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Response to Correspondence: Kim et al., "Axon Regeneration in Young Adult Mice Lacking Nogo-A/B." Neuron 38, 187–199

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Numerous in vivo pharmacological studies have demonstrated the beneficial effects of interfering with Nogo/NgR function for axonal growth and functional recovery after experimental spinal cord injury (Bregman et al., 1995; Brosamle et al., 2000; Fouad et al., 2004; GrandPre et al., 2002; Ji et al., 2005; Li et al., 2004, 2005; Li and Strittmatter, 2003; Schnell and Schwab, 1990; Wang et al., 2006). Similar benefits are noted for stroke recovery (Lee et al., 2004; Papadopoulos et al., 2002; Seymour et al., 2005). However, genetic analysis of *nogo* and *ngr* has proven inconclusive (Woolf, 2003). Two mouse lines, here termed *nogo-ab*$^{trap/trap}$ (Kim et al., 2003) and *nogo-a*$^{EIII/EIII}$ (Dimou et al., 2006; Simonen et al., 2003), show enhanced growth of corticospinal (CST) axons after dorsal hemisection. In contrast, another line, here termed *nogo-ab*$^{atg/atg}$ (Zheng et al., 2003), fails to show a regenerative phenotype. Common to each of these studies was the observation that myelin prepared from mutant mice was significantly less inhibitory to neurite outgrowth in vitro. Among the factors that might explain the varied in vivo outcomes are age at the time of lesion, mouse strain background, surgical techniques, axonal tracing methodology, and the nature of the mutant allele (Woolf, 2003). A follow-up study demonstrated that the penetrance of the *nogo-a*$^{EIII/EIII}$ phenotype for CST axonal regeneration depends on strain background (Dimou et al., 2006). Regenerative axon growth is quite prominent on the Sv129 strain background, as compared to a mixed or C57BL/6 background (Dimou et al., 2006).

Anterograde Tracing Methodology

In their Correspondence, Steward et al. present evidence that inaccurate injections of the axonal tracer biotin dextran amine (BDA) can produce erroneous labeling after spinal cord injury. They show that BDA in the CSF shortly after a spinal hemisection can label fibers in the caudal white matter of the cord, irrespective of genotype. Mislabeled fibers were distinguished by their large diameter, their enrichment in ventrolateral white matter, their linear trajectory, and their hollow appearance. This pattern occurred in one of their original cohort of mice (Zheng et al., 2003) and with much greater frequency subsequently. The second study utilized deeper injections into the cerebral cortex, injections that, in retrospect, are likely to have penetrated the ventricular wall. Since similar spinal labeling occurred after intentional ventricular injection of BDA, the authors conclude that uptake from the CSF accounts for the pattern.

Steward et al. suggest that the *nogo-ab*$^{trap/trap}$ phenotype in our study (Kim et al., 2003) is attributable to mislabeling. We are convinced that genotype-selective mistracing cannot fully explain the observations. In our studies, the vast majority of *nogo-ab*$^{trap/trap}$ mice show caudal CST fibers that bear no resemblance to the thick, linear, hollow, ventrolateral labeling pattern of Steward et al. This is clear in Figures 5A, 5B, 6C, and 6E of Kim et al. (2003), where CST axons are thin, tortuous, and situated predominantly in gray matter. We have now analyzed 58

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adult *nogo-ab*$^{trap/trap}$ mice (of various ages and strains) with dorsal hemisections and immediate BDA injection. Thirty-five of these mice (60%) exhibit thin, branched CST fibers extending caudal to the lesion. These profiles are clearly distinct from the thick, linear, hollow fibers seen in the Steward et al. study. Additional high-quality examples of clearly valid caudal CST labeling from recent *nogo-ab*$^{trap/trap}$ mice are provided in Figures 1B and 1C here. Only 3 of our 58 mice (5%) had a pattern consistent with Steward et al. We conclude that the phenomenon they describe is rare when injections are targeted to the cerebral cortex in our studies.

