another and perhaps more general mechanism, such as the neuron-intrinsic growth potential (Park et al., 2008), may be the determining factor here. It has long been hypothesized that Nogo, MAG and OMgp are functionally redundant in restricting axon regeneration (Filbin, 2003; Liu et al., 2006; Zheng et al., 2006), which predicted a synergistic effect of deleting all three inhibitors. Our results demonstrate that the limited axon regeneration after deleting any one inhibitor cannot be simply attributed to the proposed functional redundancy among the three inhibitors.

Compared with our data on axon regeneration, our finding that deleting a single inhibitor enhances the compensatory sprouting of uninjured axons expands current literature indicating a role for Nogo in axon sprouting (Cafferty and Strittmatter, 2006; Raineteau et al., 2001; Thallmair et al., 1998). Sprouting is defined here as the growth of uninjured axons in response to an injury elsewhere in the CNS. It should be noted that compensatory axon sprouting can occur spontaneously after injury (Weidner et al., 2001). Our data indicate that such naturally occurring axon sprouting can be modulated by targeting not just Nogo, but

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also MAG or OMgp. While deleting any one inhibitor enhanced the sprouting of CST or serotonergic axons, deleting all three inhibitors did not further enhance sprouting. This lack of a synergistic effect may be due to a lack of functional redundancy among the three inhibitors, and/or a ceiling effect – and thus indicates a limitation – of modulating the myelin-associated inhibitory environment.

Surprisingly, deleting MAG even reduced CST axon sprouting. This may reflect a role for MAG in axon protection (Nguyen et al., 2009), growth promotion (Hasegawa et al., 2004), or simply the possibility that deleting MAG diminishes tracer transport to the distal extremities of axons. Regardless, the reduced CST sprouting in MAG single mutants presents a caveat in interpreting the data on CST sprouting in the Nogo/MAG/OMgp triple mutants where deleting Nogo and MAG may have counteracting effects. It remains possible that deleting Nogo and OMgp but not MAG may be more effective in inducing CST sprouting than deleting Nogo alone. Future work on Nogo/OMgp double mutants is required to address this question. A second caveat in interpreting the sprouting effect in the various mutants is the possible effects of genetic background variations on injury-induced axonal growth (Ma et al., 2004). The original single mutant mice used to establish the triple mutant line had mixed background of 129S7 and C56BL/6. Although we took precautions against genetic drifts (see Experimental Procedures), our use of "cousins" rather than siblings as controls leaves open the possibility of strain-dependent effects. The use of single sex sibling controls, however, would have been practically impossible since intercrosses between triple heterozygous mice would only have a yield of 1/128 for any particular genotype. An alterative approach would be backcrossing each of the single mutant to a pure strain followed by intercrosses to generate triple mutant mice. We are currently performing these backcrosses to address this issue in future.

Both axon regeneration and axon sprouting can lead to functional recovery (Bradbury and McMahon, 2006). In this regard, however, we found that enhanced axonal sprouting in the mutants was not associated with a detectable enhancement in behavioral recovery. This result indicates that the modest degree of enhancement in axon sprouting (~50–100% increase) observed in the mutants is insufficient to elicit robust functional benefits. Therefore, the physiological significance, if any, of the enhanced sprouting after deleting Nogo, MAG and/or OMgp remains to be established.

While deleting Nogo promoted CST axon sprouting, deleting MAG or OMgp promoted serotonergic axon sprouting. This apparent differential sensitivity of an axonal tract to the deletion of different myelin inhibitors could simply reflect its differential exposure to different inhibitors. The three myelin inhibitors are differentially localized in the myelin and at axon-glial contact sites (Huang et al., 2005; Huber et al., 2002; Trapp, 1990). It is thus conceivable that this differential cellular and subcellular localization among the three myelin inhibitors may render axons of different characteristics (e.g. whether myelinated or not) a differential sensitivity to their growth-modulating effects. Further studies are required to determine the exact mechanisms for such differential sensitivity and to rule out the possibility of injury model-specific effects.

While this paper was in press, a similar genetic study by the Strittmatter group on Nogo-A,B/MAG/OMgp was published (Cafferty et al., 2010). Both papers agree on a general role for the three proteins in axon sprouting, and a more prominent role for Nogo in contributing to the inhibitory nature of CNS myelin in vitro and in inhibiting CST sprouting in vivo. However, there are notable differences between the two studies. Cafferty et al. observed significantly enhanced CST regeneration and locomotor recovery in their Nogo-A,B single mutants and Nogo-A,B/MAG/OMgp triple mutants after injury; we did not (in either our Nogo null mutants or our Nogo/MAG/OMgp triple null mutants). They observed a

