Rehabilitation after intracerebral hemorrhage in rats improves recovery with enhanced dendritic complexity but no effect on cell proliferation

Angela M. Auriata, Shannon Wowka, Frederick Colbourne

A A
Department of Psychology, University of Alberta, Edmonton, AB, Canada
" Centre for Neuroscience, University of Alberta, Edmonton, AB, Canada

ABSTRACT

Rehabilitation, consisting of enriched environment and skilled reach training, improves recovery after intracerebral hemorrhage (ICH) in rats. We tested whether rehabilitation influences dendritic morphology (Golgi–Cox staining—experiment 1) or cell proliferation (immunohistochemistry—experiment 2). In the latter experiment, BrdU was given from 14 to 18 days post stroke, and cells were labeled for BrdU along with NeuN, Iba1 or GFAP. One week after a striatal ICH, via collagenase infusion, the rats were given rehabilitation for 2 weeks or control treatment (group housing in standard cages). Behavioral outcome (e.g., skilled reaching, walking) was assessed at multiple times. Rats were euthanized at 5 (experiment 2) or 6 (experiment 1) weeks post-ICH. As expected, rehabilitation significantly improved skilled reaching and walking ability. There was also a concomitant increase in dendritic length in peri-hematoma striatum and ipsilateral cortex as well as in the contralateral striatum. Lesion volume did not differ between groups, nor did cell proliferation. There was no evidence of neurogenesis, but there was increased Iba1 and GFAP labeling in the injured hemisphere. Thus, rehabilitation likely improves outcome after ICH though a plasticity response (e.g., increased dendritic growth) that does not involve neurogenesis.

1. Introduction

An intracerebral hemorrhage (ICH) is a devastating stroke causing significant mortality and disability [23]. While substantial improvement occurs [21,23], a better mechanistic understanding is needed to further lessen residual deficits. Animal stroke models have been used to identify factors that influence recovery and to test rehabilitation strategies [10,11,18,27]. Unfortunately, most ICH studies focus on pathophysiology and neuroprotection, ignoring mechanisms of recovery. Thus, there is a paucity of animal data available for ICH.

Nonetheless, rodent experiments show that striatal ICH causes an initial atrophy of peri-hematoma neurons that subsequently appears to recover [19]. In contrast, the contralateral striatum shows a sustained increase in dendritic complexity. Greater dendritic branching, indicating enhanced synaptogenesis, has been routinely linked with recovery after brain injury, and is a likely contributor to behavioral improvement after ICH [11,18,27]. Similarly, animal studies show modestly enhanced neurogenesis after ICH [15,28], which also appears to occur in humans [25]. The contribution of these mechanisms, among others (e.g., inflammatory response), to spontaneous and rehabilitation-facilitated recovery after ICH is not well known.

A variety of rehabilitation strategies have been tested in rodent ICH models with limited success. These putative treatments include: environmental enrichment (EE) [1,2], simple exercises (e.g., treadmill) [3,7,12], and forced-use therapies (e.g., “constraint-induced movement therapy”) [1,7]. Interestingly, in some cases, rehabilitation lessened brain injury after ICH [1,7,12]. This has also been observed in some ischemia models. Delayed cell death occurs in rodent models of ICH, and is especially prominent in the collagenase model [14,19]. Thus, rehabilitation may also improve recovery through neuroprotection.

In this study, we evaluated these potential mechanisms through which rehabilitation improves recovery after collagenase-induced ICH in rat [22]. Rehabilitation (REHAB), which consisted of environmental enrichment and skilled reach training [4], started 1 week after the lesion and lasted for 2 weeks. Control rats (CONT) were group housed. In experiment 1, we assessed whether dendritic complexity was increased at 3 weeks following treatment. In experiment 2, we tested whether rehabilitation influenced cell proliferation and the inflammatory response at 2 weeks following treatment. We assessed cell proliferation and inflammation/gliosis at an earlier time point than dendritic plasticity because these factors were likely to decrease with time from injury. Multiple behavioral endpoints were used in both experiments, but the staircase test...
of skilled reaching was, a priori, the primary endpoint due to its sensitivity to striatal ICH [13] and the effects of rehabilitation [1,7].