Steward et al.'s suggestion that our observations are artifactual and that there is no injury-enhanced CST growth phenotype in *nogo-ab*$^{trap/trap}$ mice is based primarily on the similarity of fiber appearance in our Figures 4B and 6B (Kim et al., 2003) with the CSF-dependent labeling of their Correspondence. Figures 4B and 6B derive from one particular mouse in our original study that had fibers consistent with Steward et al. It is possible that the labeling in the transverse sections of Figures 4B and 6B occurred via the mechanism suggested in the Steward et al. manuscript. The origin of labeling in these two panels has to be considered uncertain in light of the follow-up studies of Steward et al. However, if the rare mice with such a pattern are excluded from the axon counts in Figures 4F and 6F (Kim et al., 2003), the same statistically valid conclusions remain. Specifically, after omitting these rare mice, labeled CST fibers outside of the DCST proper are significantly ($p \leq 0.05$) increased in the *nogo-ab*$^{trap/trap}$ mice on both sides of the spinal cord, at a level 5 mm rostral to the hemisection (as in Figure 4F). Similarly, after exclusion, labeled CST fibers in the caudal spinal cord with a thin tortuous regenerative appearance are significantly ($p \leq 0.05$) increased in the *nogo-ab*$^{trap/trap}$ mice, at a level 5 mm below the hemisection (as in Figure 6F). While the inclusion of potentially CSF-mislabeled fibers from rare *nogo-ab*$^{trap/trap}$ mice may have inadvertently overrepresented the regenerative growth in transverse sections, the *nogo-ab*$^{trap/trap}$ population exhibits injury-induced CST growth when such mice are excluded from the analysis.

Of equal importance, it should be noted that the rare BDA/CSF artifact of Steward et al. is easily distinguished and is not an issue in our analysis of parasagittal sections; it complicates observations only in transverse sections from a small subset of mice (Figures 4B and 6B of Kim et al., 2003). This is obvious in longitudinal sections from one of the three *nogo-ab*$^{trap/trap}$ mice that had the linear, hollow, thick fibers on transverse sections (Figure 2F). While weakly labeled linear profiles can be detected in white matter of these parasagittal sections, they are clearly distinguishable from the densely stained varicose and tortuous regenerating CST fibers present in the same mouse that are consistent with regenerated fibers (Steward et al., 2003). Only the strongly stained, branching CST fibers were included in our original Figure 5. When the trajectories of such regenerating fibers as in Figures 1B and 1C here and Figures 5A and 5B (Kim et al., 2003) are combined from all spinal sections from one mouse in camera lucida, the pattern is as in our original Figures 5G–5K.

Overall, we thank Steward et al. for exploring one potential artifact in BDA axonal tracing. While the veracity of tracing must be monitored, mislabeling cannot fully account for the differences between *nogo-ab*$^{trap/trap}$ mice and control mice. CST growth in *nogo-ab*$^{trap/trap}$ mice is significantly greater than in control animals, but it is also very much less than complete, as documented in our original Figures 5 and 6 and Figures 1B and 1C here.

Age Dependency of Regenerative Phenotype in nogo-ab$^{trap/trap}$ Mice

Our initial study of *nogo-ab*$^{trap/trap}$ mice raised a question as to whether age after sexual maturity might influence the degree of CST regenerative axonal growth after spinal cord hemisection. Therefore, we examined T7 bilateral dorsal hemisection lesions in 8- versus 14-week-old *nogo-ab*$^{+/trap}$ and *nogo-ab*$^{trap/trap}$ mice. All mice had normal open field locomotor

scores prelesion (Figure 2A) and showed flaccid hindlimb paralysis after surgery. Eight-week-old *nogo-ab*$^{trap/trap}$ recovered significantly greater hindlimb function in comparison to heterozygote littermates (two-way ANOVA, $p \leq 0.05$). Both groups of 14-week-old mice recovered less well than did the 8-week-old mice of the same genotype. However, the relatively improved outcome in *nogo-ab*$^{trap/trap}$ mice as compared to *nogo-ab*$^{+/trap}$ mice was maintained at 14 weeks of age. The magnitude of the age effect was approximately as great as the genotype effect, such that the 14-week-old *nogo-ab*$^{trap/trap}$ mice performed indistinguishably from the 8-week-old *nogo-ab*$^{+/trap}$ mice.

