Modifying Lipid Rafts Promotes Regeneration and Functional Recovery

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SUMMARY

Ideal strategies to ameliorate CNS damage should promote both neuronal survival and axon regeneration. The receptor Neogenin promotes neuronal apoptosis. Its ligand prevents death, but the resulting repulsive guidance molecule a (RGMa)-Neogenin interaction also inhibits axonal growth, countering any prosurvival benefits. Here, we explore strategies to inhibit Neogenin, thus simultaneously enhancing survival and regeneration. We show that bone morphogenetic protein (BMP) and RGMa-dependent recruitment of Neogenin into lipid rafts requires an interaction between RGMa and Neogenin subdomains. RGMa or Neogenin peptides that prevent this interaction, BMP inhibition by Noggin, or reduction of membrane cholesterol all block Neogenin raft localization, promote axon outgrowth, and prevent neuronal apoptosis. Blocking Neogenin raft association influences axonal pathfinding, enhances survival in the developing CNS, and promotes survival and regeneration in the injured adult optic nerve and spinal cord. Moreover, lowering cholesterol disrupts rafts and restores locomotor function after spinal cord injury. These data reveal a unified strategy to promote both survival and regeneration in the CNS.

INTRODUCTION

CNS injuries result in permanent functional loss due to the poor capacity of adult CNS neurons to regenerate axons. This is compounded by the pronounced susceptibility of adult CNS neurons to undergo apoptotic death when injured. It is therefore important to address both neuronal apoptosis and regenerative failure in the mature CNS (Schwab, 1990). The inability of axons to regenerate is largely associated with (1) a poor intrinsic ability to regenerate (Liu et al., 2011), and (2) the non-neuronal aspects of the CNS environment that inhibit axonal elongation. As a consequence, strategies that promote regeneration mainly focus on either enhancing the inner capacity of axons to regenerate or on neutralizing the negative aspects of the environment (Liu et al., 2011). Following injury, axons are confronted with inhibitors present both in the glial scar (Fawcett and Asher, 1999) and the myelin (McKerracher et al., 1994; Schwab, 2004; Wang et al., 2002). Notably, the repulsive guidance molecule a (RGMa) is present in both the glial scar and the myelin (Schwab et al., 2005a, 2005b) and accumulates at lesions after traumatic brain injury, spinal cord injury (SCI), and ischemia (Schwab et al., 2005a, 2005b).

RGMa is a potent inhibitor of axonal growth in both the adult and developing CNS (Monnier et al., 2002). It exerts its repulsive activity through the transmembrane receptor Neogenin (Rajagopalan et al., 2004). In addition to their role in axonal outgrowth, RGMa and Neogenin are involved in cell survival. Neogenin is a dependence receptor that causes death or survival when RGMa is absent or present, respectively (Matsunaga and Chédat, 2004). In the developing chick brain, Neogenin induces apoptosis in the absence of RGMa. The RGMa/Neogenin pathway is also an important trigger of neuronal death in the injured CNS (Koeberle et al., 2010). Thus, RGMa has two contradictory activities in the regenerating CNS: it promotes cell survival, which is an important positive aspect of its function, but it also inhibits axonal growth, which is undesirable. Neogenin mediates both effects; therefore, inhibiting this receptor might promote both survival and axonal regeneration. However, whether removing Neogenin would confer these benefits is unknown, and even if it did, there are no strategies to achieve this goal pharmaceutically.

The plasma membrane of cells contains a combination of glycosphingolipids and protein receptors organized in microdomains termed “lipid rafts” (Thomas et al., 2004). Lipid rafts have been implicated in regulating the activity of a plethora of extracellular receptors and diverse cellular processes. Neurons,
for example, require lipid rafts to react to guidance signals provided by the extracellular cues brain-derived neurotrophic factor (BDNF) or Semaphorin (Guirland and Zheng, 2007). Interestingly, the Netrin-1 receptor, Deleted in Colorectal Cancer (DCC), which is a homolog of Neogenin, requires rafts to guide neurons and control apoptosis (Furne et al., 2006). Although RGMa, which is a glycosylphosphatidylinositol (GPI)-anchored protein, is known to accumulate in lipid rafts (Monnier et al., 2002), it is hitherto not known if Neogenin similarly relies on its presence in these subdomains to either transmit guidance signals or control death.

