

# 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 Chédotal, 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,





### Figure 1. Neogenin Is Present in Lipid Rafts (A) Schematic representation of the sequences used to generate the N-20 and AF1079 antibodies against Neogenin.

(B) Western blot analysis of brain lysates showing that N-20 detects one 180 kDa band, whereas AF1079 recognizes the 180 kDa band and an N-terminal deletion of Neogenin at  $\sim$ 150 kDa.

(C and D) Immunofluorescence images of retinal growth cone of (C) F-actin (Alexa 488 phalloidin) and (D) Neogenin. Scale bars, 30  $\mu m.$ 

(E) Composite images of Neogenin (Neog.) staining, the raft marker CTB, and merged image; yellow indicates regions of colocalization. ICQ to quantify colocalization between Neogenin and CTB was calculated as described by Li et al. (2004) in 12 growth cones. ICQ reflects the ratio of the number of positive or negative ( $A_i - a$ ) ( $B_i - b$ ) values to the total pixel number minus 0.5 to derive a -0.5 to +0.5 range. Here, an average ICQ of 0.27 was computed, which indicates strong colocalization between Neogenin and CTB. Scale bars, 30 µm.

(F) Composite images of Neogenin (AF1079), the RGMa, and merged image; yellow indicates regions of colocalization. An average ICQ of 0.32 was computed, which indicates strong colocalization between Neogenin and RGMa. Scale bars, 30 μm.

(G) Chick brain gradient fractionation showing that Neogenin (arrowhead) localizes with RGMa and raft marker Flotillin. Transferrin receptor (TfR) was used a heavy fraction marker.

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 pep-

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 glycosylphosphatidylinisotol (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 (4lg) 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-RGMadependent relocation of Neogenin to lipid rafts, we show that all receptor functions are dependent on its association with tide-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 4lg 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



### Figure 2. *cis* Interaction between the 4lg Domain in Neogenin and the N-Terminal Part of RGMa Recruits Neogenin to Lipid Rafts

(A) RGMa proteins were incubated on plates coated with 4lg- or 6FNIII-AP (binding: –, base line; ++, >5 x base line) (quantitative analysis for this experiment is presented in Figure S2). One C- and one N-RGMa domain (N-RGMa<sup>77-113</sup>) interacted with 6FNIII. One N-RGMa domain (N-Raft) interacted with 4lg.

(B) Supernatants (Sup.) from cells expressing His-6FNIII or His-4Ig were pulled down (i.p.) with beads coated with N-Raft, N-RGMa<sup>50–99</sup>, and N-inh. Western blot was then performed with a His antibody to reveal whether or not His-6FNIII or His-4Ig interacted with the coated beads. This showed that His-6FNIII was pulled down by N-inh., whereas His-4Ig was pulled down by N-Raft. Western blot on cell supernatants was performed to demonstrate that the same amount of His-FNIII and His-4Ig was used to carry out the pull-down. IP, immunoprecipitation.

(C and D) Images (C) and quantification (D) of retinal explant outgrowth on laminin (Lam.) and laminin plus N-RGMa or N-RGMa protein domains show that only N-RGMa and N-inh. inhibit axonal growth. Data are average  $\pm$  SEM (n = 3 independent experiments). \*p < 0.0001. Scale bars, 100  $\mu m.$ 

(E) Chick brains were injected with control peptide (Ctrl; N-RGMa<sup>50-99</sup>), 4lg, N-Raft, or Noggin 1 day before preparing membrane fractionations. In control experiments, Neogenin localizes to the Flotillin/RGMa-containing lipid raft fraction (red boxes). 4lg, N-Raft, or Noggin shifts Neogenin to heavy fractions.

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; Hérincs et al., 2005) (Figure 1G). We next treated embryos with methyl-ß-cyclodextrin (MßCD) to deplete membrane cholesterol, which is known to disrupt lipid rafts (Grosse et al., 1998), and performed fractionations of brain samples. MBCD shifted

Neogenin to nonlipid raft fraction populated by transferrin receptor (Figure S1C).