Axonal growth was assessed in these mice via unilateral tracing of the CST with BDA (Figures 2B–2E). Labeled fibers can be seen as a fasciculated bundle that is completely interrupted by the lesion (Figure 2B). In this group of 8-week-old *nogo-ab*$^{trap/trap}$ mice, labeled axons are detected more than 3 mm caudal to the lesion and are significantly more numerous in *nogo-ab*$^{trap/trap}$ mice than in *nogo-ab*$^{+/trap}$ mice (Figure 2E, replicating Kim et al., 2003). Again, the caudal BDA^{+} axons have a varicose morphology, consistent with regenerated fibers (Figures 2C and 2D). Regenerating CST axons are also observed in 14-week-old *nogo-ab*$^{trap/trap}$ mice, but are significantly less frequent than in 8-week-old *nogo-ab*$^{trap/trap}$ (Figures 2E). The sum of CST axons observed caudal to the lesion in 14-week-old *nogo-ab*$^{trap/trap}$ mice is significantly greater from heterozygote littermates at 1 mm past the lesion (Figure 2E). Axons of the descending serotonergic raphespinal system were also assessed in 8- and 14-week-old *nogo-ab*$^{trap/trap}$ and *nogo-ab*$^{+/trap}$ injured mice (Figures 2F–2I). Rostral to the hemisection, there is no significant difference in 5HT-IR arborizations in the ventral horns of 8- and 14-week-old *nogo-ab*$^{trap/trap}$ and *nogo-ab*$^{+/trap}$ mice (Figure 2F). Inspection of transverse sections of the lumbar spinal cord caudal to the lesion revealed robust sprouting of 5HT-IR fibers in the ventral horns of 8-week-old *nogo-ab*$^{trap/trap}$ mice (Figure 2G). The total length of 5HT-IR fibers is sig-nificantly greater than in 14-week-old *nogo-ab*$^{trap/trap}$ mice (Figure 2H) or in 8-week-old *nogo-ab*$^{+/trap}$ mice (Figure 2I). Thus, the age after sexual maturity significantly modifies the restrictive effect of Nogo-A/B on axonal growth and on functional recovery.

Different nogo-ab Mutant Alleles Have Distinct Regenerative Phenotypes

Since our findings indicate that age modulates the *nogo-ab*$^{trap/trap}$ regenerative phenotype and since strain background alters the phenotype (Dimou et al., 2006), the results reported by different laboratories for *nogo-a*$^{-/-}$ mice might be caused by mouse age, strain background, surgical technique, postoperative care, axonal tracing method, or the nature of the mutant allele. In order to isolate the influence of different mutant alleles from other factors, we obtained the *nogo-ab*$^{atg/atg}$ mice with the least reported axonal growth (Zheng et al., 2003) and interbred them with the *nogo-ab*$^{trap/trap}$ mice with the greatest reported axonal growth (Kim et al., 2003). We completed dorsal hemi-sections on mixed heterozygote *nogo-ab*$^{trap/atg}$, *nogo-ab*$^{trap/trap}$, and *nogo-ab*$^{atg/atg}$ mice. All mice had normal hindlimb articulation presurgery and flaccid hindlimb paralysis after waking from anesthesia postsurgery. In open field locomotion, *nogo-ab*$^{trap/trap}$ mice recovered significantly more hindlimb function than did *nogo-ab*$^{trap/atg}$ and *nogo-ab*$^{atg/atg}$ mice (two-way ANOVA, $p \leq 0.05$, Figure 1D). There was no significant difference in the BBB scores of *nogo-ab*$^{trap/atg}$ versus *nogo-ab*$^{atg/atg}$ mice.

BDA-labeled CST axons were counted 1–3 mm caudal to the lesion. *Nogo-ab*$^{trap/trap}$ mice (Figures 1B, 1C, and 1E) have significantly more axons caudal to the lesion in comparison to *nogo-ab*$^{trap/atg}$ (Figure 1E) or *nogo-ab*$^{atg/atg}$ (Figures 1A and 1E) mice. Thus, measures of CST regenerative growth and locomotor BBB scores yield similar patterns. We conclude that the nature of the *nogo* mutant allele has a significant effect on the SCI phenotype when age, strain, surgeon, behavioral paradigm, and tracing method are held constant. The regenerative phenotype associated with the *nogo-ab*^{trap} allele is fully complemented by the *nogo-ab*^{atg} allele and is not dominant, even on a *nogo-ab* null background.