Here, we dissected the factors that affect localization of Neogenin in lipid rafts and the consequences for neuronal survival and axonal regeneration. We show that selective interaction between the immunoglobulin domain in Neogenin (4Ig) and the N-terminal part of RGMa (N-Raft) triggers bone morphogenetic protein (BMP)-mediated Neogenin recruitment to lipid rafts. Using a peptide strategy to prevent N-RGMa-dependent relocation of Neogenin to lipid rafts, we show that all receptor functions are dependent on its association with rafts. Moreover, we show that modifying lipid rafts by depleting cholesterol promotes neuronal survival and axonal regeneration after optic nerve injury and SCI. Strikingly, blocking Neogenin transport into lipid rafts using either our peptide-based strategy or cholesterol depletion restored locomotor functions after SCI.

RESULTS

Neogenin Is Present in Lipid Rafts in Growth Cones
To gain insights into the role of Neogenin in the developing CNS, its expression in brain membranes (embryonic day 8 [E8]) was examined by western blots. An antibody against the entire extracellular domain of Neogenin (AF1079) detected two bands at 180 and 150 kDa, whereas the antibody against the N terminus (N-20) identified only the 180 kDa band (Figures 1A and 1B), suggesting that brain membranes contain full-length Neogenin (180 kDa) and an N-terminal truncation (150 kDa) that lacks some of the 4Ig repeats. Recent studies indicate that diverse receptors concentrate in cholesterol-rich raft microdomains to elicit cellular response to extracellular signals (Guirland et al., 2004). So far, there is no direct evidence that Neogenin localization in lipid rafts is critical to growth cone response to RGMa. To test this possibility, we first examined Neogenin membrane

[Diagram and images as described in the text]
localization. In confocal images, staining performed with an antibody against the N-terminal part of Neogenin exhibits a punctate staining pattern in the growth cone of retinal ganglion cells (RGCs) (E8; Figures 1A–1D). These punctate structures colocalized with the raft markers, cholera toxin subunit B (CTB) and RGMa (Figures 1E and 1F), with computed intensity correlation quotients (ICQs) of 0.27 and 0.32, respectively, suggesting that Neogenin significantly associates with RGMa (p = 0.005) and rafts (p = 0.0002) (Li et al., 2004). In control experiments, in which the axons were stained with CTB and F-actin, the computed correlation quotient was 0.05, indicating segregation of both stains (Figure S1). To corroborate these findings, we characterized the abundance of Neogenin in isolated lipid rafts from chick brain membrane. Indeed, Neogenin was recovered in fractions 2–4 that are enriched in two lipid raft markers, Flotillin (Maliszewska-Cyna et al., 2010) and RGMa (Monnier et al., 2002), but not in the nonraft fractions that contain the heavy fraction marker, transferrin receptor (Guirland et al., 2004; Herincs et al., 2005) (Figure 1G). We next treated embryos with methyl-β-cyclodextrin (MβCD) to deplete membrane cholesterol, which is known to disrupt lipid rafts (Grosse et al., 1998), and performed fractionations of brain samples. MβCD shifted Neogenin to nonlipid raft fraction populated by transferrin receptor (Figure S1C).

Interaction between N-Raft and Neogenin-4Ig Regulates Neogenin Presence in Lipid Rafts

In chondrocytes, there is evidence that RGMs may regulate Neogenin association with lipid rafts; however, the mechanisms that regulate this process remain elusive (Zhou et al., 2010). We hypothesized that a yet unidentified interaction between Neogenin and RGMa is involved in Neogenin recruitment toward lipid rafts. To search for such a domain, we fused the 4Ig and 6FNIII domains of Neogenin to alkaline phosphatase (AP) and analyzed binding to various RGMa peptides in an ELISA (Figure 2A). Similar to published data (Rajagopalan et al., 2004), 6FNIII, but not 4Ig, interacted with full-length RGMa (Figure 2A). However, we discovered an interaction between the 4Ig domain and the N-terminal part of RGMa (Figure 2A). Because we found that the N-terminal part of RGMa also interacts with the fibronectin domain in Neogenin (Tassew et al., 2012), we asked whether distinct N-terminal regions bind 4Ig and 6FNIII. Interestingly, a peptide spanning aa 28–73 (which we will refer as N-Raft) interacted with 4Ig-AP, whereas a peptide spanning aa 77–113 interacted with 6FNIII. We next treated embryos with MβCD to deplete membrane cholesterol, which is known to disrupt lipid rafts (Grosse et al., 1998), and performed fractionations of brain samples. MβCD shifted Neogenin to nonlipid raft fraction populated by transferrin receptor (Figure S1C).
aa 77–113 (N-inh.) interacted with 6FNIII-AP. In complementary experiments, N-Raft-AP bound to 4Ig and not 6FNIII, whereas N-inh.-AP interacted with 6FNIII but not 4Ig (Figure S2). Consistent with the ELISA data, 6FNIII and 4Ig were pulled down by N-inh.- and N-Raft-coated beads, respectively (Figure 2B). To assess the specificity of the interaction between Neogenin 4Ig and N-Raft, we checked whether DCC, the closest Neogenin homolog, interacted with N-Raft. No significant interaction was observed between DCC-4Ig and N-Raft (Figure S2C).