## Interaction between N-Raft and Neogenin-4lg 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 4lg 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 4lg, interacted with full-length RGMa (Figure 2A). However, we discovered an interaction between the 4lg 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 4lg 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 (N-inh.) interacted with 6FNIII-AP. In complementary experiments, N-Raft-AP bound to 4lg and not 6FNIII, whereas N-inh.-AP interacted with 6FNIII but not 4lg (Figure S2). Consistent with the ELISA data, 6FNIII and 4lg were pulled down by N-inh. and N-Raft-coated beads, respectively (Figure 2B). To assess the specificity of the interaction between Neogenin 4lg and N-Raft, we checked whether DCC, the closest Neogenin homolog, interacted with N-Raft. No significant interaction was observed between DCC-4lg 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 (51.5.5  $\pm$  3.0  $\mu$ m and 88.1  $\pm$  13.3  $\mu$ m versus 238.1  $\pm$  9.3  $\mu$ m; 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 4lg portion of Neogenin does not inhibit axonal outgrowth in *trans*.

To assess whether the purpose of the N-Raft and 4lg 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 (4lg-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 4lg and N-Raft to compete with endogenous domains should displace Neogenin from rafts toward the nonraft fractions. N-Raft, 4Ig, or control peptide (N-RGMa<sup>50-99</sup>) 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-RGMa<sup>50-99</sup>). 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 4lg 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 4lg 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 RGMa $\Delta$ ) 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 4lg 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 RGMa $\Delta$  (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  $\mu$ g/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 M $\beta$ CD 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 markedly reduced by N-RGMa, C-RGMa, and RGMa $\Delta$ 



(Figures 3A and 3F). Noggin restored outgrowth in retinal explants (E8) exposed to each individual RGMa to control values (Figures 3A and 3F). These data suggest that a similar mechanism, which is dependent on BMP, underlies the Neogeninmediated response to all three RGMa proteins. Collectively, these results indicate that RGMa together with BMP(s) regulates Neogenin transport into lipid rafts, and this link is required to mediate axon growth inhibition by RGMa.

# Neogenin Requires Lipid Rafts for Correct Axonal Pathfinding

To assess the biological relevance of the interaction between 4lg and N-Raft on axonal paths in vivo, we ectopically expressed both peptides and compared pathfinding to controls (N-RGMa<sup>50-99</sup>). To ensure that treatments affect growing axons and not their environment, we performed electroporation and narrowed the application of the electric field to the optic vesicle at E2, thus restricting expression to the eye and to retinal axons (Figure 3G). In control experiments, all retinal axons established arborization within a well-defined terminal zone (Figure 3H). As a positive control, we electroporated an shRNA that silences RGMa, which we have shown perturbs axonal paths (Figure 3I; Tassew et al., 2008). Consistent with our hypothesis, 100% (eight of eight) of the 4lg and 85.7% (six of seven) of the N-Raft electroporations caused aberrant paths (Figures 3J and 3K). Thus, the 4lg-N-Raft interaction that is critical for Neogenin localization to lipid rafts in vitro is also essential for axonal pathfinding in vivo. Together with the outgrowth experiments (Figures 3A-3F), these data suggest a model for the BMP, RGMa, and Neogenin action in which RGMa binding in cis to Neogenin, aided by BMP, translocates the receptor to lipid rafts, and this is essential for the subsequent trans RGMa-Neogenin interaction that inhibits axons (Figure 3L). Blocking translocation to lipid rafts with Noggin, N-Raft peptide, 4lg peptide, RGMa knockdown in the growth cone, or cholesterol depletion provides five strategies for stimulating axonal growth (Figure 3L).

### **Neogenin Requires Lipid Rafts to Induce Cell Death**

Although neutralizing RGMa increases axonal growth, this also has the undesirable effect of activating the prodeath function of Neogenin (Koeberle et al., 2010). We therefore reasoned that preventing Neogenin transport into lipid rafts might simultaneously promote regeneration and survival. To determine whether Neogenin requires lipid rafts to induce apoptosis, we transfected HEK293 cells with Neogenin and measured death by trypan blue exclusion assay. In this assay, we used HEK293 cells that stably express RGMa (Figure S4). Neogenin elevated cell death by 2-fold above that seen in mock cells (Figure 4A). When Noggin, 4Ig, N-Raft, or M $\beta$ CD was added to the medium of Neogenin-transfected cells, cell viability was improved  $\sim$ 2-to 3-fold, indicating that lipid rafts are required for Neogenin-induced death (Figure 4B).