Discussion

Strikingly, the studies here demonstrate that different mutant *nogo-ab* alleles produce distinct phenotypes when other variables are controlled. Even in the *nogo-ab*$^{trap/trap}$ mice, a minority of CST fibers exhibit long-distance regenerative growth after dorsal hemisection, and this percentage is modified by age. The molecular basis for the variable penetrance of a growth phenotype within one strain and between two mutant alleles is not yet clear. Both mutations eliminate detectable Nogo-A protein, but preserve brain *nogo-c* mRNA levels. Because one mutant allele targets exon I and the other targets exon III with a different insertion, there may be different effects on the *nogo-c* promotor or on the expression of adjacent genes. In considering the hypothesis that the expression of neighboring genes might be altered by these mutations, we have noted no alteration of brain expression for the six genes closest to the *nogo* locus in RT-PCR studies (data not shown). Since cell-type-specific expression has not been assessed, it remains possible that there is differential low-level but compensatory expression of Nogo-C or a similarly sized isoform selectively in oligodendrocytes. The *nogo-ab*$^{trap/atg}$ mixed heterozygote phenotype matches most closely the dorsal hemisection phenotype of the *nogo-ab*$^{atg/atg}$ mice. The complementation of the *nogo-ab*^{trap} allele by the *nogo-ab*^{atg} allele is consistent with hypomor-phic expression of a Nogo protein in oligodendrocytes. In contrast, the hypothesis that an amino-terminal protein fragment derived from the *nogo-ab*^{trap} allele has a dominant proregenerative effect on a Nogo-A null background (Kim et al., 2003) is not supported by the *nogo-ab*$^{trap/atg}$ phenotype.

Crucially, the existence of an allelic difference between the *nogo-ab*^{trap} and *nogo-ab*^{atg} mice poses the question of whether the *nogo-ab*$^{atg/atg}$ mice are devoid of enhanced injury-induced axonal growth. Despite the absence of a CST growth phenotype after dorsal hemisection, the *nogo-ab*$^{atg/atg}$ mice of Zheng et al. (2003) do show marked injury-induced CST axonal growth after the more discrete CNS lesion of pyramidotomy (Cafferty and Strittmatter, 2006). Therefore, the lack of CST growth in the *nogo-ab*$^{atg/atg}$ mice is relative, and not absolute. Thus, strain, age, mutant allele, and lesion model all have an influence on the adult CNS axonal growth phenotype of mice lacking Nogo-A.

References

Bregman BS, Kunkel-Bagden E, Schnell L, Dai HN, Gao D, Schwab ME. Nature 1995;378:498–501. [PubMed: 7477407]

Brosamle C, Huber AB, Fiedler M, Skerra A, Schwab ME. J Neurosci 2000;20:8061–8068. [PubMed: 11050127]

Cafferty WBJ, Strittmatter SM. J Neurosci 2006;26:12242–12250. [PubMed: 17122049]

Dimou L, Schnell L, Montani L, Duncan C, Simonen M, Schneider R, Liebscher T, Gullo M, Schwab ME. J Neurosci 2006;26:5591–5603. [PubMed: 16723516]

Fouad K, Klusman I, Schwab ME. Eur J Neurosci 2004;20:2479–2482. [PubMed: 15525289]

GrandPre T, Li S, Strittmatter SM. Nature 2002;417:547–551. [PubMed: 12037567]

Ji B, Li M, Budel S, Pepinsky RB, Walus L, Engber TM, Strittmatter SM, Relton JK. Eur J Neurosci 2005;22:587–594. [PubMed: 16101740]

Kim JE, Li S, GrandPre T, Qiu D, Strittmatter SM. Neuron 2003;38:187–199. [PubMed: 12718854]

Lee JK, Kim JE, Sivula M, Strittmat-ter SM. J Neurosci 2004;24:6209–6217. [PubMed: 15240813]

Li S, Strittmatter SM. J Neurosci 2003;23:4219–4227. [PubMed: 12764110]

Li S, Liu BP, Budel S, Li M, Ji B, Walus L, Li W, Jirik A, Rabacchi S, Choi E, et al. J Neurosci 2004;24:10511–10520. [PubMed: 15548666]

Li S, Kim JE, Budel S, Hampton TG, Strittmatter SM. Mol Cell Neurosci 2005;29:26–39. [PubMed: 15866044]

Papadopoulos CM, Tsai SY, Alsbiei T, O'Brien TE, Schwab ME, Kartje GL. Ann Neurol 2002;51:433–441. [PubMed: 11921049]

Schnell L, Schwab ME. Nature 1990;343:269–272. [PubMed: 2300171]

Seymour AB, Andrews EM, Tsai SY, Markus TM, Bollnow MR, Brenneman MM, O'Brien TE, Castro AJ, Schwab ME, Kartje GL. J Cereb Blood Flow Metab 2005;25:1366–1375. [PubMed: 15889044]