To assess whether these Neogenin-RGMa interactions are biologically relevant, we performed outgrowth experiments using RGMa peptides as substrates (Figure 2C). Full-length N-RGMa and the N-inh.-fragments caused a 3- to 4-fold decrease in axonal growth compared to controls (51.5 ± 3.0 μm and 88.1 ± 13.3 μm versus 238.1 ± 9.3 μm; Figures 2C and 2D). Because both N-RGMa and N-inh. interact with 6FNIII, this agrees with our previous findings that RGMa proteins must interact with the 6FNIII domain to inhibit retinal axon growth (Tassew et al., 2012). When the N-raft fragment was used as a substrate, it had no effect on axonal outgrowth (Figures 2C and 2D). These data indicate that, unlike the N-inh.-6FNIII interaction, the contact described here between N-Raft and the 4Ig portion of Neogenin does not inhibit axonal outgrowth in trans.

To assess whether the purpose of the N-Raft and 4Ig interaction might be to recruit Neogenin into lipid rafts, we tested whether deleting these RGMa and Neogenin domains would prevent Neogenin association with rafts (Figure S2). In human embryonic kidney 293 (HEK293) cells cotransfected with Neogenin and RGMa and treated with BMP2, which promotes Neogenin recruitment to lipid rafts (Zhou et al., 2010), Neogenin was found in the raft fraction. In contrast, although deletion mutants of Neogenin (4Ig-del) and RGMa (N-Raft-del) were normally targeted toward the cell surface (data not shown), they were not recruited to the raft fractions (Figure S2). We surmised that if the interaction between 4Ig and N-Raft is indeed required for Neogenin presence in lipid rafts, overexpressing recombinant 4Ig and N-Raft to compete with endogenous domains should displace Neogenin from rafts toward the nonraft fractions. N-Raft, 4Ig, or control peptide (N-RGMa50-99) was injected into the optic tectum at E8, and the tecta were collected a day later for fractionation analyses. As expected, full-length Neogenin localized exclusively to the raft fraction in controls (N-RGMa50-99). Strikingly, N-Raft and 4Ig relocalized Neogenin from rafts to the heavy fractions (Figure 2E). Because Neogenin presence in lipid rafts is BMP dependent, we extended our analysis by injecting the BMP chelator Noggin into chick brains. Similar to the effects of 4Ig and N-Raft, Noggin evicted Neogenin from rafts (Figure 2E). Together with the in vitro findings, these in vivo results indicate that an interaction between the 4Ig domain in Neogenin and the N-Raft domain in RGMa is necessary to recruit Neogenin to lipid rafts.

Knockdown Analysis Reveals a cis Interaction between RGMa and Neogenin

Previously, we showed that the CNS contains three RGMa isoforms (N-RGMa, C-RGMa, and RGMaΔ) that inhibit axonal outgrowth in trans (Tassew et al., 2012). Thus, when provided as the growth substrate, each of these RGM peptides blocks axon growth. Moreover, they achieve this by interacting with the Neogenin fibronectin domain (Figures 2A–2D) (Tassew et al., 2012). Our peptide and deletion analysis (Figures 2E and S2) indicate that interaction between Neogenin 4Ig and N-Raft recruits Neogenin into lipid rafts. We wondered whether this interaction occurs in cis or trans. RGMa localizes to lipid rafts through its GPI anchor (Monnier et al., 2002); thus, it follows that it would recruit Neogenin through a cis interaction. Consistent with this notion, our data show that Neogenin and RGMa are colocalized in the growth cone (Figure 1F), and when the N-Raft peptide is provided as a growth substrate (i.e., in trans), it does not block axonal outgrowth (Figure 2D). As a further test, we silenced RGMa in the growth cone by delivering small hairpin RNA (shRNA) into the E2 chick eye and measured outgrowth on each of the three RGMa proteins using explants from transfected E7 retinas. Transduction of shRNA in the eye would only disrupt putative cis RGMa interaction with Neogenin in RGCs without affecting trans interactions between the growth cone and RGMa provided exogenously as substrate.

