Research Report

Failure of estradiol to improve spontaneous or rehabilitation-facilitated recovery after hemorrhagic stroke in rats

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Abstract

Estrogen influences not only the incidence of stroke, but also the amount of injury sustained from a stroke including intracerebral hemorrhage (ICH). In this study we tested whether delayed 17β-estradiol (E2) treatment affects recovery following striatal ICH. Female rats were trained and tested on several behavioral tests to assess skilled reaching, spontaneous forelimb usage and walking ability. Two weeks following ovariectomy, rats were subjected to a moderate-sized ICH via infusion of collagenase into the striatum. One week later they were implanted with either an E2 pellet (0.36 mg; 60-day release) or they underwent a sham procedure. They were further divided into groups that received either environmental enrichment (EE) rehabilitation therapy (group housing in a complex cage with ramps, tunnels, etc.) or a control condition (group housing in a standard cage). Rats were then behaviorally evaluated out to 8 weeks post-ICH and then euthanized. Neither EE nor E2 affected lesion size, which averaged 62.8 mm³ across all groups. The EE therapy improved recovery on some tests (e.g., traversing a horizontal ladder) whereas E2 treatment did not notably affect either spontaneous or EE-facilitated recovery. Thus, E2 fails to improve recovery or protect against brain injury when given after a 1-week delay in contrast to its clear neuroprotective effects when given before or soon after ICH.

Keywords: Environmental enrichment Striatum Motor system Intracerebral hemorrhage

1. Introduction

The incidence of stroke is lower in pre-menopausal women than in men, which has been largely attributed to the high levels of estrogen, predominantly 17β-estradiol (E2), circulating in women (Ayala et al., 1995; Bushnell et al., 2006; Prentice, 2007; Wise et al., 2005). Unexpectedly, the Women’s Health Initiative (WHI) trial reported that estrogen replacement therapy actually increased stroke incidence in postmenopausal women (Wassertheil-Smoller et al., 2003); although it has been argued that this trial has serious design flaws (Bushnell et al., 2006; Klaiber et al., 2005; Wise et al., 2005). Regardless, the potential of using E2 to protect the brain (neuroprotection) in both women and men following stroke is of great interest. Indeed, in experimental models of global cerebral ischemia E2 treatment has been repeatedly shown to attenuate hippocampal CA1 sector injury (Jover et al., 2002; Shughrue and Merchenthaler, 2003; Sudo et al., 1997). Similarly, E2 reduces infarct size following focal ischemia (Dubal et al., 1998; McCullough et al., 2001; Simpkins et al., 1997). It is not...
surprising then that E2 pretreatment reduces injury in models of intracerebral hemorrhage (ICH) that target the striatum (Auriat et al., 2005; Nakamura et al., 2005). Estrogen is thought to exert its neuroprotective actions against ischemia via numerous mechanisms (Gibson et al., 2006). Direct mechanisms include preserving cerebral blood flow and enhancing post-ischemic reperfusion (Hurn and Brass, 2003) as well as suppression of pro-apoptotic signals (Dubail et al., 1999; Harms et al., 2001). While similar mechanisms likely contribute to E2’s neuroprotective effects in ICH models, estrogen may further affect ICH by promoting hemostasis and lessening the hematoma size following vessel rupture (Auriat et al., 2005). While ischemia and ICH share many mechanisms of injury, fundamental differences remain (Xi et al., 2006). Thus, it is important to test putative treatments in ICH models rather than rely on findings in ischemia models.