Simonen M, Pedersen V, Weinmann O, Schnell L, Buss A, Ledermann B, Christ F, Sansig G, van der Putten H, Schwab ME. Neuron 2003;38:201–211. [PubMed: 12718855]

Steward O, Zheng B, Tessier-Lavigne M. J Comp Neurol 2003;459:1–8. [PubMed: 12629662]

Wang X, Baughman KW, Basso DM, Strittmatter SM. Ann Neurol 2006;60:540–549. [PubMed: 16958113]

Woolf CJ. Neuron 2003;38:153–156. [PubMed: 12718850]

Zheng B, Ho C, Li S, Keirstead H, Steward O, Tessier-Lavigne M. Neuron 2003;38:213–224. [PubMed: 12718856]

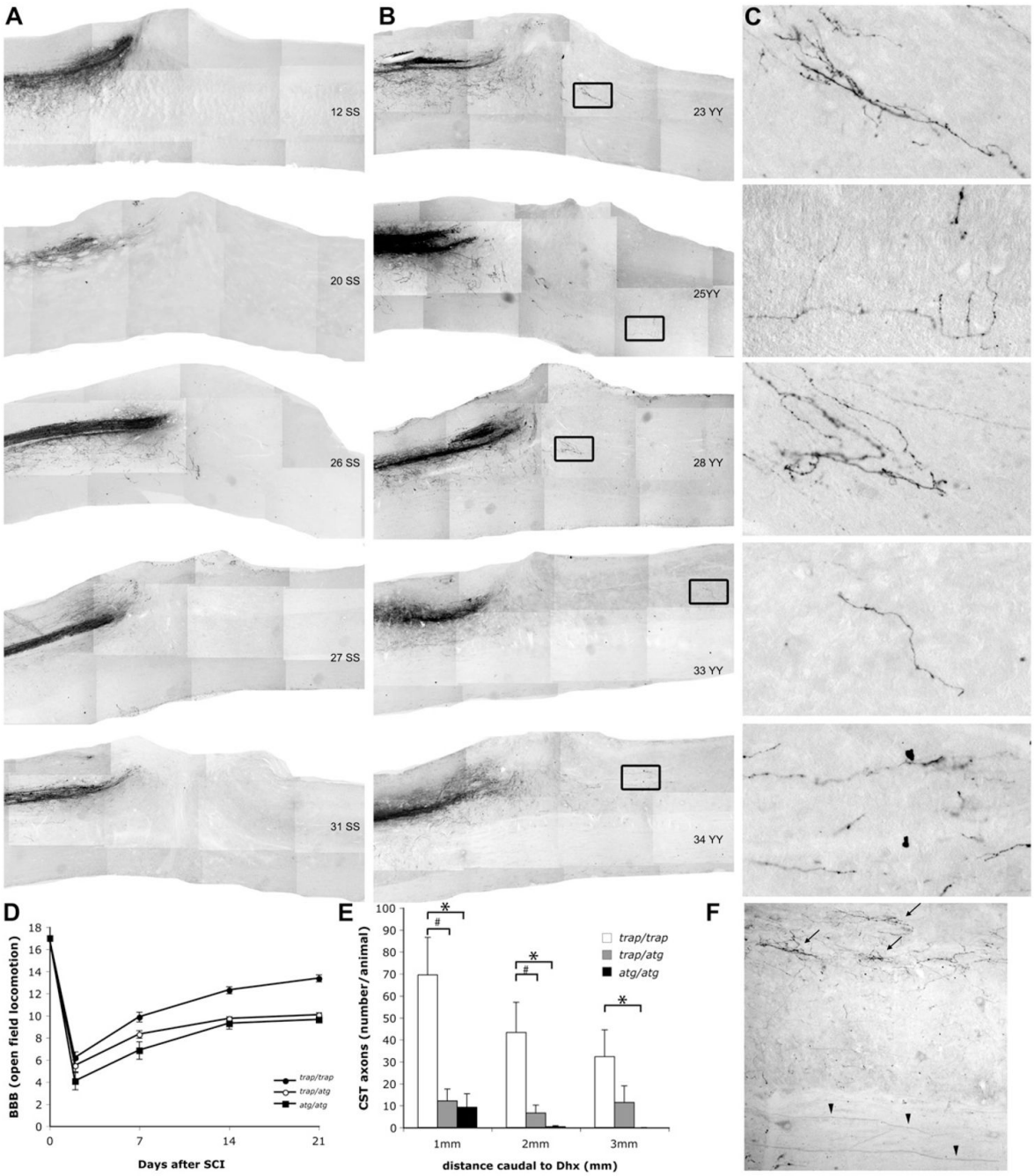

Figure 1. CST Axonal Tracing in Different nogo-a/b$^{-/-}$ Mice after Dorsal Hemisection