Two different shRNAs that suppressed endogenous RGMa (shRNA21 and shRNA37; Figure S5) enhanced retinal explant outgrowth on N-RGMa substrate by 3.1 and 2.8 times, respectively, relative to control shRNA (Figures 3A and 3B). Similarly, silencing of RGMa led to a 2.8- to 3.4-fold increase in axonal growth on C-RGMa and RGMaΔ (Figures 3A and 3B). In rescue experiments, cotransfection with mouse RGMa, resistant to shRNA37, restored the inhibitory activity of RGMa proteins in dissociated RGCs treated with shRNA37 (Figure S3). Therefore, the result obtained with RGMa shRNA is not an off-target effect. Altogether, our data indicate that in addition to the established trans interaction, a cis interaction between RGMa and Neogenin is also required to inhibit axonal outgrowth.

We hypothesized that this cis interaction between RGMa and Neogenin occurs between 4Ig and N-Raft. If so, these peptides could be used as blocking peptides and should restore axonal growth in retinal explants grown on RGMa proteins. Indeed, the addition of 5 μg/ml of either 4Ig (Figures 3A and 3C) or N-Raft (Figures 3A and 3D) to the medium blocked the inhibitory effects of RGMa proteins and restored outgrowth. Together, these data suggest that a cis interaction between the Neogenin 4Ig domain and N-Raft is required to inhibit axonal growth.

RGMa Inhibition Requires Neogenin Association with Lipid Rafts and BMPs

Because the interaction between 4Ig and N-Raft regulates Neogenin association with lipid rafts (Figure 2E), the experiments presented above suggest that Neogenin must be present in lipid rafts to inhibit axonal growth. To address this issue, we used agents that disrupt lipid rafts: 10 mM MβCD or 2 U/ml of cholesterol oxidase (CO). Both treatments restored outgrowth to control values, when axons were cultured on RGMa proteins (Figures 3A, 3E, and S3D). Thus, the axonal response to RGMa is triggered by Neogenin transit to rafts.

Our data suggest that in the developing CNS, Neogenin recruitment into lipid rafts depends on BMPs (Figure 2E). If so, Noggin should affect the axonal response to RGMa proteins. As expected, axonal outgrowth was unaffected by laminin alone but was reduced by N-RGMa, C-RGMa, or RGMaΔ.
Figure 3. Neogenin Association with Lipid Rafts Is Required for RGMa Inhibitions

(A) Temporal explants cultured on N-RGMa and treated with 4 Ig (1 μg/ml), N-Raft (1 μg/ml), RGMa shRNAs (shRNA21 and shRNA37), MjiCD (10 mM), CO (2 U/ml), and Noggin (100 nM). Scale bar, 60 μm. (B–F) Explants were grown on laminin (Ctrl), on laminin plus N-RGMa, C-RGMa, or RGMaΔ, and treated with (B) RGMa shRNA21 and shRNA37, (C) 4Ig, (D) N-Raft, (E) MjiCD and CO, and (F) Noggin (Nog.). Data are average ± SEM (n = 3 independent experiments). *p < 0.005.

(G) Schematic representation of in vivo experiments in the chick visual pathway. The right eye was electroporated at E2 with plasmid expressing RGMa/Neogenin constructs, and at E17, tracing was performed from the eye to the optic tectum. The inset denotes the area presented in (H)–(K).

(H) Control experiments using N-RGMa50–99, which does not interact with Neogenin. Axonal path is intact, and all fibers established terminal arbors into the predicted zone (T2). Scale bar, 100 μm.

(I–K) Electroporation of (I) RGMa-shRNA-, (J) N-Raft-, or (K) 4Ig-perturbed axonal paths with numerous fibers establishing terminal arbors outside the predicted zone (arrowheads). Scale bar, 100 μm.

(L) Schematic representation of the action of treatments presented above. The 4Ig domain in Neogenin interacts with the N-terminal part in RGMa (N-Raft) to allow a BMP-dependent recruitment of Neogenin into lipid rafts. Once in lipid rafts, Neogenin can transduce axonal inhibition. Treatments that prevent Neogenin association with lipid rafts abolish axonal inhibition. These treatments are (1) chelating of BMPs with Noggin; (2) masking of the 4Ig site on Neogenin with the addition of N-Raft peptides; (3) masking of the N-Raft site on RGMa with the addition of 4Ig peptides; (4) silencing of RGMa with shRNAs (shRNA21 and ShRNA37); and (5) disrupting lipid rafts using cholesterol depletion (MjiCD and CO).
similarly enhanced cell survival by ~50% versus control (Figures 4E and 4F).