We have previously shown that RGMa is expressed in the developing chick neural tube (Matsunaga et al., 2004). Thus, to investigate whether Neogenin requires rafts for its prodeath function in vivo, we analyzed apoptosis in neural tubes of chick embryos (E2). A GFP construct was electroporated with plasmids that expressed either mock or Neogenin, and apoptosis was assayed by TUNEL staining. In contrast to mock expression, which did not induce apoptosis (Figure S4), numerous TUNELpositive cells were observed in GFP-Neogenin-transfected cells (Figures 4C and 4D). This proapoptotic effect of Neogenin was abolished when either 4Ig or N-Raft was injected into the tecta following Neogenin electroporation. 4Ig or N-Raft reduced the number of TUNEL-positive cells by  ${\sim}18\text{-}\text{fold}$  (from 432  $\pm$  23 to 23  $\pm$  4) and  $\sim$ 4-fold (from 432  $\pm$  23 to 103  $\pm$  8), respectively (Figure 4D). These results show that Neogenin presence in lipid rafts is mandatory to cause apoptosis in the developing brain.

## Neogenin Requires Lipid Rafts to Induce Apoptosis in Injured Retinal Neurons

Our data underpin the obligatory role of lipid rafts for RGMa/ Neogenin function during brain development. Because the RGMa/Neogenin pathway is a critical trigger of neuronal death in the injured CNS (Koeberle et al., 2010), localization of Neogenin in rafts might also be essential to its pathophysiological roles. We tested this notion in retinal organotypic cultures wherein axotomy of RGCs deprives cells of trophic factors, thus inducing apoptosis. Cell death was detected using propidium iodide (PI), and all images were taken at the same intensity with the RGC layer in focus. Cell death was gauged by measuring the relative PI intensity of whole mounts. The presence of either 4Ig or N-Raft in the medium significantly reduced cell death (by  $\sim$ 40%) (Figures 4E and 4F). Perturbing rafts with either M $\beta$ CD or CO



<sup>(</sup>A) Temporal explants cultured on N-RGMa and treated with 4lg (1 μg/ml), N-Raft (1 μg/ml), RGMa shRNAs (shRNA21 and shRNA37), MβCD (10 mM), CO (2 U/ml), and Noggin (100 nM). Scale bar, 60 μm.

<sup>(</sup>B–F) Explants were grown on laminin (Ctrl), on laminin plus N-RGMa, C-RGMa, or RGMa $\Delta$ , and treated with (B) RGMa shRNA21 and shRNA37, (C) 4lg, (D) N-Raft, (E) M $\beta$ CD and CO, and (F) Noggin (Nog.). Data are average ± SEM (n = 3 independent experiments). \*p < 0.005.

<sup>(</sup>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).

<sup>(</sup>H) Control experiments using N-RGMa<sup>50-99</sup>, which does not interact with Neogenin. Axonal path is intact, and all fibers established terminal arbors into the terminal zone (TZ).

<sup>(</sup>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 TZ (arrowheads). Scale bar, 100  $\mu$ m.

<sup>(</sup>L) Schematic representation of the action of treatments presented above. The 4lg 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 4lg site on Neogenin with the addition of N-Raft peptides; (3) masking of the N-Raft site on RGMa with the addition of 4lg peptides; (4) silencing of RGMa with shRNAs (shRNA21 and ShRNA37); and (5) disrupting lipid rafts using cholesterol depletion (MβCD and CO).



Figure 4. Neogenin Requires Lipid Rafts to Induce Cell Death

(A) HEK293 cells that stably express RGMa (Figure S4) were transfected with empty plasmid (Ctrl) or Neogenin (Neo). Neogenin induced cell death (arrows) when compared to control.