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synergistic effect of deleting all three inhibitors both in vitro and in vivo; we did not. In fact, our data indicate that MAG may even be protective or growth-promoting for CST axons. The two studies also differ in the assessment of the relative contribution among the three proteins in inhibiting 5-HT axon sprouting. Genetic backgrounds and the exact nature of the mutations might affect mutant phenotypes. We used a Nogo mutation that is null for all known Nogo isoforms (Lee et al., 2009b), whereas Cafferty et al. did not; we used an OMgp mutation that did not disrupt NF1 gene expression (Lee et al., 2009a), whereas it was not clear if this was the case for the one they used. Nevertheless, in the case of Nogo we previously also assessed the very same Nogo-A,B gene trap mutant line analyzed by Cafferty et al. and failed to observe any enhancement of CST regeneration (Lee et al., 2009b). There are also differences in the injury models used. We used dorsal hemisection and complete transection to study the regeneration of CST and 5-HT axons respectively, and pyramidotomy and lateral hemisection to study the sprouting of CST and 5-HT axons respectively. Cafferty et al. used a single dorsal hemisection model to study regeneration/sprouting of both CST and 5-HT axons, and in the case of 5-HT axons they did not distinguish between “regeneration” and “sprouting”. Thus, the definition of regeneration vs. sprouting, the nature of the mutations, the injury model used, and likely the exact method to produce the injury may impact experimental outcomes. Future work is required to resolve these issues.

In summary, our data demonstrate that deleting the three major myelin inhibitors is insufficient to promote the regeneration of injured axons in the adult CNS. While deleting Nogo, MAG and/or OMgp may enhance the sprouting of uninjured axons, there was no associated behavioral improvement. Thus, the therapeutic potential of targeting these three molecules alone to promote axonal repair after CNS injury may be limited.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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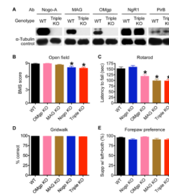

Figure 1. Basic characterization of Nogo/MAG/OMgp triple mutants

(A) Western blot analysis of Nogo-A, MAG, OMgp, NgR1 and PirB on total brain extracts from WT and Nogo/MAG/OMgp mutant mice. WT, wild type; KO, knockout (mutant); Ab, Antibody. Representative results are shown from one out of 2–3 independent biological replicates that gave similar results. (B–E) Baseline behavioral performance of WT, single and triple mutants in various locomotor tasks used in the spinal cord injury models (B–D, n = 17–24) or the forepaw preference test used in the pyramidotomy model (E, n = 8–11). *$P < 0.05$ compared with WT. One-way ANOVA with Tukeys post-test. All error bars are s.e.m.

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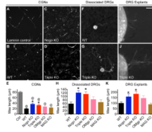

Figure 2. Lack of a synergistic effect of deleting Nogo, MAG, and OMgp in releasing myelin inhibition *in vitro*

(A-E) Representative images (A–D) and quantification (E) of neurite outgrowth from WT postnatal mouse cerebellar granule neurons (CGNs) plated on laminin or CNS myelin from mice of various genotypes. (F–K) Representative images (F, G, I, J) and quantification (H, K) of neurite outgrowth from dissociated adult WT mouse dorsal root ganglion (DRG) neurons (F, G, H) or DRG explants (I, J, K) cultured on top of adult spinal cord sections of various genotypes. Max length, longest neurite length. Results are shown from one out of three experiments that gave similar results. All error bars are s.e.m. n > 120 (E); n > 30 (H); n > 25 (K). @$P < 0.05$ compared with Laminin control. *$P < 0.05$ compared with WT. One-way ANOVA with Tukeys post-test. Scale bars: 100 μm (A–D, I, J), 50 μm (F, G).

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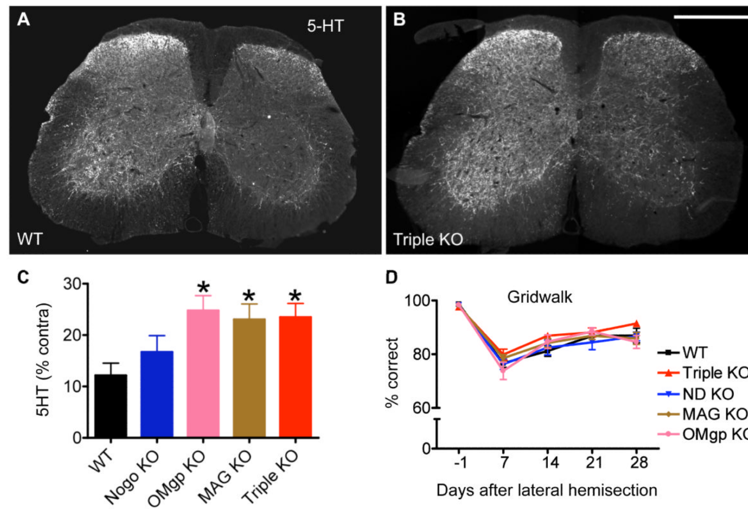

