Chondroitinase ABC Enhances Pericontusion Axonal Sprouting But Does Not Confer Robust Improvements in Behavioral Recovery

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Abstract

Traumatic brain injury (TBI) results in enduring functional deficits. Strategies aimed at promoting plasticity within the injured brain may aid in enhancing functional outcome. We have previously shown that spontaneous pericontusional axon sprouting occurs within 7–14 days after controlled cortical impact injury in the adult rat, but ultimately fails due to an increasingly growth-inhibitory environment. We therefore sought to determine whether acute infusion of chondroitinase ABC into the site of the cortical contusion, to further reduce pericontusional growth-inhibitory chondroitin sulfate proteoglycans (CSPGs), would enhance and prolong the sprouting response. We also wanted to determine if chondroitinase-enhanced sprouting would ameliorate the behavioral deficits in forelimb function that occur in this model. Acute chondroitinase infusion decreased intact CSPGs and significantly increased pericontusional cortical grey and white matter growth-associated protein 43 (GAP43)-positive axon sprouting at 7 days post-injury. A return of intact CSPGs at later time points likely contributed to the absence of persistently increased levels of axon sprouting by 14–21 days post-injury. There was no overall benefit on forelimb function during the time of maximal sprouting or at any subsequent times in three of four behavioral outcome measures. However, there was a chondroitinase-induced improvement in recovery from unskilled limb use deficits on the staircase forelimb reaching test toward sham-injured values at 28 days, which was not achieved by the vehicle-treated rats, indicating that there is some minor functional benefit of the increased sprouting induced by chondroitinase treatment. The current results, together with data from spinal cord injury models after chondroitinase intervention, suggest that a combinatorial approach with the addition of neurotrophins and rehabilitation would result in more robust axon sprouting and consequently improve behavioral outcome.

Key words: chondroitin sulfate proteoglycans; cortex; growth-associated protein 43; immunohistochemistry; plasticity; recovery of function; sprouting; traumatic brain injury

Introduction

DESPITE OVER TWO DECADES OF RESEARCH focused on preventing secondary damage or cell death there is still no effective clinical treatment for the alleviation of enduring functional deficits that are induced by traumatic brain injury (TBI). While acute interventional neuroprotective strategies are undoubtedly important in preventing further loss of function, strategies aimed at promoting plasticity within the injured brain may also aid in enhancing functional outcome. Numerous types of interventions have been used in the CNS to promote axon sprouting, based on the idea that improving brain circuitry, either by enhanced sprouting of existing cir-

cuits or by the creation of new pathways around the injury site, will improve function after debilitating injury (Bradbury et al., 2002; Seymour et al., 2005; Smith et al., 2007).

Although some level of spontaneous regeneration occurs after brain trauma (Christman et al., 1997; Emery et al., 2000; Harris et al., 2010; Hulsebosch et al., 1998), major impediments to more robust sprouting include the non-growth-permissive extracellular environment due to endogenous levels of growth inhibitory molecules, their upregulation after injury in association with glial scar formation, and the discrete window of enhanced neurotrophic support that occurs during the growth program initiated after injury. Chondroitin sulfate proteoglycans (CSPGs) are a major class of growth-inhibitory

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molecules that, unlike in other types of injury (Asher et al., 2002; Matsui et al., 2005; Tang et al., 2003), are initially downregulated in pericontused regions behind the developing glial scar after experimental TBI (Harris et al., 2009a). It is during this initial 2- to 3-week period that spontaneous pericontusional axon sprouting occurs after TBI, until CSPGs and other inhibitory factors reassert a balance favoring growth inhibition (Harris et al. 2010).

Methods to increase axon sprouting are an important area of research for both brain and spinal cord injury, and some functional benefits have been achieved and attributed solely to sprouting after spinal cord injury (Bradbury et al., 2002; Houle et al., 2006). Numerous studies have shown that blocking of inhibitory molecules after brain trauma results in enhanced sprouting at the level of the spinal cord or brainstem and provide some functional benefit (Lee et al., 2004; Wiessner et al., 2003). While these and other sites up and down the CNS axis are all potentially important for making contributions to plasticity to aid functional recovery, relatively few studies have looked at pericontusional regions or sites closer to the primary injury site. In a preliminary study using deterministic and probabilistic diffusion tractography methods, we found that both intra- and interhemispheric connectivity is severely disrupted as early as 7 days after cortical contusion injury, with connectivity deficits persisting to at least until 4 weeks post-injury (Harris et al., 2009b). These deficits occur despite the corpus callosum appearing normal on T2 anatomical images in less severely injured rats. Since spontaneous sprouting occurs in these regions where CSPGs are initially low (Harris et al., 2009a), it seemed logical that a potential therapy to enhance sprouting should be studied at this level to capitalize on an environment that is already primed for growth-promotion through the natural endogenous response to TBI.