Higher levels for both Neogenin and RGMa have been observed in the retina following injury (Schnichels et al., 2012). Furthermore, we have shown that RGMa and Neogenin cause apoptotic death of RGCs in vivo following axotomy (Koeberle et al., 2010). To determine whether Neogenin requires lipid rafts in this context, 4Ig or N-Raft was injected intraocularly, and cell survival was measured 14 days after axotomy. Both 4Ig (682 ± 21 cells/mm²) and N-Raft (652 ± 24 cells/mm²) increased RGC survival ~2-fold versus control (361 ± 19 cells/mm²) (Figures 4G–4I). Likewise, systemic application (intraperitoneal [i.p.]) of MbCD also increased RGC survival ~2-fold (Figures 4G and 4J). CO is not stable and could not be tested in vivo. Collectively, these results support our hypothesis that Neogenin-induced apoptosis in the injured CNS relies on its association with lipid rafts, which is governed by interaction between its 4Ig domain and N-Raft.

**Neogenin Requires Lipid Rafts to Hamper Axonal Regeneration after SCI**

Thus far, our in vitro results show that segregating Neogenin from lipid rafts supports axonal outgrowth and halts neuronal death in the developing CNS (Figures 3 and 4). We also observed improved survival of RGCs following axotomy in vivo (Figures 4G–4J). Although RGMa is expressed by radial glia cells during development and acts as a guidance molecule (Monnier et al., 2002), it is expressed by reactive astrocytes and oligodendrocytes in the injured CNS (Hata et al., 2006; Schwab et al., 2005a). In both the developing and the regenerating CNS, RGMa works as an inhibitor of axonal growth. Thus, having shown that preventing Neogenin association with lipid rafts suppresses the inhibitory function of the RGMa/Neogenin pathway in developing RGCs, we extended our study to models of injuries in which this pathway impedes regeneration. We studied regeneration after SCI in a rat spinal cord compression model that closely mimics human SCI (Rivlin and Tator, 1978). All animal procedures were reviewed and approved by local animal care committees.
Neuronal Sparing and Motor Axon Regeneration

Neuronal loss after SCI results in loss of muscle control and eventual paralysis. Furthermore, RGMa/Neogenin triggers neuronal death in injured CNS (Koeberle et al., 2010). Thus, we asked if blocking Neogenin association with lipid rafts could halt neuronal loss and accelerate axonal regeneration after SCI and the ensuing functional recovery. Injured rats received systemic application of either 4Ig or MjICD for 6 weeks, and perilesional neurons were quantified with the neuronal marker NeuN. 4Ig or MjICD treatment increased the number of perilesional neurons compared to controls by ~2-fold (Figures 5I–5M). Intrathecal application of 4Ig over a 6-week period resulted in a similar increase (~2-fold) of NeuN-positive cells in cross-sections of the cord (Figures 5N–5P and S5E). Measurements revealed that the lesion volumes for 4Ig- and MjICD-treated animals did not significantly vary when compared to controls (Figure S5D). Thus, this supports the notion that blocking Neogenin association with lipid rafts attenuates neuronal loss following SCI, which parallels our findings after optic nerve injury.

The inability of CNS axons to regenerate is largely due to inhibitors present in the myelin that are not conducive to regeneration (Fawcett and Asher, 1999). To evaluate whether the improved behavioral outcome resulted from axonal regeneration, we performed anterograde tracing of cortico-spinal tracts (CSTs) by injecting biotin dextran amine (BDA) into the motor cortex (Figure 6). Only one control animal showed a single regenerating fiber that extended beyond the injury site (cavity). By contrast, all 4Ig- and MjICD-treated animals had fibers protruding beyond the cavity, and some extended a few millimeters beyond this site (Figures 6A and 6B). The average length of the longest axon was drastically increased in animals treated with 4Ig (2,875 ± 228 μm) or MjICD (2,195 ± 335 μm) compared to its respective controls (18 ± 7 μm and 128 ± 118 μm) (Figures 6C and 6D). Although axons were rarely seen beyond the lesion site in controls, many fibers in the 4Ig or MjICD group spanned beyond 3,000 μm of the lesion site (Figures 6E and 6F). We used a SCI model of impact/compression injury in which both the dorsal and ventral aspects of the spinal cord are simultaneously compressed. The severity of SCI used in the present study results in central cavitation of the gray matter and adjacent white matter that severs all CST axons leaving a spared rim of subpial white matter (Figure S6C). Moreover, in 4Ig and MjICD animals, we observed fibers that displayed aberrant paths (Figure S6D), also indicative of regeneration. To ensure that BDA fibers were regenerating axons, we analyzed the cord from 4Ig (i.v. injection)-treated animals at different time points (n = 5 for each) and found that fibers were significantly longer at 6 weeks when compared to 4 weeks following injury (Figure 6G). In particular, we observed a significantly higher number of fibers between 2 and 3 mm caudal from the lesion in 6-week versus 4-week animals (Figure 6H). We also examined cross-sections of the spinal cord at 3 mm rostral and 5 mm caudal to the lesion. In sham animals, spared fibers were apparent at both locations showing the continuity of the CST in the uninjured spinal cord. In contrast, in 4Ig (i.v.)-treated animals, fibers were observed rostral to the lesion, but not at 5 mm caudal to the lesion, indicating the lack of spared fibers. In 4Ig (i.v.)- or MjICD (i.p.)-treated rats, labeled fibers were detected at a maximal distance of ~4 mm caudal to the lesion site, suggesting that these fibers are regenerating and not spared. Taken together, these findings suggest that excluding Neogenin away from rafts promotes axonal regeneration after SCI and the attendant functional recovery.