Figure 3. Sprouting of raphespinal serotonergic axons and locomotor recovery after lateral hemisection

(A, B) Representative images of transverse sections of lumbar spinal cord immunostained for serotonergic (5-HT) axons in a WT mouse (A) and a triple mutant (B). (C) Quantification of 5-HT immunoreactivity at the lumbar enlargement ipsilateral to the lateral hemisection (n = 9–13). *$P < 0.05$ compared with WT, one-way ANOVA with Tukeys post-test. (D) Gridwalk behavioral recovery. Two-way repeated measures ANOVA with Bonferonni post-test. All error bars are s.e.m. Scale bars: 500 μm. See also Figure S1.

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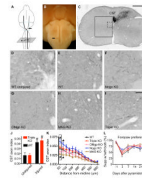

Figure 4. Sprouting of CST axons and recovery of forepaw preference after pyramidotomy
(A) Illustration of the pyramidotomy model (dorsal view). Arrow, site of pyramidotomy; shaded area, the plane of section for (C); arrowhead, axonal sprouts from the uninjured side. (B) Representative ventral view of the boxed area in (A) to show the site of pyramidotomy (arrow). (C) Representative transverse spinal cord section labeled for uninjured CST axons at the cervical enlargement following pyramidotomy. Solid rectangle represents the region quantified in (J, K). (D–I) Representative higher magnification images corresponding to the dotted area in (C) from mice of various genotypes. (J, K) Quantification of labeled uninjured CST axons at cervical levels comparing uninjured and injured mice (J), or comparing all genotypes following pyramidotomy (K). Rectangle in (K) indicates the data points depicted for injured mice in (J). Uninjured mice: n = 2–3 mice/genotype; pyramidotomized mice: n = 8–11 mice/genotype. (L) Recovery of forepaw preference. *P < 0.05 compared with WT; #P < 0.05 compared with uninjured. Two-way repeated measure ANOVA with Bonferonni post-test. All error bars are s.e.m. Scale bars: 500 μm (C), 50 μm (D–I). See also Figures S2 and S3.

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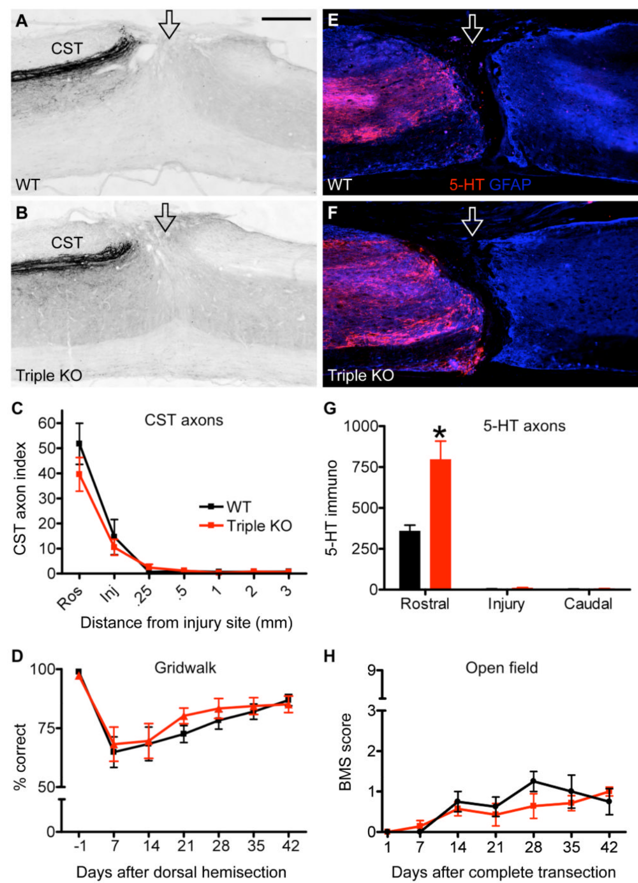

Figure 5. Lack of regeneration of CST and raphespinal serotonergic axons in triple mutant mice
(A–D) Representative images (A, B) and quantification (C, n = 10–12) of traced CST axons in sagittal sections, and gridwalk behavioral recovery (D) following a dorsal hemisection injury. Arrows indicate the injury site. Rostral is to the left. (E–H) Representative images (E, F) and quantification (G, n = 4–7) of 5-HT immunostained serotonergic axons (red) co-stained for GFAP (blue) in sagittal sections, and locomotor recovery as assessed by the BMS open field test (H) following a complete transection spinal cord injury. *$P < 0.05$ compared with WT, two-way repeated measures ANOVA with Bonferonni post-test. All error bars are s.e.m. Scale bars: 500 μm. See also Figures S3, S4 and S5.