We therefore designed a study to potentiate the reduction in growth-inhibition within the injured region using the bacterial enzyme chondroitinase ABC. We used the adult rat cortical contusion model, which we have tailored to produce a pericontusional region over the forelimb cortex sensory-motor representation, leaving the underlying corpus callosum anatomically intact but deficient in its connectivity (Harris et al., 2009b). We hypothesized that intervening acutely with chondroitinase, a method previously shown to digest growth-inhibitory CSPGs in the CNS (Bradbury et al., 2002; Massey et al., 2006; Yick et al., 2003), would enhance the normal spontaneous increase in axonal sprouting, and alleviate some of the functional sensorimotor deficits that occur in this TBI model.

Methods

Experimental groups

Adult male Sprague-Dawley rats weighing 250–275 g were used for all experiments. Three separate cohorts of rats were randomized to nine groups for immunohistochemistry analysis for axon sprouting and enzyme detection at post-injury days 7, 14, and 21 (injury + chondroitinase treatment, $n = 10$, 7 and 5/time point; injury + vehicle treatment, $n = 6$, 6 and 5/time point). For behavior analysis of injury and treatment effects using the staircase forelimb reaching test, rats were randomized to groups receiving injury + chondroitinase ($n = 8$), injury + vehicle ($n = 9$), injury only ($n = 5$), or sham-injury ($n = 12$). Behavior analysis using the grid walk and cylinder tests was conducted using a different cohort of rats

randomly assigned to injury + chondroitinase ($n = 11$), injury + vehicle ($n = 11$), and injury only ($n = 5$) groups, with end-point immunohistochemistry analysis at 28 days.

All procedures were approved by the UCLA Chancellor's Committee for Animal Research.

Surgery for controlled cortical impact and post-injury treatments

The previously characterized controlled cortical impact (CCI) injury model (Chen et al., 2003; Harris et al., 2009a) was used to create a unilateral cortical contusion injury to the left hemisphere. Briefly, a 6-mm-diameter circular craniectomy was made over the left sensorimotor cortex, centered at the bregma and 3.5 mm lateral to the midline, while animals were under 1.5–2% isoflurane anesthesia in 100% oxygen flowing at 1.0–1.5 L min⁻¹. CCI was produced using a 4-mm-diameter, flat-tipped metal impactor attached to a pneumatically-controlled piston on a stereotaxic micromanipulator. The piston was angled at 22.5° from vertical to make contact perpendicularly to the surface of the brain at 20 psi and to a depth of 2 mm below the dura. For injury-only control rats, the bone flap was replaced and sealed in place with cyanoacrylate glue and the skin incision was sutured closed. For the injury + treatment groups, immediately after injury either 750 nL of chondroitinase ABC dissolved in artificial cerebrospinal fluid (aCSF; 48 U/mL; Sigma-Aldrich, St. Louis, MO), or aCSF vehicle alone was slowly infused at 100 nL/min into the center of the injury site at a depth of 1 mm below the dura. Injections were accomplished with a 33-g needle attached to a microsyringe (Hamilton Co., Reno, NV), and a digital micro-infusion pump (Stoelting, Wood Dale, IL) mounted on a stereotaxic arm. After waiting 3 min for diffusion from the injection site, the needle was slowly withdrawn. An osmotic pump (#1007D; Alzet, Cupertino, CA), filled with either chondroitinase dissolved in aCSF (10 U/mL; 0.5 μ L/h for 7 days) or aCSF alone, was placed subcutaneously at the shoulders and was attached to a metal cannula that was positioned 1 mm below the dura in the center of the injury site (Brain Infusion Kit 3; Alzet). The concentration of chondroitinase for both the initial injection and the combined volume of the injection plus that infused using the osmotic pump were chosen to approximate those used previously to reduce endogenous, intact CSPGs in the normal rat brain (Pizzorusso et al., 2002), and to suppress injury-induced upregulation of growth-inhibitory glycosaminoglycan (Lin et al., 2008). The cannula and its retaining pedestal assembly were held rigidly in place using dental acrylic cement that anchored it to two skull screws placed anterior and posterior to the craniotomy site. The skin wound was sutured closed over the pedestal assembly and dental acrylic, which further protected and stabilized the implant. Sham-injury control rats were subjected only to craniotomy and the bone flap was replaced. Bupivacaine (0.05–0.07 mg/kg SC) was infiltrated into all wound margins prior to the animals recovering from anesthesia. Antibiotics were given in the drinking water for the first 5 days following surgery.

Immunohistochemistry

Rats with CCI or sham injury were overdosed with sodium pentobarbital (100 mg/kg IP), and transcardially-perfused with 0.1 M phosphate-buffered saline (PBS) at approximately mean arterial blood pressure, followed by 4% paraformaldehyde.