Neogenin Requires Lipid Rafts to Hamper Axonal Regeneration after Optic Nerve Crush

It may be difficult to differentiate between spared and regenerating fibers in SCI; however, GAP-43 only stains regenerating fibers, and the optic nerve crush model would be a confirmation that our treatments promote axonal regeneration (Meyer et al., 1994; Monnier et al., 2011). Following injury, animals received i.v. injections of 4Ig or N-Raft, and axonal regeneration was examined 21 days later (Figure 7). Regeneration was visualized by GAP-43 immunostaining that only localizes to regenerating adult RGC axons of the proximal and distal nerve stump (Meyer et al., 1994; Monnier et al., 2011). In controls, few axons crossed the lesion site within the optic nerve, and growth cones stopped abruptly at the crush site (Figure 7A). Regenerating axons in controls were limited to <250 μm in length, and an average of ~408 regenerating axons per optic nerve were observed beyond the lesion site (Figures 7A and 7E; <250 μm, 224 ± 36; 250–500 μm, 108 ± 12; and >500 μm, 76 ± 24). In stark contrast, animals treated with 4Ig showed increased regeneration beyond...
the lesion site and more pronounced axon sprouting in the proximal segment of the nerve (Figures 7B and 7E). Quantification of axon numbers by length showed that 4Ig significantly increased the number of regenerating axons by 5.7-fold relative to controls (Figures 7B and 7E; <250 μm, 1.134 ± 0.68; 250–500 μm, 640 ± 128; and >500 μm, 410 ± 64). Animals that received N-Raft also showed robust regeneration beyond the lesion site to a degree comparable to 4Ig (Figures 7C and 7E). Thus, both 4Ig and N-Raft enhanced axon regeneration in the myelinated adult CNS.

We next examined whether cholesterol depletion could promote regeneration after optic nerve crush. Axonal length was measured in rats that were treated over a 28-day period with MjCD, previously used to study metastasis in vivo (Grosse et al., 1998) (Figures 7D and 7E). For all distances considered, the average number of axons per section in animals treated with MjCD (i.p.) injection was significantly higher than controls by 7.1-fold (Figures 7D and 7E; <250 μm, 23.7 ± 1.2; 250–500 μm, 10.7 ± 0.9; and >500 μm, 8.4 ± 1.4). We also examined the intraretinal integrity of RGC axons after optic nerve crush to look for signs of increased axon/soma preservation by our treatments 4 weeks following injury. The cell bodies of surviving RGCs with intact axons were visibly labeled with CTB-fluorescent labels 4 weeks after injury (Figure S7C). Treatments with 4Ig, N-Raft, and MjCD resulted in (1) thicker axon bundles throughout the retina, and (2) increased labeling of RGC soma, which is consistent with a role of these treatments in both cell survival and axonal regeneration. Overall, these findings show that Neogenin plays an important role in the failure of axons to regenerate after optic nerve injury and that preventing its association with lipid rafts results in robust axonal regeneration. Because astroglia cells were not affected, the prorgerative effect of our treatments most likely resulted from an effect on regenerating axons (Figure S7). In summary, these data demonstrate that altering Neogenin presence in lipid rafts promotes axonal regeneration after CNS injury.

DISCUSSION

RGMa has two contradictory actions in the regenerating CNS, i.e., it inhibits axonal growth, which is undesirable, and promotes cell survival, which is valuable (Koeberle et al., 2010). Here, we solve this dilemma by developing an approach that inhibits the RGMa receptor, Neogenin, to promote both cell survival and axonal regeneration. We show that Neogenin is a constitutive part of lipid rafts and that this association is required to inhibit axons and induce apoptosis. Multiple approaches to prevent Neogenin transport to rafts promoted axonal growth on inhibitory RGMa and blocked Neogenin-induced apoptosis. Both peptide-mediated blockade of Neogenin translocation into rafts and cholesterol depletion to disrupt rafts improved axonal regeneration in the injured optic nerve and the spinal cord. Importantly, these strategies resulted in significant functional recovery after SCI. Thus, our data uncover an unappreciated role for lipid rafts and Neogenin in regeneration following CNS injuries.