While the ability of E2 to reduce injury, such as CA1 sector cell loss after global ischemia, has translated into improved functional recovery (e.g., memory tasks) (Auriat et al., 2005; Gulinello et al., 2006; Li et al., 2004a,b), it is also well known that E2 affects brain function in ways that should independently improve functional recovery. For example, E2 promotes the formation of new dendrites and excitatory synapses in the hippocampus (Woolley and McEwen, 1992), and this effect is correlated with improvements in hippocampal-dependent memory (McEwen and Woolley, 1994; Packard and Teather, 1997). As well, E2 affects the expression of brain-derived neurotrophic factor (BDNF) (Berchtold et al., 2001; Sohrabji et al., 1995), which not only promotes neuronal survival but also regulates plasticity (Alderson et al., 1990; Lindsay, 1988). Interestingly, E2 receptors in the forebrain are co-localized with BDNF (Miranda et al., 1993), and E2 replacement in ovaricectomized (OVX) female rats increases BDNF expression in the forebrain (Allen and McCar son, 2005; Jeziorski and Sohrabji, 2000). Accordingly, E2 may affect recovery by enhancing dendritic arborization and spine density (Berchtold et al., 2001; Jeziorski and Sohrabji, 2003; McEwen, 2001), among other mechanisms, and these may be used to improve spontaneous recovery after stroke. Effective rehabilitation therapies also promote increases in dendritic arborization and spine density following stroke (Biernaskie and Corbett, 2001). Thus, it is possible that E2 treatment may further enhance rehabilitation efforts.

In this study, we assessed whether delayed E2 treatment affects spontaneous and rehabilitation-facilitated recovery after ICH in rats (Fig. 1—timeline of events). The collagenase model of ICH (Rosenberg et al., 1993) was used because it produces relatively consistent bleeding within the striatum along with well-characterized behavioral deficits (Auriat et al., 2008; MacLellan et al., 2006). Beginning 1 week after ICH, rats were group housed in either standard (STD) or EE cages and received either E2 pellets or they underwent sham procedures (SH). We used environmental enrichment (EE) as a rehabilitation treatment as it improves functional recovery following several types of brain injury (for a review, see Will et al., 2004), including ICH in rats (Auriat et al., 2008). Rats were group housed in the EE cages, which contained ramps, tunnels, beams and various objects to explore. Estrogen was administered continuously, via an implanted pellet, until the end of the study to mimic estrogen replacement therapy. Furthermore, the onset of E2 treatment was delayed to 1 week post-ICH to isolate E2’s ability to independently promote functional recovery from its direct effects on cell death and hematoma size found with pretreatment (Auriat et al., 2005). Comprehensive histological and functional evaluation at long survival times has been highly recommended in the assessment of putative stroke therapies (Recommendations for standards regarding preclinical neuroprotective and restorative drug development, 1999; MacLellan et al., 2006). Thus, we gauged recovery at 2, 5 and 8 weeks after ICH with several well-characterized behavioral tests that included: the tray task to assess skilled reaching (Whishaw et al., 1986), the cylinder test of forelimb use asymmetry (MacLellan et al., 2006; Shanina et al., 2006), the horizontal ladder walking test (MacLellan et al., 2006; Metz and Whishaw, 2002) and the elevated beam task to assess balance and locomotion (Feeney et al., 1982; MacLellan et al., 2006). We predicted that delayed EE would promote functional recovery and that delayed E2 would facilitate recovery independently of and in combination with EE. Neither treatment was expected to alter lesion size.

2. Results

2.1. Protocol violations and mortality

Thirteen rats were excluded entirely from this study. Of these, 7 died during surgery of unknown causes, likely due to anesthetic complications. Six others were excluded due to surgical errors (e.g., no lesion likely due to a blocked injection needle). The remaining group sizes were as follows: STD-SH (n = 16), STD-E2 (n = 12), EE-SH (n = 14), and EE-E2 (n = 17). Some data for several other rats were excluded from one or more behavioral tests for failing to meet criteria stated in Experimental procedures.

2.2. Body weight

Rat body weight (Fig. 2) was initially analyzed with a 3-factor ANOVA (Housing and Hormone factors; Time factor: OVX, ICH, and weeks 1, 5 and 8 post-ICH); owing, however, to significant Day interactions in this analysis (p < 0.003), the data were analyzed with 2 between factor ANOVAs at each time. There were no significant Housing or Hormone main effects or interactions (p ≥ 0.196) on the days of OVX, ICH and 1 week post-
post-ICH. However, both main effects were significant ($p<0.001$) at 5 and 8 weeks post-ICH as E2 and EE treatments caused rats to have a lower body weight.