2. Materials and methods

2.1. Subjects

We used 64 male Sprague–Dawley rats obtained from the Biosciences's colony at the University of Alberta. All procedures were approved by the Biosciences Animal Care and Use Committee at the University of Alberta and were in accordance with the Canadian Council on Animal Care guidelines. Rats were kept 4 per cage with free access to water and food, except as noted below, under 12-h light cycles (on from 7 a.m. to 7 p.m.). Cages of 4 rats were randomly assigned to either REHAB or CONT treatment (n = 16 per experiment), thereby avoiding stress associated with changing cage mates.

2.2. Behavioral training and testing

2.2.1. Skilled reaching

All rats were food deprived to ∼90% of their free feeding weight and maintained at this level adjusting for normal growth. Rats were trained twice daily 5 days a week over 3 weeks. During each 15-min training session the rat was placed in a staircase box (length: 30 cm; width: 6.8 cm; height: 12 cm) containing a central platform and descending stairs on each side [17]. On each of 7 steps per side there were 3 food pellets (45 mg, Bio-Serv, Frenchtown, NJ), which the rat grasped with its forepaw. Rats were excluded if they did not obtain an average of 8 pellets per session with at least one paw over the final 3 training days. Afterwards, rats were returned to ad lib feeding until they were food deprived for testing at 6 days, and 5 weeks (experiment 2) or 6 weeks (experiment 1) post-ICH.

2.2.2. Walking

Rats walked across a 1 m long horizontal ladder [16] with randomly spaced rungs (1–4 cm apart; diameter: 3 mm). Rats were trained for 3 days (4 crossings per day) and video recorded on the final day. The contralateral to ICH forelimb was analyzed to determine walking ability (error = (number of slips/total steps) × 100). Walking ability was assessed 3 days prior to injury and at 6 days and 32 days (experiment 2) or 39 days (experiment 1) following ICH.

2.2.3. Spontaneous limb use

Rats were placed in a Plexiglas cylinder (diameter: 20 cm; height: 45 cm) and video recorded for 10 min. Spontaneous forelimb use was measured for each initial contact and movement on the cylinder wall, scored as being made by the ipsilateral or contralateral (to ICH) limb or as both. Contralateral forelimb use was expressed as: ((contralateral forelimb contacts + 1/2 bilateral contacts)/total contacts) × 100 [24]. Animals were tested 3 days before and at 6 and 32 days (experiment 2) or at 39 days (experiment 1) following ICH.

2.2.4. Trop task

Rats in experiment 2 were also trained and tested on a tray task where they reach through vertical bars at the front of a cage (length: 27 cm; width: 19 cm; height: 25 cm) to retrieve food placed on an external tray (17% Layer Prostock feed; Masterfeeds, Edmonton, AB). Rats were given a daily 30-min training session following the completion of staircase training. Reaching was video recorded 3 days before ICH, and at 6 and 32 days post-ICH. The % success was determined as: (successful/total reaches) × 100 [26].

2.3. ICH surgery

Rats weighed ~350 g on the day of surgery. Procedures were similar to those used previously [1]. Briefly, they were anesthetized with 4% isoflurane (25% maintenance; 60% N2O and remainder O2) for aseptic surgery and placed in a stereotaxic frame. Body temperature was maintained between 36.5 and 37.5 °C. A hole was drilled at 3.5 mm lateral and 1.0 mm anterior to Bregma on the side contralateral to the ICH. Weight was monitored daily to ensure no animal dropped below 90% of its free feeding weight. The CONT rats remained group housed in standard cages for the duration of the experiment with the same feeding schedule and cage change cycles. Furthermore, they were given an equivalent amount of sugar pellets scattered on the floor of their home cage as obtained by the REHAB group.

2.5. BrdU injections

All rats in experiment 2 received daily injections of BrdU (100 mg/kg i.p., Sigma, St. Louis, MO, USA, B5002) on days 14 through 18 after ICH.

2.6. Histology

In experiment 1, rats were euthanized at 39 days post-ICH via an overdose of pentobarbital (80 mg/kg, i.p.). They were then perfused with 0.9% saline. The brains were removed and placed in Golgi–Cox solution for 14 days. Brains were then immersed in 30% sucrose in distilled water for 2 days. Tissue was Vibratome-sectioned at 200 µm and stained according to established methods [9,19]. Four brain areas were assessed: (1) medium spiny neurons in the peri-hematomata region, (2) the corresponding area in the contralateral striatum, (3) basilar dendrites of layer 5 pyramidal neurons in the ipsilateral, and (4) contralateral forelimb motor cortex. For each region of interest (ROI) 5 cells were drawn per animal. Neurons were traced via Camera Lucida using a 20× objective by a researcher blinded to treatment conditions. Only those neurons that were fully impregnated with Golgi–Cox solution and unobscured were drawn. Sholl analysis of ring intersections was used to estimate dendritic length and complexity [9]. Briefly, a transparent grid of concentric rings was used to assess the number of cell branches that intersected each ring. Finally, spines on the terminal tips of neurons in the same ROIs were drawn at 1000× (oil immersion). The number of spines and length of each segment were determined and the spine density was expressed as the number of spines per unit length.