hyde in PBS. Following cryoprotection in 25% sucrose in PBS until the brain sank, the brain was frozen and coronal sections were cut at 50 μm . Standard free-floating, single-label immunohistochemistry (IHC) was performed as described previously (Chen et al., 2003; Harris et al., 2010) on one series of sections that were 600 μm apart for each primary antibody used. In brief, after antigen retrieval, a 1-h quench step, blocking in TXTBS (TBS containing 0.2% Triton X-100; Sigma-Aldrich) with 10% normal horse serum (NHS; Vector Laboratories, Burlingame, CA), and 1% bovine serum albumin (BSA; Vector Laboratories), adjacent sections were incubated overnight at room temperature on a shaker in: mouse growth-associated protein 43 (GAP43) primary antibody (1:1500, #MAB347; Chemicon/Millipore, Billerica, MA) in TBS containing 5% NHS, mouse anti-2b6 (1:200; Associates of Cape Cod, East Falmouth, MA), or mouse anti-CSPG (1:200, CS-56; Sigma-Aldrich). After three washes in TBS, the sections were incubated with biotinylated rat-adsorbed anti-mouse IgG (1:500; Vector Laboratories) in TBS with 5% NHS for 1 h, followed by three washes in TBS. Signal amplification was achieved using the Vectastain Elite ABC kit (Vector Laboratories) for 30 min following the manufacturer's instructions, followed by three washes in TBS. Antibody binding was then visualized with diaminobenzidine in distilled water containing 0.03% hydrogen peroxide. Finally, the sections were mounted on gel-coated slides, dehydrated, and cover-slipped with DPX mounting medium.

Cell density counts and cortical atrophy measures

GAP43-positive cell profile counts were completed as described previously (Harris et al., 2010) on 3–5 IHC sections/brain that contained the injured/ipsilateral sensorimotor or parietal cortex, using tissue sections spaced at least 600 μm apart. Sections were analyzed using a 40 $\times$ objective, and the locations of positive cell profiles were recorded digitally using StereoInvestigator Software (MicroBrightfield, Williston, VT). The total number of GAP43-positive profiles, defined as either a stained cell body or a neurite/fibril-like segment, were counted in the entire ipsilateral grey and white matter (corpus callosum) encompassing the region from the midline to the rhinal fissure. Large, tangentially-lying GAP43-positive processes were counted once. The counts were expressed as the average density of GAP43-positive profiles by measuring the area of tissue where the counts were performed, and calculating the tissue volume based on the inter-slice distances.

Cortical atrophy was assessed on IHC GAP43-stained sections from 7- to 21-day injured rats using the brain contours used to delimit the counting area described above. For each rat, the average volume of ipsilateral tissue loss over the 3–5 sections analyzed through the injury site was computed as the section-level difference between ipsilateral and contralateral cortical grey + white matter volumes, expressed as a percentage of the contralateral cortical volume. Atrophy in post-injury day 28 brains after behavioral assessment was computed in a similar manner from scanned images of Nissl-stained, 50- μm coronal brain sections using ImageJ software (Rasband, 2008).

Group-level image computation of axonal sprouting

In order to provide a visual representation of the overall degree and extent of sprouting within each group, semi-

quantitative images of the distribution of GAP43-positive cells were created in a group-wise fashion by digitally aligning and superimposing images of cell counts exported from StereoInvestigator software. Single, representative, coronal sections at the level of the bregma from each rat were overlaid and then rigidly aligned using the contours of the brain previously drawn within the counting software as reference points (Rasband, 2008). For the 7- and 14-day groups containing unequal numbers of rats, sections were taken from six rats that faithfully encompassed the quantified distributions of GAP43-positive profiles in order to illustrate the data in the most representative way. The resulting maps depict the summed result of a single representative section from each of six rats within each group (see Figs. 4 and 5). The aligned maps of cell counts were then manually contoured at the individual rat level to obtain the minimum distance, region of interest (ROI) that best fit the counted cells. The ROIs were then recombined for group level display as layered contoured maps. Data were superimposed on the section-average brain and contusion contour in order to semi-quantitatively represent the degree of overlap between the population of GAP43-positive profiles within each group.

Behavioral outcome measures

Staircase test. At 1 week prior to surgery the rats were pre-trained daily on the staircase apparatus (Lafayette Instrument, Lafayette, IN) to retrieve food pellets for 10 min per day. The apparatus is an acrylic glass box with a removable, baited, double staircase on either side of a central platform. A sugar pellet (45 mg; Noyes, Lancaster, NH) is placed on each step of the left and right staircase and presented bilaterally at seven graded stages of forelimb reaching difficulty to provide an objective measure of side bias. The design of the apparatus prevents any dropped pellets from being retrieved. The number of steps cleared within a 10-min period (pellet retrieved and eaten) is used as an objective measure of forelimb skilled reaching ability. The number of pellets dropped is used as a measure of unskilled limb performance. Two trials were run per testing day and averaged. The investigator conducting the testing was blinded to the rat group assignments for the entire study. Rats were food-restricted throughout the study to maintain 90–95% of average body weight for age-matched male Sprague-Dawley rats. After surgery, the rats were re-tested in the staircase pellet retrieval task at 7, 14, 21, and 28 days.

A separate cohort of rats was used to assess two additional measures of forelimb function to confirm the staircase findings in animals not subjected to food restriction. Once again the behavioral testers were blinded to rat group assignments for the duration of the study and data analysis.

