Regeneration of CNS axons back to their target following treatment of adult rat brain with chondroitinase ABC

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Following CNS injury in the adult mammal, axon regeneration fails in scar regions containing a number of different chondroitin sulfate-bearing proteoglycans (CSPGs)[1]. Degradation of chondroitin sulfate using chondroitinase ABC reduces growth inhibition associated with many CSPGs[2–13]. Here we demonstrate that it is possible to enhance CNS axon regeneration in the adult rat nigrostriatal tract following chondroitinase ABC degradation of chondroitin sulfate.

In a preliminary experiment to determine the time course of CSPG deposition following nigrostriatal tract axotomy, six anesthetized adult rats were given unilateral nigrostriatal axotomy lesions[14] using a Scouten wire knife, and killed after 1, 2 or 4 weeks. Immunolabeling using various antibodies against chondroitin sulfate and against CSPG core proteins showed that in this model of injury, as in others[3–5], peak levels of many CSPGs were detected at injury sites between one and two weeks post-axotomy (data not shown).

To degrade chondroitin sulfate throughout this period, we repeatedly delivered chondroitinase ABC to the site of axotomy. Twenty-three anesthetized adult rats were given unilateral nigrostriatal axotomy lesions, as above. Cannulae were secured transcranially to allow sham infusion or repeated perilesional infusions of 3 µl saline containing 600 ng of either high-purity, protease-free chondroitinase ABC (Seikagaku, Japan), or penicillinase (control bacterial protein, Roche, UK), on days 0, 3, 7 and 10 post-axotomy (Fig. 1a).

To assess regeneration of dopaminergic nigrostriatal axons, series of parasagittal sections of brains from animals killed 11, 18 or 100 days post-axotomy were immunolabeled using antibodies against tyrosine hydroxylase (TH). At all times examined following axotomy and treatment with chondroitinase ABC, dopaminergic nigral axons had grown through the injury site (Fig. 1d) and along the course of the original nigrostriatal tract (Fig. 1e) back to their original principal target (the ipsilateral striatum), where they branched (Fig. 1f). Axons did not always grow directly toward the target but often grew ectopically, branching extremely frequently. In contrast, at no time did dopaminergic nigral axons regenerate beyond the site of axotomy following axotomy with either sham infusion or treatment with control protein (Fig. 1g–i). The few TH-immunoreactive processes observed in control animals beyond the site of axotomy and within occasional striatal foci (Fig. 1b and c) were not thought to be degenerating or regenerating nigral axons, because the number of these axons did not change with time; further, these axons are seen following either complete axotomy[14] or chemical ablation of the substantia nigra[15]. Estimates for the number of regenerating dopaminergic nigral axons in axotomized animals treated with chondroitinase ABC have been adjusted accordingly.

The number of TH-immunoreactive processes observed growing 1 mm beyond the site of axotomy toward the original principal target differed greatly between treatment groups (Kruskal Wallis, $H_2 = 16.9$, $p < 0.001$; Dunn's *post hoc* tests, $p < 0.05$; Fig. 1b). Following treatment with chondroitinase ABC, the mean number of dopaminergic nigral axons growing this far was estimated as 1914 on day 11, 2162 on day 18 and 2020 on