In experiment 2, rats were euthanized 32 days after ICH via pentobarbital. They were perfused with 0.1 M phosphate-buffered saline (PBS) followed by fixation with 4% paraformaldehyde. Brains were further fixed overnight and then transferred to 30% sucrose in PBS. Brains were cryostat sectioned at 50 µm. Every fourth section was placed on slides for cresyl violet staining and lesion volume assessment. The volume of each hemisphere was calculated as: (average area of complete coronal section of the hemisphere–area of damage–area of ventricle) × interval between sections × number of sections. Lesion volume was then calculated by subtracting the remaining volume of injured hemisphere from the volume of the normal hemisphere [1,13].

Sections from a random subset of animals (n = 8 per group) were collected at the level of maximal lesion and placed in centrifuge tubes containing PBS for immunohistochemistry. Three sections per brain were saved; one was double labeled for BrdU and a mature neuronal marker (NeuN), one for BrdU and a macrophage/microglia marker (Iba1), and one for BrdU and an astrocyte marker (GFAP). Prior to BrdU labeling, tissue was exposed to 2.5% H2O2 for 30 min at room temperature. Free-floating sections were incubated in blocking solution (10% donkey serum and 0.3% Triton-X 100 in PBS) for 1 h and then incubated with primary antibodies in 2% blocking solution for 24 h. Sections were then placed in secondary antibodies (1:500) with DAPI (1:500; nuclear label, Pierce, Rockford, IL, USA, 46190) for 1 h.

The following primary antibodies were used in this study: rat anti-BrdU (1:1000; Serotec, Raleigh, NC, OBIT0030-1), rat anti NeuN (1:500; Millipore, Temecula, CA, MAB377(CH)), mouse anti GFAP (1:400; Sigma, St. Louis, MO, G3893), and rabbit anti Iba1 (1:1000, Wako, Richmond, VA, 019-19741). The secondary antibodies used in this study, all from Jackson ImmunoResearch (West Grove, PA, USA) are: donkey and rat Cy3 (712-165-153), donkey anti mouse Cy5 (715-485-151) and donkey anti rabbit biot 488 (711-485-152). Control sections were incubated without primary antibodies, and no nonspecific labeling was observed in these sections.

Six regions (450 µm × 350 µm) per brain were identified for quantification of GFAP+ and Iba1 + cells at 20× magnification (Olympus BX51), BrdU + cells in the ipsilateral and contralateral ventricles, as well as in the striatum of a single section per brain were counted. All BrdU+ cells in each section were examined for double labeling.

2.7. Statistics

Data are expressed as mean ± SEM and were analyzed by repeated measures and/or 1-way analysis of variance (ANOVA) using SPSS (v. 17.0, SPSS Inc, Chicago, IL).

3. Results

3.1. Exclusions

Five rats in experiment 1 did not reach baseline criteria for staircase training and were excluded. Nonetheless, they received surgery and remained housed with their cage mates to reduce the stress of changing the housing arrangement. One rat in the CONT...
group was euthanized early due to excessive weight loss following ICH. These factors left group sizes of 13 in each group. There were no exclusions from experiment 2.

3.2. Behavioral testing

In experiment 1, there were significant Day \((p < 0.001)\) and Group \((p = 0.018)\) main effects, and a Day \(\times\) Group interaction \((p = 0.001; \text{Fig. 1a})\) in the staircase test. The groups were not different prior to ICH, or after ICH but prior to intervention \((p \geq 0.714)\). However, rehabilitation improved skilled reaching on all test days \((p \leq 0.019)\) by ~22\% (vs. CONT). Similar results were observed in experiment 2 (Fig. 2a) where there was a significant Day main effect \((p < 0.001)\) and a Day \(\times\) Group interaction \((p = 0.001)\), but no Group main effect \((p = 0.803)\). Rehabilitation significantly reduced impairment by ~29\% on the test days \((p \leq 0.044)\), but, as expected, there were no group differences prior to treatment \((p \geq 0.244)\). Rehabilitation successfully reduced skilled reaching impairments in both experiments.