(A) Parasagittal sections of thoracic spinal cord from five different *nogo-ab*$^{atg/atg}$ mice. The CST is traced from BDA injections at the time of a dorsal hemisection. Rostral is left, dorsal is up. There is no evidence of CST growth past the lesion.

(B) Sections from five different *nogo-ab*$^{trap/trap}$ mice. These mice were littermates of the mice in (A) and were lesioned, traced, and processed as in (A). Note the evidence of CST fiber growth caudal to the lesion site.

(C) For each section in (B), the boxed region is magnified.

(D) Open field locomotor scores (BBB) were recorded 3–21 days after bilateral dorsal hemisection in *nogo-ab*$^{trap/trap}$, *nogo-ab*$^{atg/atg}$, and mixed heterozygote *nogo-ab*$^{trap/atg}$

animals (n = 8/group). Two-way ANOVA revealed that *nogo-ab*$^{trap/trap}$ recovered significant hindlimb function in comparison to *nogoA*$^{trap/atg}$ and *nogo-ab*$^{trap/atg}$ ($p < 0.05$). All data are mean ± SEM.

(E) BDA-labeled CST axons were counted 1, 2, and 3 mm caudal from the epicenter of the lesion (denoted by asterisk). *nogo-ab*$^{trap/trap}$ had significantly more CST axons caudal to the lesion in comparison to *nogo-ab*$^{trap/atg}$ and *nogo-ab*$^{atg/atg}$ animals (*$p < 0.01$; #$p < 0.05$; ANOVA, n = 8/group). All data are mean ± SEM.

(F) Distinction between CST tracing and mislabeling in one mouse with both patterns. A parasagittal section of the thoracic spinal cord in a region 2–3 mm caudal to a dorsal hemisection is shown. This image is from the problematic mouse of Figures 4B and 6B in Kim et al. (2003). While BDA labeling of the CST is evident as tortuous finely branched varicose fibers (arrows), weaker BDA labeling of the linear, thick, hollow type described by Steward et al. is clearly distinct (arrowheads).