(B) Quantification of cell death in Neogenin-transfected cells that were incubated in BSA (Neo+Ctrl), 4Ig (1 µg/ml), N-Raft (1 µg/ml), Noggin (100 nM), or M $\beta$ CD (10 mM). Treatments that prevent Neogenin association with lipid rafts significantly rescued Neogenin-induced cell death. Data are average ± SEM (n = 3 independent experiments). \*p < 0.0001.

similarly enhanced cell survival by  ${\sim}50\%$  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, 4lg or N-Raft was injected intraocularly, and cell survival was measured 14 days after axotomy. Both 4lg (682  $\pm$ 21 cells/mm<sup>2</sup>) and N-Raft (652 ± 24 cells/mm<sup>2</sup>) increased RGC survival  $\sim$ 2-fold versus control (361 ± 19 cells/mm<sup>2</sup>) (Figures 4G-4I). Likewise, systemic application (intraperitoneal [i.p.]) of M $\beta$ CD also increased RGC survival  $\sim$ 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 4lg 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

(C and D) Representative images (C) and quantification (D) of cell death in chick E5 tecta electroporated with GFP- and a Neogenin-expression plasmid and then injected with N-RGMa<sup>50-99</sup> (Ctrl; 1 µg/ml), 4lg (1 µg/ml), or N-Raft (1 µg/ml) and subjected to TUNEL assay. 4lg or N-Raft significantly reduced the number of TUNEL-positive cells caused by Neogenin. Data are average  $\pm$  SEM from three different tecta/conditions (\*p < 0.001). Scale bars, 100 µm.

(E and F) Representative images (E) and quantification (F) of retinal whole mounts incubated for 4 days with control peptide (1  $\mu$ g/ml), 4lg (1  $\mu$ g/ml), N-Raft (1  $\mu$ g/ml), or M $\beta$ CD (10 mM), and dead cells were stained with PI. Images from nine whole mounts per condition from three independent experiments were taken at the same intensities, and the fluorescence was measured using ImageJ. 4lg, N-Raft, and M $\beta$ CD significantly reduced cell death in retinal whole mounts. Scale bars, 500  $\mu$ m.

(G) Fluorescence micrographs of flat-mounted retinas depicting FluoroGold-labeled RGCs in injured retinas at 14 days following axotomy. Control retinas (Ctrl) had few surviving RGCs. 4Ig, N-raft, and M $\beta$ CD increased cell survival. Scale bar, 200  $\mu$ m.

(H) Representations of the retinal areas selected in our analyses.

(I and J) Quantification of the density of surviving RGCs at 14 days postaxotomy showing that (I) 4Ig and N-raft and (J) M $\beta$ CD significantly increased RGC survival. Data are average  $\pm$  SEM (n = 6 eyes). \*p < 0.01. committees in accordance with guidelines established by the Canadian Council on Animal Care. Functional recovery was monitored weekly using the Basso, Beattie, Bresnahan (BBB) locomotor rating scale, motor subscore, and ladder walk tests (Basso et al., 1995; Metz and Whishaw, 2002). Treatment with either 4lg (intravenous [i.v.]) or MBCD (i.p.) showed significant functional recovery as early as 2-3 weeks post-SCI, as evidenced by a higher BBB score and motor subscore compared to controls (Figures 5A, 5B, 5D, and 5E). Hindlimb coordination was assessed using the ladder walk test where footfalls are considered errors, and thus, a higher score reflects poorer coordination. Compared to controls, rats injected with 4lg had fewer footfalls (Figure 5C). A similar pattern of functional improvement was also observed in rats injected with MβCD (Figures 5D-5F). Importantly, staining for astrocytes indicated that treatments with 4lg and MBCD did not affect populations of these cells. Thus, improvement of motor functions seen could have resulted from improved neuronal survival or axon regeneration (Figure S5).

To ensure that the effect observed with 4lg resulted solely from an action of this peptide at the injury site, we performed local intrathecal application using ALZET osmotic minipumps. To lower spontaneous recovery, a stronger clip was used to perform compression SCI (26g versus 20g). Similar to systemic application, local application of 4lg showed significant functional recovery as early as 2–3 weeks post-SCI as evidenced by a higher BBB score (Figure 5G). Here also, hindlimb coordination was significantly improved, and rats locally injected with 4lg had fewer footfalls (Figure 6H). We also tested the effects of M $\beta$ CD on locomotor recovery in older animals (>7 months; n = 4), post-SCI. Similar to the results with young animals, M $\beta$ CD treatment led to marked improvements (+6.8 BBB points versus control; Figure S6C). Thus, hindering the association of Neogenin with lipid rafts robustly stimulates functional locomotor recovery following SCI.