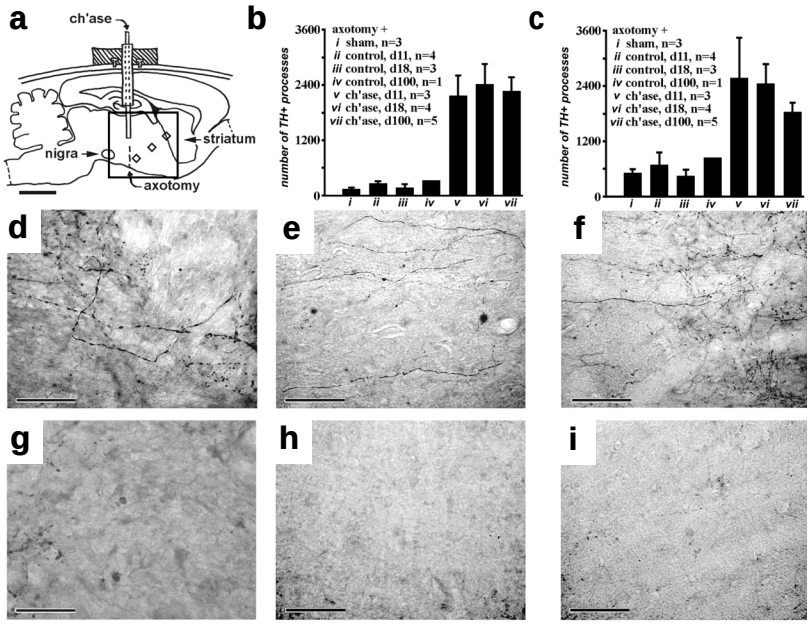

Fig. 1. Treatment with chondroitinase ABC enhanced dopaminergic nigrostriatal axon regeneration *in vivo*. All animals were treated in accordance with the UK Animals (Scientific Procedures) Act 1986. Axotomized, sham-infused animals examined 11, 18 or 100 days post-axotomy did not differ significantly one from another and were combined. Error bars, standard error; *n*, number of animals per group. (**a**) Parasagittal schematic showing the site of axotomy and transcranial infusion. Three small diamonds indicate regions shown at higher magnification in (**d–f**) and (**g–i**). Scale bar, 2 mm. (**b**) The estimated mean number of tyrosine hydroxylase (TH)-immunoreactive axons counted 1 mm anterior to the site of the axotomy was significantly greater in axotomized animals treated with chondroitinase ABC than in those treated with control protein. (**c**) The estimated mean number of TH-immunoreactive processes entering the ipsilateral striatum was significantly greater in animals treated with chondroitinase ABC than those treated with control protein. (**d–i**) TH immunolabeling examined 11 days post-axotomy indicated that following treatment with chondroitinase ABC (**d–f**), but not following treatment with control protein (**g–i**), dopaminergic nigral neurons regrew through the site of axotomy (**d**, **g**) and along the course of the original nigrostriatal tract (**e**, **h**) and into the striatum (**f**, **i**), where they arborized. Scale bars, 50 µm.

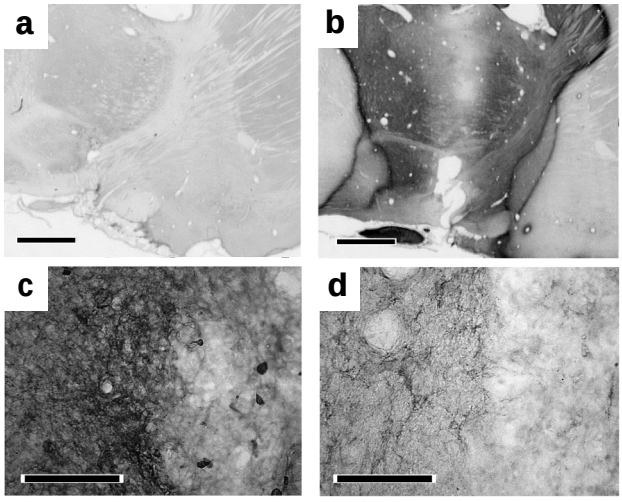

Fig. 2. Treatment with chondroitinase ABC degraded chondroitin sulfate *in vivo*, examined 11 days post-axotomy. (**a**, **b**) 2B6 immunolabeling indicated that chondroitin-4 sulfate was degraded between the site of axotomy and the original target following treatment with chondroitinase ABC (**b**) but not control protein (**a**). Fields of view correspond to large rectangle shown in Fig. 1a. The region lacking 2B6 immunoreactivity in (**b**) is due to cavitation. Scale bars, 2 mm. (**c**, **d**) CS-56 immunolabeling indicated that chondroitin sulfate was degraded at the site of axotomy following treatment with chondroitinase ABC (**d**), but not control protein (**c**). Right half of image corresponds to lesion core, left half corresponds to gliotic surround. Scale bars, 100 μm.

day 100 (Fig. 1b). However, because these numbers include numerous axon collaterals, they may overestimate the number of primary regenerating axons. In intact brains, approximately 50,000 TH-immunoreactive axons were counted at this level.