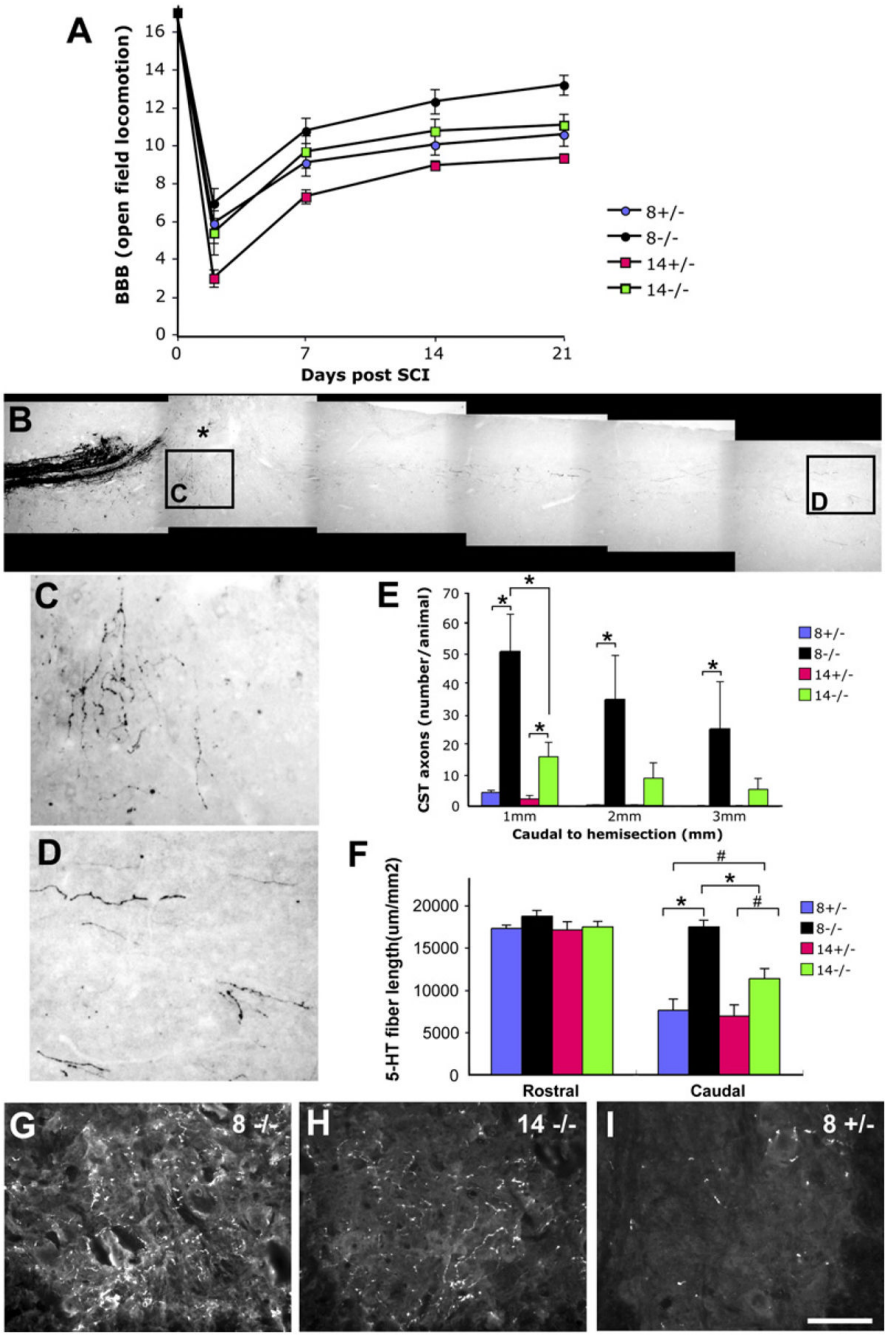

Figure 2. Age Dependence of Regenerative Phenotype in nogo-ab$^{trap/trap}$ Mice

(A) Open field locomotor scores were recorded 3–21 days after dorsal hemisection in 8-week-old *nogo-ab*$^{+/trap}$, *nogo-ab*$^{trap/trap}$, 14-week-old *nogo-ab*$^{+/trap}$, and 14-week-old *nogo-ab*$^{trap/trap}$ mice. Within each age group, there is significantly better walking in the *nogo-ab*$^{trap/trap}$ mice as compared to the *nogo-ab*$^{+/trap}$ mice ($p < 0.05$; ANOVA, n = 12 per group). All data are mean ± SEM. (B) Sagittal photomontage through the lesion site (denoted by asterisk) shows a typical example of CST axon tracing after unilateral cortical BDA infusion in an 8-week-old *nogo-ab*$^{trap/trap}$ mouse. Scale bar, 500 μm.

(C and D) High-magnification views of BDA-labeled CST fibers from (B) reveal contorted and varicose axonal profiles consistent with regenerated axons. Scale bar, 100 μm.

(E) Counts of BDA-labeled profiles observed 1–3 mm caudal to the lesion site in the indicated mice from micrographs as in (B). Axons caudal to the lesion site were observed more frequently in 8-week-old *nogo-ab*$^{trap/trap}$ mice than in 8-week-old *nogo-ab*$^{+/trap}$, 14-week-old *nogo-ab*$^{trap/trap}$, or *nogo-ab*$^{+/trap}$ mice (*$p < 0.05$, t test). All data are mean ± SEM.

(F–I) Photomicrographs of transverse sections through the ventral horn show significantly greater 5HT-IR axon sprouting caudal to a dorsal hemisection in 8-week-old *nogo-ab*$^{trap/trap}$ mice (G) than in 14-week-old *nogo-ab*$^{trap/trap}$ mice (H) or in *nogo-ab*$^{+/trap}$ littermates (I) ([F]; *$p < 0.0005$, t test). The 14-week-old *nogo-ab*$^{trap/trap}$ mice exhibit a greater degree of caudal 5HT axon arbor than do 14-week-old *nogo-ab*$^{+/trap}$ and 8-week-old *nogo-ab*$^{+/trap}$ mice ([F]; #$p < 0.05$, t test). Quantifi-cation of axon density in the ventral horn rostral to the lesion shows no difference between age and genotype groups (F). Scale bar, 200 μm. All data are mean ± SEM.