Grid-walk test. Rats were habituated to walking on a raised, 38 mm² metal grid, 36 cm wide and 72 cm long, for 4 days prior to injury. Behavior was videotaped for 2.5 min/trial for offline analysis. Right-limb faults (the future affected limb) were scored as "1" by a missed foot placement on the grid resulting in limb placement below the grid to a distance of approximately half the length of the forelimb. Half-faults (scored as "0.5") were used to describe foot-slips when only the forepaw or less than half of the limb was placed below the grid. After habituation to the task, the rats were tested for two

trials/day on the day prior to surgery, at 4 days after, and then weekly on days 7, 14, 21, and 28 days after injury. The rats were trained to walk the whole length of the grid in order to return to the home cage. Daily trials were averaged and then grouped.

Cylinder asymmetry test. Rats were not habituated to the apparatus, but were tested on the day prior to injury and at weekly intervals thereafter until 28 days. Each day's testing was performed immediately before the grid-walk task. The rats were placed in a transparent Perspex 18-cm-diameter cylinder and videotaped for 10 rears onto the cylinder wall. Limb-use asymmetry scores were computed from the difference in the number of unaffected (left) and affected (right) initial limb placements onto the cylinder wall.

Statistical analysis

For all cell count data the Kolmogorov-Smirnov with Lillie correction and Levene median tests were used to describe the distribution of the data and to determine the equality of the variances, respectively. Having passed these normality tests, group numerical data were expressed as the means $\pm$ standard error of the mean (SEM). Cell density count differences were explored using one-way analysis of variance according to our *a priori* hypothesis of chondroitinase-induced increased sprouting. Statistical significance (α) was set at $p < 0.05$ for all comparisons. Linear mixed-effects modeling statistics were used for all behavior group comparisons (Hornik, 2008). Normally distributed data were inferred by the degree of kurtosis and skewness (~ 1), as well as by using the Shapiro normality test. Different models were assessed that best fit the data (fixed and random effects of time-by-group multiplet of interaction, time-by-group additive interaction, and an autoregressive model). The resulting Akaike and Bayesian information criteria were used to determine the best fit for each parameter tested. Group-by-time interactions as a function of time were used to drill down to group level differences at specific levels of time.

Results

Tissue effect of chondroitinase

At 7 days after injury the severity of brain injury was similar between chondroitinase- and vehicle-infused rats, as assessed by loss of ipsilateral cortical plus white matter tissue volume (18 ± 4.2 and $15 \pm 3.5 \text{ mm}^3$, respectively). Regional proteolysis due to chondroitinase ABC action can be identified using the "stub" antibody 2b6 against the protein core regions newly deficient in side-arm glycosaminoglycans. We evaluated all brains for signs of enzyme action and found 2b6-positive staining in all brains at 7 days after injury, although it was variable. Staining consisted of either hyperintensity at the site of injection pump infusion in less-severely injured brains extending over both grey and white matter (Fig. 1A), or more often as regions of hyperintensity along the edges of the contusion (Fig. 1B). Within these regions, immunostaining for intact CSPGs in chondroitinase-treated animals was reduced compared to vehicle-infused rats (Fig. 1C–F), confirming the potency of enzyme activity at 7 days post-injection. However, at days 14 or 21, we were unable to reliably identify 2b6-positively-stained areas in all brains analyzed, although fol-

lowing behavioral testing we did find minor evidence of 2b6 in 4 of 9 chondroitinase-infused brains at 28 days after injury (data not shown). The absence of greater evidence for enzyme digestion in brains after day 7 is likely due to either a lack of sustained enzyme activity within the brain, leading to renewed CSPG expression, or to expansion of the cortical cavitation due to tissue atrophy at the later time points. Gross volume analysis of the amount of tissue lost following injury revealed that atrophy was indeed likely to be responsible for at least some of the loss of enzyme-digested tissue, as the hemispheric tissue loss increased from 29% at 7 days to 38% at 21 days. In addition, at 14 days (Fig. 1G and H), and at all later time points examined (data not shown), the quantity of intact CSPGs indicated by CS-56 staining in chondroitinase-infused rats was similar to that seen in vehicle-infused animals.

Chondroitinase enhances axon sprouting

Analysis of interleaved sections at the same anteroposterior plane used for enzyme analysis revealed that injury plus vehicle alone resulted in spontaneous grey and white matter axon plasticity, as indicated by GAP43-positive axonal sprouting at 7 days after injury (Figs. 2B, 3B, and 4), to levels similar to those reported after CCI injury alone (Harris et al., 2010). Chondroitinase increased sprouting in surviving grey matter at the pericontusional edge (Fig. 2), particularly in white matter underlying the contusion (Figs. 3 and 4), compared to vehicle-treated injured animals. Quantification of this effect revealed a variable increase compared to the six vehicle-infused rats of between 0% ($n = 3$) and $>20\%$ ($n = 7$) more cortical axonal sprouting, although this increase did not reach statistical significance due to the large variability seen among the chondroitinase-treated rats ($p > 0.05$, $n = 10$). However, within this group there was a significant association between the degree of axonal sprouting and the severity of injury, as assessed by the volume of tissue lost (Pearson $r = -0.71$, $p = 0.011$; Fig. 4C). When mildly-injured rats sustaining $<20\%$ tissue loss at day 7 were excluded from the analysis, sprouting was $60 \pm 16.8\%$ higher in chondroitinase-treated ($n = 7$) than in vehicle-infused rats ($n = 4$), and this difference was significant (Fig. 4D, $p < 0.05$). The extent of sprouting was also significantly different between the two levels of tissue injury ($>20\%$ versus $<20\%$ tissue volume loss) within the chondroitinase-treated group ($p < 0.05$, $n = 7$ and 3, respectively), lending further credence to the association between injury severity and effect of treatment on sprouting. The association between sprouting and injury severity was specific to the injury plus chondroitinase group, as there was no significant correlation between axon sprouting and injury severity in the injury plus vehicle group ($r = -0.52$, $p = 0.15$).