### **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 4lg or MBCD for 6 weeks, and perilesional neurons were quantified with the neuronal marker NeuN. 4Ig or MBCD treatment increased the number of perilesional neurons compared to controls by ~2-fold (Figures 5I-5M). Intrathecal application of 4lg over a 6-week period resulted in a similar increase ( $\sim$ 2-fold) of NeuN-positive cells in crosssections of the cord (Figures 5N-5P and S5E). Measurements revealed that the lesion volumes for 4lg- and M $\beta$ CD-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 MBCD-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 4lg (2,875  $\pm$  228  $\mu$ m) or M $\beta$ CD (2,195 ± 335  $\mu$ m) compared to its respective controls  $(18 \pm 7 \ \mu m \text{ and } 128 \pm 118 \ \mu m)$  (Figures 6C and 6D). Although axons were rarely seen beyond the lesion site in controls, many fibers in the 4lg or MBCD group spanned beyond 3,000  $\mu m$  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 4lg and MβCD 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 4lg (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 4lg (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 4lg (i.v.)- or M<sub>β</sub>CD (i.p.)-treated rats, labeled fibers were detected at a maximal distance of  $\sim$ 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 4lg 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  $\sim$ 408 regenerating axons per optic nerve were observed beyond the lesion site (Figures 7A and 7E; <250  $\mu$ m, 224  $\pm$  36; 250-500  $\mu$ m, 108  $\pm$  12; and >500  $\mu$ m, 76  $\pm$  24). In stark contrast, animals treated with 4lg showed increased regeneration beyond



(legend on next page)

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 4lg significantly increased the number of regenerating axons by 5.7-fold relative to controls (Figures 7B and 7E; <250  $\mu$ m, 1,134 ± 68; 250–500  $\mu$ m, 640 ± 128; and >500  $\mu$ m, 410 ± 64). Animals that received N-Raft also showed robust regeneration beyond the lesion site to a degree comparable to 4lg (Figures 7C and 7E). Thus, both 4lg 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 M<sub>β</sub>CD, 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 MBCD (i.p. injection) was significantly higher than controls by 7.1-fold (Figures 7D and 7E; <250  $\mu m,$  23.7  $\pm$  1.2; 250-500  $\mu$ m, 10.7  $\pm$  0.9; and >500  $\mu$ m, 8.4  $\pm$  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-fluorescein isothiocyanate (FITC) (Figure S7C). Treatments with 4lg, N-Raft, and MBCD resulted in (1) thicker axon bundles throughout the retina, and (2) increased labeling of RGC somata, 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 proregenerative 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 4lg-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 Sema3A are added to the medium, they interact in trans 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.,



(A-F) Behavioral assessment using the BBB (A and D), motor subscore (B and E), and average number of footfalls (C and F) shows that systemic treatment with 4lg (i.v.) or M $\beta$ CD (i.p.) resulted in marked functional improvement in spinal cord-injured rats versus control (Ctrl). Data are average  $\pm$  SEM (control, n = 7; 4lg, n = 8; M $\beta$ CD, n = 8) (\*p < 0.005).

(N and O) Neu-N staining of cross-sections of injured spinal cords after intrathecal treatment with 4Ig. The insets display cord from rats treated with (N) vehicle and (O) 4Ig. Arrowheads point to neurons.

(P) Quantification of experiments presented in (N) and (O) shows that intrathecal treatment with 4lg resulted in a significant increase in the number of neurons after SCI. Quantification at different distances to the lesion site is presented in Figure S5D. Data are average  $\pm$  SEM (n = 8 animals/condition). \*p < 0.001. Scale bars, 100  $\mu$ m.

<sup>(</sup>G and H) Behavioral assessment using the BBB (G) and average number of footfalls (H) shows that intrathecal application of 4lg resulted in marked functional improvement in spinal cord-injured rats versus control. Data are average  $\pm$  SEM (control, n = 10; 4lg, n = 10) (\*p < 0.005).