We identified an RGMa domain (N-Raft) that interacts with the 4Ig-Neogenin to regulate Neogenin targeting into lipid rafts. At first glance, this mechanism resembles how DCC, TrkA, and Neuropilin are recruited to rafts, i.e., when their respective ligands Netrin-1, BDNF, and Sem3A are added to the medium, they interact in cis with their receptors thereby increasing their association with rafts (Guirland et al., 2004). However, unlike the mechanism mentioned above, trafficking of Neogenin to rafts by RGMa appears to require additional molecular mechanisms. Indeed, both Neogenin and RGMa reside on the same membranes, and we show that a cis interaction between these proteins is necessary to localize Neogenin to rafts. A similar cis interaction between EphA3 receptor and its ligand, ephrin, has been reported, but it negatively regulates EphA3 (Carvalho et al., 2006), which is in contrast to RGMa-Neogenin interaction, which activates Neogenin, Thus, our data evoke a paradigm by which an extracellular guidance cue “primes” its receptor through a cis interaction.

BMPs have been shown to regulate axonal growth by (1) regulating the expression of guidance cues such as ephrin/Eph proteins (Sakuta et al., 2006) or (2) acting as a repulsive guidance cue (Phan et al., 2010). Here, we uncover a role for BMPs in regulating axonal growth by enabling Neogenin function. This paradigm may explain some recent studies, which at first view appear contradictory. One study demonstrated that BMP2 and BMP4 injection activated Smad1, thereby promoting axonal regeneration (Zou et al., 2009). However, another study showed that abolishing BMP activity promoted regeneration (Matsuura et al.,...
Thus, activating and neutralizing the same pathway seemed to promote regeneration. Our data reconcile this discrepancy and suggest that the proregenerative effect of BMP neutralization with Noggin may not result from a silencing of the BMP/Smad pathway but rather from a neutralization of the RGMa/Neogenin pathway.

Because cholesterol is an important constituent of growing axons, it has been assumed that it is necessary for axonal growth/regeneration (Posse De Chaves et al., 2000). Our data defy the notion that cholesterol is merely a “building brick” required for the biogenesis of axonal membranes. A recent study suggests that cholesterol may directly modulate growth by a Fyn-dependent mechanism and thereby play an active role in axonal elongation (Ko et al., 2005). Interestingly, during peripheral nerve regeneration, cholesterol synthesis in the nerve is downregulated (Goodrum, 1990), which fits with our data indicating that lower cholesterol-levels in membranes promote regeneration. There are other indications that lipid rafts may be a critical regulator of axonal regeneration. For instance, the Nogo receptor is a GPI-anchored protein and is naturally present in rafts (Fournier et al., 2001). Ephrin-As, which are strong axon inhibitors, are also GPI anchored and require oligomerization in rafts.
to inhibit growing fibers. Therefore, several CNS inhibitors rely on lipid rafts to prevent regeneration. Although the above evidence was suggestive, our data demonstrate that cholesterol depletion promotes regeneration.

Neutralizing inhibitory proteins is a well-established strategy to promote regeneration. For instance, antibodies have been used to neutralize myelin, Nogo-A, and RGMa, thus promoting some recovery after injury (Hata et al., 2006). Thus far, none of these approaches has led to the development of therapeutics, perhaps because besides promoting regeneration, they also have unwanted effects (e.g., promote apoptosis). We present an alternative strategy that counteracts inhibitory pathways by modifying receptor membrane localization.

Because many receptors are present in lipid rafts, treatment with MβCD will not only affect Neogenin presence in these structures. For instance, cyclodextrins could influence signaling of receptors for Nogo, Semaphorin, and BDNF, which are present in lipid rafts (Furne et al., 2006; Guirland and Zheng, 2007). This lack of specificity may prevent the activation of pathways that could promote neuronal survival and regeneration in the injured CNS. On the other hand, this may well be an advantage because it may allow for the neutralization of receptors for multiple growth inhibitors. Regardless of the specificity of cyclodextrin, the effects that we observed strongly suggest that the benefit of lipid raft depletion largely outweighs some of its possible negative aspects on regeneration.