By 14 days after injury the mean number of GAP43-positive profiles was not significantly lowered from 7 days in either treatment group ($p > 0.05$). Similarly to 7 days, sprouting remained increased in the chondroitinase-treated group ($n = 7$) at 14 days, by up to 45% over vehicle-treated ($n = 6$) levels (Fig. 5A, B, and E). However, this effect was variable in both injury groups, and the difference between the absolute number of GAP43 profiles remained outside significance ($p > 0.05$). There was no association of sprouting with the amount of hemispheric tissue loss in either the chondroitinase- or vehicle-treated group at this time point ($r = -0.34$ and -0.72 , respectively, $p > 0.05$). By 21 days after

FIG. 1. Representative sensorimotor cortical coronal immunohistochemistry brain sections at 7 days (**A–F**) and 14 days (**G** and **H**) after injury, illustrating the degree of chondroitin sulfate proteoglycan (CSPG) digestion by chondroitinase in three different rats (**A**, **B**, **C**, **E**, and **G**), compared to vehicle-injected rats (**D**, **F**, and **H**). At 7 days after injury, sections stained with 2B6 primary antibody to visualize the CSPG “stubs” that remain after digestion revealed that in relatively intact tissue immediately anterior to the contusion, wide areas were affected by the enzyme (**A**), while tissue remaining in more severely-affected brains was visible along the pericontusional edge (**B**). In these same regions, staining for intact CSPGs showed a corresponding reduction in CSPGs in intact regions anterior to the contusion after chondroitinase (**C**), compared to vehicle-injected injured rats (**D**), and in more severely-affected rats after chondroitinase (**E**), compared to vehicle injection (**F**). Staining for intact CSPGs at 14 days after injury (**G** and **H**) revealed a developing glial scar in pericontusional tissue (left side of images) in both chondroitinase- (**G**) and vehicle-injected rats (**H**), together with similar levels of staining intensity and numerous CSPG-positive cells in both. This indicates reduced enzyme action and a return of inhibitory glycosylated CSPGs (scale bars = 250 μm for all images except insets [25 μm]).

injury, sprouting was significantly decreased in both groups compared to 7 and 14 days ($p < 0.05$), but there was no longer an effect of chondroitinase treatment on sprouting (Fig. 5C, D, and F).

Injury-induced behavioral deficits were not significantly ameliorated by chondroitinase treatment

The staircase forelimb reaching task (Montoya et al., 1991) was used as an initial measure of functional outcome, since we have previously shown it to be a sensitive indicator of both contusion injury and recovery due to axon sprouting (Smith

et al., 2007). There was no significant effect of aCSF infusion on this behavior outcome compared to injury alone ($p > 0.05$). Similarly, there was no difference in contusion size between the injury-plus-aCSF and injury-alone groups as assessed by the percentages of ipsilateral tissue volume loss on post-injury day 28 ($31 \pm 4.2\%$ versus $34 \pm 8\%$, respectively), and therefore the data were pooled into a single group referred to hereafter as “injury plus vehicle.” Injury plus vehicle treatment ($n = 14$) resulted in a significant increase in the number of pellets dropped during staircase testing compared to sham-injured controls ($n = 12$), indicating impairment of unskilled limb use that endured at least until 4 weeks post-injury ($p < 0.001$;

FIG. 2. Representative coronal immunohistochemistry brain sections of growth-associated protein 43 (GAP43) staining in grey matter sensorimotor cortex at 7 days after injury, illustrating enhanced axon sprouting after chondroitinase administration in two different brains at low power (A and C), and high power (A₁–A₄, C₁–C₃, and D), compared to a vehicle-treated, injured brain (B). No positive staining was ever noted in sham-injured rats (data not shown). The diagram at top left shows the approximate position of the stained regions (asterisk) relative to the injury site. Many of the stained grey matter fibers arose from GAP43-positive cell bodies in the deeper cortical layers (C and C₃), and were often branched and appeared to extend dendrites (D). Other fibers followed a more tortuous route (A₂), and showed bulbed endings (A₃), especially close to the injury site (A₄; scale bars = 250 μ m in low-power images and 25 μ m in high-power images).