<sup>(</sup>I-M) Longitudinal sections of an injured spinal cord stained with the neuronal marker NeuN. The insets display peri-lesional neurons in the injured spinal cord from rats after systemic treatment with (I) vehicle control, (J) 4Ig, and (K) M $\beta$ CD. Arrows point to neurons after 4Ig and M $\beta$ CD treatments. Dotted line indicates border between white and gray matter. Quantification of experiments presented in (I)–(K) shows that treatment with (L) 4Ig and (M) M $\beta$ CD resulted in a significant increase in the number of spared neurons after SCI. For each animal, the absolute number of neurons was quantitated from five sections at equivalent anterior-posterior distance through the thickness of each cord encompassing equivalent gray matter tissue.



### Figure 6. Altering Neogenin Presence in Lipid Rafts Promotes Axonal Regeneration in the Myelinated Spinal Cord

To visualize axons from the CST, anterograde axonal tracing with BDA was performed at 6 weeks post-SCI.

(A) Top panel displays a control spinal cord after injury. The insets represent higher magnifications. The lesion cavity is presented on the left.

(B) Injured spinal cord treated with 4Ig. Tracing with BDA revealed fibers caudal to the cavity in 4Ig experiments, but not in Control. The longest regenerating axons extended several millimeters beyond the caudal end of the cavity. Arrows indicate fibers. Arrowheads in the insets indicate turning fibers. Scale bars, 100  $\mu$ m.

(C and D) Average maximal length post-SCI was increased following (C) 4lg and (D) M $\beta$ CD treatments. Data are average  $\pm$  SEM (n = 4 independent rats/condition; three to four sections/rat). \*p < 0.001.

(E and F) The average number of regenerating axons/section was measured, which shows a greater number of regenerating axons in injured rats treated with (E) 4lg and (F) M $\beta$ CD. Data are average ± SEM (n = 4 independent rats/condition; three to four sections/rat). \*p < 0.005.

(G and H) Injured spinal cord treated with 4lg for 4 or 6 weeks before animals were sacrificed and axonal length was measured. (G) The average axonal length was significantly greater at 6 weeks when compared to 4 weeks. (H) The average number of regenerating axons/section was measured, which shows a significantly higher number of axons of 2–3 mm when cords were treated for 6 weeks when compared to 4 weeks. Data are average  $\pm$  SEM (n = 5 independent rats/condition; three to four sections/rat). \*p < 0.005.

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

2008). 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.

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





## Figure 7. Altering Neogenin Presence in Lipid Rafts Promotes Axonal Regeneration after Optic Nerve Injury

(A–D) GAP-43 immunostaining in longitudinal sections of optic nerve at 21 days after optic nerve crush and various treatments. Compared to controls (A), altering Neogenin presence in lipid rafts with (B) 4lg and (C) N-Raft or (D) disrupting lipid raft with M $\beta$ CD enhances axonal regeneration past the crush site (yellow dotted line) and into the distal myelinated optic nerve. Arrows demarcate some of the regenerating axons in each nerve section. Inset images are higher-magnification views of regenerating axons from the corresponding section.

(E) Quantification of axonal length showing that 4lg, N-Raft, and M $\beta$ CD significantly enhanced axonal regeneration after optic nerve injury. Data are average  $\pm$  SEM (n = 7 optic nerves/condition). \*p < 0.001.

Scale bar, 200 µm.

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 MBCD 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.

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 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 M $\beta$ CD 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 mg/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 (4lg, 2 mg/ml; N-raft, 1 mg/ml; M $\beta$ CD, 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 4lg or vehicle was injected intraspinally immediately following SCI. Two injections (250 ng/µl, 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 4lg-treated rats also received a 1 mg/kg dose of 4lg i.v. via the intrajugular vein and subsequent i.v. injections weekly for 2 more weeks. Rats in the M $\beta$ CD group received i.p. injection of M $\beta$ CD at 1,000 mg/kg/week immediately following SCI and then daily i.p. injections until sacrifice. Control rats received equivalent vehicle volumes as described above.

#### Intrathecal Infusion

To assess local delivery of 4lg, 19 adult female Wistar rats were injured and injected rostral and caudal to the lesion site with 4lg or PBS. Immediately after the intraspinal injections, 4lg or PBS was delivered intrathecally and then continuously for 14 days (0.5  $\mu$ l/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 unimpaired 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 <a href="http://dx.doi.org/10.1016/j.celrep.2014.06.014">http://dx.doi.org/10.1016/j.celrep.2014.06.014</a>.

### **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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