In experiment 2, reaching ability was also assessed in the tray task (Fig. 2b). Those rats that did not make more than 10 reach attempts with their impaired limb were excluded from analysis leaving CONT and REHAB groups with the following group sizes: baseline \((16, 15)\), day 6 \((12, 9)\) and day 32 \((12, 10)\). There was an effect of Day \((p < 0.001)\), but not of Group \((p = 0.762)\), and no interaction \((p = 0.099)\). Owing to the data loss in the repeated measured design, we proceeded with a planned comparison that revealed a significant effect of rehabilitation on the test day \((p = 0.039)\). Adding further support to the staircase finding that also found improved reaching ability in REHAB treated rats.

In experiment 1, analyzing walking ability for the contralateral forelimb revealed a significant Day \((p < 0.001)\) and Day \(\times\) Group effect \((p = 0.016)\), but there was no Group main effect \((p = 0.222)\). Additional one-way comparisons indicated that although the performance between groups did not differ at baseline or day 6 \((p \geq 0.568)\), the REHAB group was significantly better at day 39 \((p = 0.009; \text{Fig. 1b})\). In experiment 2 (Fig. 2c), there were significant Day \((p < 0.001)\) and Group \((p = 0.016)\) main effects and a Day \(\times\) Group interaction \((p < 0.001)\). There were no pre-treatment differences \((p \geq 0.365)\), but the REHAB group was less impaired post-treatment \((p < 0.001)\). Thus, rehabilitation improved ladder-walking ability with the impaired forelimb in both experiments.

In both experiments (Fig. 1c; Fig. 2d), cylinder test analysis indicated that there was a Day effect \((p \leq 0.005)\), but no Group \((p \geq 0.573)\) or interaction effects \((p \geq 0.447)\). Rehabilitation did not significantly alter the spontaneous usage of the impaired forelimb.

3.3. Golgi–Cox analysis

Poor staining and technical errors resulted in the loss of some tissue from analysis. Thus, cortical tissue analysis was completed on 12 rats per group whereas the striatal analysis was completed on 11–12 rats per group. Rehabilitation increased the overall dendritic length compared to the CONT group in ipsilateral cortex, peri-lesion striatum and contralateral striatum \((p < 0.012)\), but not in the contralateral cortex \((p = 0.217)\). Dendritic length in the contralateral hemisphere was greater than in the ipsilateral side in both the cortex and striatum \((p < 0.023; \text{Fig. 3})\).

Due to uneven staining several brains were excluded from spine density analysis, leaving the following group sizes for CONT and REHAB hemispheres for each location: cortical contralateral (8, 11) ipsilateral (9, 11), and striatal ipsilateral (6, 6) and contralateral (6, 5). There were no significant differences in spine density between groups for any ROI \((p \geq 0.732; \text{data not shown})\). Nor did spine density differ across hemispheres in either cortex or striatum \((p \geq 0.362)\).

3.4. Lesion volume and cell counts

There was significant striatal injury at 32 days following ICH (experiment 2), as illustrated in our previous work [7,14,19]. Injury volume did not differ between CONT \((27.79 \pm 4.50)\) and REHAB \((24.46 \pm 3.40)\) groups \((p = 0.435)\). There were more BrdU+ cells near the ventricle of the injured than uninjured hemisphere \((p < 0.001)\). However, rehabilitation did not significantly affect these numbers or those in the striatum \((p \geq 0.362)\).

3.5. Discussion

Our rehabilitation intervention, which started 1 week after ICH, significantly reduced functional impairment (skilled reaching, walking) similar to that we previously reported [1], although benefit was neither complete nor obtained on all tests. Such findings were associated with significantly increased dendritic length in peri-hematoma striatal neurons, the ipsilateral motor cortex and the contralateral striatum. Rehabilitation did not appear to alter
Fig. 2. Data in experiment 2 for the staircase test (A), tray task (B), horizontal ladder (C) and cylinder task (D). Rehabilitation improved outcome on several tests (*p < 0.05), but not on the cylinder task (50% = normal performance).

neurogenesis or cell proliferation, suggesting that, in this model, a more important contributor to behavioral recovery is synaptogenesis, not replacement of lost cells or alterations in the response of astrocytes and microglia/macrophages.