Fig. 6A). Skilled limb use indicated by the number of pellets successfully retrieved and eaten was similarly affected by injury plus vehicle treatment ($p < 0.001$; Fig. 6B). Despite these data confirming that the test is highly sensitive to the effect of CCI injury alone, there were only trends in the data suggestive of beneficial effects of chondroitinase treatment after injury; unskilled and skilled limb use were consistently improved by chondroitinase treatment, but there was no significant effect at any time point assessed between the two injury and treatment groups for either parameter (Fig. 6A and B). However, by 4 weeks after injury the level of unskilled limb use in the injury plus chondroitinase group was not significantly different from sham-injured performance ($p > 0.05$; Fig. 6A). Interestingly, the same parameter assessed in injury-plus-vehicle rats showed that significant deficits remained ($p < 0.01$ versus sham animals) at day 28 post-injury.

We next evaluated potential treatment effects by testing additional injured rats with or without chondroitinase treatment using the grid-walk and cylinder tasks. Three vehicle-treated and two chondroitinase-treated rats were removed from the study due to their head-caps becoming dislodged during the final 2 weeks of the study. Similarly to the staircase test, there was no significant effect of aCSF infusion on either behavioral test or on contusion size at 28 days, and therefore the data for the injury-plus-aCSF ($n = 8$) and injury-only ($n = 5$) groups were pooled to form an injury-plus-vehicle group ($n = 14$), for comparison with the injury-plus-

chondroitinase group ($n = 9$). As was seen for staircase reaching, although minor data trends were present to indicate a slight degree of benefit from chondroitinase, there was no significant difference between the treatments on either the grid-walk or cylinder tasks at any time point (Fig. 7A and B).

Finally, we assessed tissue atrophy after behavioral testing on Nissl-stained tissue sections to rule this out as a potential confound. Similarly to our earlier atrophy data from IHC-stained sections at 21 days, ipsilateral cortical hemispheric tissue loss was $32 \pm 4.0\%$ in vehicle-treated and $38 \pm 10.0\%$ in chondroitinase-treated animals, indicating that atrophy was not significantly different between the groups ($p > 0.05$).

Discussion

The current data show that acute parenchymal administration of chondroitinase ABC to reduce the accumulation of CSPGs that occurs in this model of TBI (Harris et al., 2009a) significantly increases both cortical grey and white matter GAP43-positive axon sprouting at 7 days post-injury. Re-normalization of intact CSPG levels at later time points likely contributed to the lack of persistently increased levels of axon sprouting. Despite the early increase in chondroitinase-induced sprouting seen in sensorimotor cortical regions, there was no overall benefit on forelimb function during the time of maximal sprouting, or at any subsequent time points, in three

FIG. 3. Representative coronal immunohistochemistry brain sections of growth-associated protein 43 (GAP43) staining in the corpus callosum directly below the injury site at 7 days post-injury (**A** and **B**), or sham-injury (**C**), illustrating increased axon sprouting after chondroitinase treatment (**A**) compared to vehicle (**B**). Numerous GAP43-positive profiles were seen as black dots at low power after injury (**A** and **B**) compared to sham (**C**), and they were more prevalent after injury plus chondroitinase treatment (**A**) compared to injury plus vehicle (**B**). At high power, these positive profiles were observed as axonal fibers and as small bleb-like protuberances (**A**₁–**A**₄ and **B**₁) that were not present in sham-injured rats (**C**₁; scale bars = 250 μ m in low-power images and 25 μ m in high-power images).

of four behavioral outcome measures. However, there was an improvement in recovery from unskilled limb use deficits toward sham-injured values at 28 days for the staircase test compared to the vehicle-treated injury group, indicating that there was a minor benefit from the increased sprouting induced by chondroitinase treatment.

Axon sprouting

We observed significant increases in axon sprouting in 7 of 10 rats infused with chondroitinase, and this effect was generally greater in white matter than in grey matter. Similar effects of chondroitinase on sprouting have previously been noted after spinal cord injury (Bradbury et al., 2002; Ikegami et al., 2005; Massey et al., 2006; Tom et al., 2009; Yick et al., 2003), and after denervation or lesioning of white matter tracts in the brain (Moon et al., 2001; Tropea et al., 2003). Functional improvements due to axon sprouting of either new or existing circuits in the spinal cord have been confirmed by re-lesioning (Bradbury et al., 2002). However, it is

unknown whether spontaneous sprouting that occurs in the brain contusion model (Harris et al., 2010), or that due to chondroitinase, specifically improves corpus callosum function. The electrical activity of the callosum has been shown to be severely impacted after fluid percussion-induced brain injury (Baker et al., 2002), especially in small, unmyelinated fibers (Reeves et al., 2005). We have also shown in the CCI injury model that there is a >50% reduction in fiber tracts connecting under the contusion, and ~30% reduction in interhemispheric connectivity, in the first 4 weeks following injury (Harris et al., 2009b). The spontaneous establishment of novel patterns of grey matter activation in response to forelimb stimulation at 28 days after trauma (Harris et al. 2003) might be due at least in part to intracortical or callosal sprouting. It would seem likely that any increases in sprouting at the level of the corpus callosum, however subtle, would have the potential to reconnect large regions of cortical grey matter, which could then support improvements in neurobehavioral function. Despite the lack of greater behavioral gain of function seen in the current