In the past decade, there has been some level of success in promoting axonal regeneration after CNS injuries. In particular, two studies obtained robust axonal regeneration after optic nerve injury (Fischer et al., 2004; Sun et al., 2011). However, although these studies demonstrate that regeneration is feasible in the injured CNS, they will be difficult to translate into human therapies because they utilized injection of lentiviral vectors several weeks before injury (Fischer et al., 2004). Moreover, the
first study (Fischer et al., 2004) was done by knocking down PTEN and SOCS3 together with ciliary neurotrophic factor injection. In the second study (Sun et al., 2011), regeneration occurred when a DN-NogoR vector was injected together with lens injury to “activate” RGCs. In contrast, we performed one treatment at a time and initiated each strategy after injury. Nevertheless, the above-mentioned studies show that combinatorial therapies potently induce regeneration. It will be important to combine our treatment with other treatments. For instance, combining our peptide treatments, which suppress RGMa inhibition, with treatments that will promote the intrinsic ability of neurons to regenerate should result in robust regeneration.

**EXPERIMENTAL PROCEDURES**

Detailed procedures are presented in Supplemental Experimental Procedures.

**Retinal Explants, Outgrowth Assay, and Immunolocalization**

Poly-L-Lysine (Sigma-Aldrich; 10 μg/ml)-coated glass coverslips were treated with Laminin (Invitrogen; 10 μg/ml) and RGMa proteins (5 μg/ml) and incubated for 3 hr at room temperature. Temporal retinal explants were cultured on protein-coated surfaces for 18 hr. To disrupt rafts, explants were pretreated with 10 mM MJiCD for 12 min or 2 U/ml CO for 1 hr at 37°C. Explants were then fixed with 4% paraformaldehyde and stained with Alexa 488 phalloidin. Fiber length was quantified using Image-Pro 5.0. For immunolocalization, explants were treated with CTB-FITC (C1655; Sigma-Aldrich; 10 μg/ml) and patched, fixed, and stained with Neogenin antibody. RGCs were electroporated with RGMa-His and cultured for 18 hr. The cells were fixed, and stained with Neogenin and His-tag antibodies.

**Lipid Raft Fractionation of Cells and Tecta**

Injected chick E8 tecta (4Ig, 2 mg/ml; N-raft, 1 mg/ml; M-JiCD, 10 mM) or transfected cells (Neogenin and RGMa) were collected 24 hr later, lysed, and placed at the bottom of a sucrose density gradient (0.9–0.8–0.75–0.7–0.6–0.5–0.4–0.2 M) and centrifuged at 38,000 rpm for 16 hr in a SW 60 rotor (Beckman Instruments).

**Spinal Cord Injury**

The spinal cord was injured by clip compression at spinal cord level T8 with a 20 g force. The 4Ig or vehicle was injected intraspinally immediately following SCI. Two injections (250 ng/ml, 3 μl each) were made 1 mm rostral and 1 mm caudal to the lesion site and adjacent to the midline vein. Immediately following SCI, the 4Ig-treated rats also received a 1 mg/kg dose of 4Ig i.v. via the intrajugular vein and subsequent i.v. injections weekly for 2 more weeks. Rats in the MJiCD group received i.p. injection of MJiCD at 1,000 mg/kg/week immediately following SCI and then daily i.p. injections until sacrifice. Control rats received equivalent vehicle as described above.

**Intrathecal Infusion**

To assess local delivery of 4Ig, 19 adult female Wistar rats were injured and injected rostral and caudal to the lesion site with 4Ig or PBS. Immediately after the intraspinal injections, 4Ig or PBS was delivered intrathecially and then continuously for 14 days (0.5 μl/hr) via the catheter and pump system.

**Functional Analysis**

Functional tests were performed before the injury, 1 day after, and then weekly for 6 weeks post-SCI. Locomotor function was evaluated using the BBB locomotor rating scale. A score of zero (“0”) indicates no hindlimb movement; a score of 21 indicates unpaired locomotion as observed in control. Motor subscores were determined to assess toe clearance, predominant paw position, and absence of instability. A maximal motor subscore of seven means normal locomotion.

Ladder walk analysis was done to assess fine motor functions. At 1 week post-SCI and weekly thereafter, rats with a BBB score ≥11 were placed on the horizontal ladder walk apparatus, and three test runs were recorded. Recordings were analyzed in slow motion; the number of footfalls per hindlimb was recorded, and the average was calculated for each rat per week.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.06.014.

**AUTHOR CONTRIBUTIONS**

N.G.T. did all the biochemical, molecular, and axonal guidance experiments presented in this article. A.J.M. performed spinal cord injury experiments as well as tracing and behavioral monitoring of spinal cord-injured animals. A.P.S. performed retinal whole-mount as well as optic nerve injury/regeneration experiments.

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