The number of TH-immunoreactive processes entering the original target, 4 mm beyond the site of axotomy, also differed greatly between treatment groups (Kruskal Wallis, $H_2 = 15.3$, $p < 0.001$; Dunn's *post hoc* tests, $p < 0.05$; Fig. 1c). Following treatment with chondroitinase ABC, the mean number of dopaminergic processes entering the original target was estimated as 1786 on day 11, 1665 on day 18 and 1045 on day 100 (Fig. 1c). In intact brains, approximately 70,400 TH-immunoreactive processes were counted at this level.

Finally, the mean number of TH-immunoreactive cell bodies in the substantia nigra either ipsilateral or contralateral to the lesion did not differ between groups (data not shown). Taken together, these results indicate that treatment with chondroitinase ABC promotes long-distance regeneration of cut dopaminergic nigral axons.

To demonstrate that chondroitinase ABC degraded chondroitin sulfate in the region of axon regeneration, series of sections were immunolabeled using three different monoclonal antibodies. Antibody 2B6 (Seikagaku, Japan) recognizes an epitope created following chondroitinase ABC degradation of chondroitin-4 sulfate. (2B6 does not recognize intact chondroitin sulfate.) At all times examined, 2B6 immunoreactivity was absent in controls (Fig. 2a), whereas there was intense 2B6 immunolabeling within 4 mm of the infusion site in axotomized animals treated with chondroitinase ABC (Fig. 2b). Thus, chondroitinase ABC (but not control protein) effectively degraded chondroitin sulfate from the entire region between the site of axotomy and the proximal part of the original target. Further, the same staining patterns were observed following immunolabeling using the 3B3 antibody (Seikagaku, Japan), which recognizes an epitope created following chondroitinase ABC degradation of chondroitin-6 sulfate. (3B3 does not recognize intact chondroitin sulfate.) Finally, immunolabeling using the CS-56 antibody (Sigma, UK), which recognizes an epitope on some intact chondroitin sulfate glycosaminoglycan chains, showed intense reactivity surrounding the site of axotomy in axotomized animals treated with control protein (Fig. 2c), whereas in axotomized animals treated with chondroitinase ABC, CS-56 immunoreactivity was much reduced (Fig. 2d). Differences between groups in labeling (2B6, 3B3 and CS-56) were less pronounced at 18 and 100 days post-axotomy, indicating that CSPGs depleted of chondroitin sulfate are removed slowly from injury sites. These results clearly indicate that treatment with chondroitinase ABC (but not control protein) effectively degraded chondroitin sulfate *in vivo* from the site of axotomy and the surrounding tissue.

Finally, on an animal-by-animal basis, following axotomy and treatment with chondroitinase ABC (examined 11, 18 or 100 days post-axotomy), the extent of chondroitin sulfate degradation (quantified by optical densitometry of 2B6 immunoreactivity) correlated significantly and positively with the number of TH-immunoreactive processes entering the original target (Pearson product moment correlation, $r = 0.70$, $p = 0.011$). This indicates that with an increase in chondroitin sulfate degradation, the number of dopaminergic nigral axons able to grow long distances increased as well.

Our results show that degradation of chondroitin sulfate using chondroitinase ABC can render the environment of the damaged CNS more permissive to axon regeneration. This result confirms that CSPGs are a significant source of inhibition after CNS injury and suggests that the effects of chondroitinase ABC should be evaluated in other models of CNS injury, with the long-term intention of treating human spinal cord injuries.

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