FIG. 4. Co-registered semi-quantitative summed images constructed from six representative rats at the level of the bregma from post-injury day 7 injury plus vehicle (**A** and **A'**) and injury plus chondroitinase groups (**B** and **B'**), illustrating the difference in raw numbers of growth-associated protein 43 (GAP43)-positive profiles between groups (**A** and **B**), and the color-coded degree of overlap between the location of the positive cells among the six rats within each group (**A'** and **B'**); see methods section for construction details. (**C**) Linear fit of a scatterplot showing the higher degree of correlation between injury severity (hemispheric loss of cortical volume) and the degree of axonal sprouting at 7 days after injury plus chondroitinase (solid squares, $r = -0.71$, $p = 0.011$, $n = 10$), compared to injury plus vehicle (open circles, $r = -0.52$, $p = 0.15$, $n = 6$). (**D**) Plot of numbers of GAP43-positive profiles in ipsilateral cortical grey and white matter, showing a significant increase in the number of profiles due to chondroitinase treatment (Ch'ase, $n = 7$) at 7 days after injury compared to vehicle ($n = 4$), after removing less-severely-injured rats (3 chondroitinase and 2 vehicle rats with $<20\%$ tissue loss, indicated above the dashed line in **C**) from both groups ($*p < 0.05$). Color image is available online at www.liebertonline.com/neu

study, it will be important in future work to determine any direct connectivity and functional significance due to the increased sprouting in the corpus callosum.

Enhanced sprouting was not significantly prolonged beyond the period of chondroitinase infusion in the current study, although it was still higher than vehicle-treatment levels at 2 weeks following injury. There are several possibilities that might explain this finding. We observed a lack of sustained deficits in CSPGs, due either to the normal tissue atrophy process in the contusion core, which likely included enzyme-treated tissue around the infusion cannula, and/or to the absence of continued chondroitin sulfate proteolysis. Our tissue atrophy measurements certainly support the former conclusion. After a single chondroitinase injection, enzyme activity is sufficient to digest newly synthesized CSPGs for 7–14 days (Lin et al., 2008), and this corresponds to the peak of axonal sprouting seen in the present study. Despite our observations of enzyme-digested products remaining even at 28 days in some animals, this is not an indicator of ongoing enzyme activity, since the level of newly synthesized CSPGs with intact glycosamino side chains normalized by 28 days (Lin et al., 2008). In the current study, enzyme activity within the pumps would have significantly decreased over time, so that CSPG digestion probably resulted only from the initial injection and from 1–3 days of pump infusion (Lee et al., 2010). It is therefore not surprising that axonal sprouting was not prolonged. Furthermore, it appears unlikely that additional, more chronic interventions to maintain reduced intact glycosaminoglycan-containing CSPG levels would be beneficial, since acute rather than delayed intervention with chondroitinase is more optimal

for axonal sprouting (Mingorance et al., 2006), and for some but not all tests of behavioral outcome (García-Alías et al., 2009). Combinatorial therapies with neurotrophins are required to induce significant increases in synapse formation of newly-sprouting axons after denervation (Tropea et al., 2003). Indeed, experiments involving chondroitinase administration in the uninjured brain (Corvetto and Rossi, 2005) suggest that despite reductions in CSPGs, continued synergistic action by the injury-initiated growth program of neurotrophins and other stimuli is likely required to sustain long-lasting synaptic contacts. These data might also help to explain the lack of increased sprouting above spontaneous levels seen in 3 of 10 chondroitinase-treated rats (those with milder injury) at day 7 in the current study. If increased sprouting occurs due to a combination of chondroitinase and an extracellular environment primed by injury for growth-promotion, then only more moderate-to-severe injury levels might afford this level of synergy. Finally, it should also be noted that it remains to be seen whether delayed intervention with chondroitinase in the brain, or interventions in other brain regions, would promote greater plasticity and functional outcome. It is quite likely that the acute brain injury environment prevented some chondroitinase action due to degradation of the enzyme itself, so delayed intervention may well be more efficacious.

Behavioral outcome

We did not observe any significant functional benefits in skilled limb use due to intervention with chondroitinase. Similar findings of no functional benefit despite significant

FIG. 5. Co-registered semi-quantitative summed images constructed from five representative rats at the level of the bregma from post-injury day 14 (A, B, A', and B') and day 21 (C, D, C', and D'), from injury plus vehicle (A, A', C, and C'), and injury plus chondroitinase groups (B, B', D, and D'), illustrating the difference in raw numbers of growth-associated protein 43 (GAP43)-positive profiles between the groups at 14 days (A, B, C, and D) and the color-coded degree of overlap between the locations of the positive cells among the five rats within each group (A', B', C', and D'). (E) Quantitative numbers of GAP43-profiles in ipsilateral grey and white matter at 14 days after injury. There was no significant difference at 14 days, although there was a trend towards increased sprouting due to chondroitinase treatment. (F) By 21 days there were reduced numbers of GAP43-positive profiles present in both groups, with no effect of treatment (Ch'ase, chondroitinase). Color image is available online at www.liebertonline.com/neu

axonal regeneration have been noted with either a single injection of chondroitinase or through genetic ablation of CSPGs following spinal cord injury (Cafferty et al., 2007; Tom et al., 2009). However, repeated injections or continuous infusion have resulted in some behavioral improvements after spinal lesioning (Bradbury et al., 2002; Houle et al., 2006; Massey et al., 2006), while others have shown that only a regimen of simultaneous rehabilitation training during the time of chondroitinase intervention is capable of improving forelimb use (García-Alías et al., 2009).