Our dendritic structure findings with rehabilitation concur with previous work in ischemia and related models indicating that ipsilateral and contralateral structures are altered following injury and rehabilitation [10,11,18,27]. However, not all studies show a total increase in dendritic branching in peri-infarct areas. Indeed, Brown and colleagues, using two-photo imaging, report that there are no net changes in apical dendrites bordering an ischemic infarct; although individual dendrites remodel by shortening tips near the infarct and lengthening branches extending away from the infarct [6]. Unlike this imaging technique, Golgi–Cox labeling cannot be

Fig. 3. (A) An example of Sholl analysis where the number of ring intersections is assessed for each neuron, this is a standard measure of dendritic length for Golgi–Cox stained neurons. (B) The mean number of circle crossings in each group were taken in experiment 1 (*p < 0.05 for group comparisons). The ipsi- and contralateral hemispheres were significantly different. (C) Representative examples of camera lucida drawings of neurons from the ipsilateral primary motor cortex (C) and striatum (D).
used to longitudinally monitor individual dendritic branches, but it has the advantage of allowing one to assess deeper cortical layers as well as subcortical structures. Thus, differences among studies may relate to the region evaluated (e.g., cortex vs. striatum; apical vs. basilar dendrites), as well as to the type of insult, animal model, etc. Regardless, such studies, including our findings, cannot causally link increased dendritic branching, indicative of enhanced synaptogenesis, with behavioral recovery. Furthermore, our study does not sort out the contribution of ipsilateral vs. contralateral structures to recovery and compensation, which has been widely studied after ischemia with contradictory findings (e.g., [5, 8]). It is likely that issues, such as dependence upon initial lesion volume [13], are also relevant to recovery after ICH as after ischemic stroke.

We observed BrdU-labeled cells migrating toward the injured striatum, which supports previous observations [15, 28]. However, there are contradictory findings with regard to the effects of rehabilitation after injury. Some report increased proliferation and neurogenesis [29] while others report a decrease [20]. Many factors such as type, location and extent of injury as well as the timing, duration, intensity and method of rehabilitation likely influence neurogenesis. Thus, whereas the current rehabilitation intervention did not alter cell proliferation, and we found no co-labeling to indicate that new cells become neurons, astrocytes or microglia, one cannot exclude the possibility that other rehabilitation treatments would influence these measures after ICH. Furthermore, it should be noted that we administered BrdU relatively late after ICH, and we did not assess multiple survival times. Thus, while our results suggest that rehabilitation does not work through enhancing cellular proliferation and differentiation, the findings certainly do not exclude a possible role, especially in spontaneous recovery. Indeed, this is indicated by the significantly increased number of GFAP+ and Iba1+ cells found in the injured hemisphere. Finally, while there were significantly fewer GFAP+ cells in the ipsilateral cortex of REHAB rats, it seems unlikely that this lone difference accounts for the behavioral effects, but further study is warranted.

We did not observe a protective effect of rehabilitation on lesion volume (experiment 2), which we had previously found [1, 7]. Given the general similarity in methods, it is difficult to reconcile these findings, but it might relate to differences in initial insult severity and/or the greater demands of testing (tray task) used in the second experiment. We also cannot exclude the possibility that rehabilitation reduced tissue loss in experiment 1. This was not measured due to processing the tissue with Golgi–Cox stain. Nonetheless, the present findings and our previous work [7] suggest that the neuroprotective effect of rehabilitation, which sometimes occurs, does not solely account for the functional benefit of rehabilitation.
cells in the ipsilateral cortex (*p < 0.05). There were significantly more of these cells in the contralateral cortex (Fig. 5).

The numbers of BrdU+ (A), GFAP+ (B) and Iba1+ (C) cells counted in experiment 2 (see Fig. 4a for ROIs). There were significantly more of these cells in the contralateral cortex (*p < 0.05).

In summary, rehabilitation improves functional recovery after ICH in rodents and this effect has at least some overlapping mechanisms to those engaged after ischemic and traumatic brain injury. Further study is needed to identify factors unique to ICH so that the efficacy of rehabilitation can be maximally enhanced thereby minimizing disability in survivors of ICH.

Acknowledgements

Research supported by a grant to FC from the Heart and Stroke Foundation (HSF) of Alberta, Northwest Territories, and Nunavut. FC is supported by a senior scholar award from the Alberta Heritage Foundation for Medical Research. AA is supported by a scholarship from the Canadian Institutes of Health Research, the Canadian Foundation for Medical Research. FC is supported by a senior scholar award from the Alberta Heritage Foundation (HSF) of Alberta, Northwest Territories, and Nunavut.

References