At the more acute time points, chondroitinase-treated rats systematically trended towards better forelimb neurobehavioral scores in all four of our behavioral outcome measures compared to vehicle-treatment. This occurred despite there being equivalent levels of injury severity between the treatment groups, as indicated by the similar degrees of tissue atrophy seen at 28 days. While acute improvements in function would seem temporally unlikely to be due to axon sprouting, which peaked at 7–14 days, the reduction in CSPG-containing perineuronal nets (PNNs) that accompany chondroitinase interventions (Bruckner et al., 1998), might also enhance the acute formation of new synapses in existing circuitry. However, at least at more chronic time points after spinal cord injury, this does not appear to be the case (Tom et al., 2009).

Recent evidence from a transgenic mouse line exhibiting reduced PNNs, but no difference in CSPG content, suggests that the organization of CSPGs into perineuronal nets, rather than CSPG concentration, is the key event affecting CNS plasticity (Carulli et al., 2010). Following trauma, PNNs remain reduced at least for 4 weeks after insult, but the sprouting response is largely complete by this time (Harris et al., 2010). This suggests that other mechanisms, in addition to sprouting, contribute to plasticity after trauma. However, chondroitinase-treated rats showed normal unskilled reaching behavior by day 28 post-injury on the staircase test, while vehicle-treated rats were still significantly impaired, despite there being similar gross decreases in cortical PNNs at 28 days in both groups (data not shown). This behavioral improvement, albeit minor, would suggest that earlier sprouting contributes to later gains in function. More robust improvements might result from the addition of simultaneous rehabilitation or trophic support, as has been shown after brain lesion or spinal cord injury (García-Alías et al., 2009; Karimi-Abdolrezaee et al., 2010; Tropea et al., 2003).

In conclusion, the current data show that acute intervention with chondroitinase significantly increases cortical sprouting after experimental TBI. Although some measurable improvements in forelimb neurological function did occur, a robust improvement in functional outcome was not achieved.

FIG. 6. Plots of unskilled (A) and skilled (B) affected forelimb function testing as evaluated by the staircase test in rats before and after injury plus chondroitinase treatment (Injured + Ch'ase, filled circles, $n = 8$), and injury plus vehicle (Injured + vehicle, open triangles), pooled vehicle treatment ($n = 9$), and injury only ($n = 4$) group after statistical testing, and in sham-injured rats (Sham, open squares, $n = 9$). Regardless of treatment, injury significantly increased the number of pellets retrieved and then dropped (unskilled limb use) at 7–21 days after injury (A), and decreased the number of pellets successfully retrieved and eaten at 7–28 days (B), compared to sham-injured rats ($***p < 0.001$). There was no significant difference between the two injury plus treatment groups when assessed by either parameter. However, unskilled use of the affected forelimb in the injury plus chondroitinase group was initially reduced relative to injury plus vehicle treatment, and improved considerably over time so that at 28 days the number of pellets dropped was not significantly different from the sham-injured group ($**p < 0.01$, $*p < 0.05$).

Combinatorial therapies, such as those designed to widen the temporal window for permissive growth and provide the requisite neurotrophic growth stimulus or rehabilitation training during the period of maximal sprouting, may be required to more effectively alleviate the functional deficits present after TBI.

FIG. 7. Tests of forelimb function using the grid-walk (A) and cylinder tests (B) in chondroitinase- ($n = 9$) and vehicle-treated injured rats ($n = 14$, solid circles and open triangles, respectively) in a separate cohort of rats from those used for the staircase test of forelimb reaching. Despite both tests yielding significant deficits in affected forelimb function out to 4 weeks post-injury when compared to the zero values seen pre-injury, indicating sustained sensitivity to injury, there was no significant benefit to chondroitinase administration seen at any time point post-injury. Similarly to the staircase test, there were minor trends in the data towards a positive effect of chondroitinase, particularly in the first week of treatment.

Acknowledgments

We wish to thank Ms. Jessica Harnisch and Mr. Shant Damian for technical assistance, and Dr. David McArthur for help with statistics. This work was supported by the UCLA Brain Injury Research Center, and Award Number R01 NS055910 from the National Institute of Neurological Disorders and Stroke (NINDS). The content is the sole responsibility of the authors and does not represent the official views of the NINDS or the National Institutes of Health.

Author Disclosure Statement

No competing financial interests exist.

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