2.3. Uterine weight

Uterine weights at euthanasia were 0.035 g±0.048 (mean±SD), 0.184±0.247, 0.055±0.071 and 0.165±0.126 in the STD-SH, STD-E2, EE-SH and EE-E2 groups, respectively. With a 2-way ANOVA we found that the Hormone main effect was significant ($p=0.001$), but the Housing main effect ($p=0.997$) and the interaction ($p=0.583$) were not. Thus, E2 treatment promoted excessive uterine growth.

2.4. Behavioral outcome

2.4.1. Tray task

Most rats showed a clear limb preference for grasping food in the tray task. For instance, during the last training session (a 10-minute baseline session) rats reached an average of 83.0% of the time with one limb (Fig. 3A). A 3-way ANOVA (Housing and Hormone factors; Time factor: baseline, weeks 2, 5 and 8 post-ICH), for which the interactions were non-significant ($p>0.070$), showed that asymmetry scores were similar among groups (Housing main effect: $p=0.593$; Hormone main effect: $p=0.594$) and significantly lower at 2, 5 and 8 weeks post-ICH (simple within-subjects contrasts: $p<0.001$ vs. baseline). Thus, following ICH rats altered their limb preference to more frequently use their ipsilateral-to-stroke limb instead of their contralateral, “preferred” limb, but neither EE nor E2 affected this change.

Unfortunately, many rats used their contralateral-to-stroke limb too infrequently (<5 reaches) after ICH to adequately gauge their reaching success at obtaining food. While the loss of data was approximately equal over the weeks and among groups, it amounted to an averaged loss of ~42% of data points, and, therefore, a loss of statistical power in this analysis. Furthermore, the loss of data was inconsistent as some animals did not attain our criterion on only 1 or 2 test times. Therefore, the reaching success data were analyzed with 2-way ANOVAs (Housing and Hormone factors) at the four measurement times. Reaching success with the preferred limb during baseline was similar among groups and averaged 61.9% (Housing main effect: $p=0.733$; Hormone main effect: $p=0.389$; interaction: $p=0.456$; Fig. 3B). Following ICH there was a Housing main effect favoring EE treatment on week 2
ICH (Fig. 4). A 2-between (Housing and Hormone factors) 1-within (Time factor: baseline, weeks 2, 5 and 8 post-ICH) ANOVA revealed a significant Time effect ($p<0.001$), with significant forelimb use asymmetry favoring the ipsilateral-to-stroke limb following ICH (each post-ICH week vs. baseline; $p<0.001$). The Housing ($p=0.275$) and Hormone ($p=0.315$) main effects were not significant nor were any of the interactions ($p \geq 0.321$). Thus, neither E2 nor EE treatment affected asymmetry scores, which were significantly affected by ICH.

2.4.2. Cylinder task

As expected asymmetry scores were close to 50 during baseline testing in the cylinder task and these scores decreased following ICH (Fig. 4). A 2-between (Housing and Hormone factors) 1-within (Time factor: baseline, weeks 2, 5 and 8 post-ICH) ANOVA revealed a significant Time effect ($p<0.001$), with significant forelimb use asymmetry favoring the ipsilateral-to-stroke limb following ICH (each post-ICH week vs. baseline; $p<0.001$). The Housing ($p=0.275$) and Hormone ($p=0.315$) main effects were not significant nor were any of the interactions ($p \geq 0.321$). Thus, neither E2 nor EE treatment affected asymmetry scores, which were significantly affected by ICH.

2.4.3. Beam task

Beam traversing scores were normal (i.e., 7) at baseline for all animals. The median score of 5 traverses was significantly worse (lower score) at 2 weeks post-ICH ($p=0.001$ vs. baseline, Wilcoxon Signed Ranks Test; Fig. 5), although many animals performed normally. The lower median scores observed at 5 ($p=0.066$) and 8 ($p=0.109$) weeks post-ICH were not significantly worse than baseline scores (data not shown). Thus, with this analysis, ICH seemed to only transiently impair performance on this test. Furthermore, there were no group effects at 2 ($p=0.851$; Kruskal–Wallis Test), 5 ($p=0.630$) or 8 weeks ($p \geq 0.298$). The sum score of the first 2 beam sessions was also analyzed as performance would be worse on these trials (vs. median score) and this might help reveal group effects. With this analysis there were significant impairments after ICH (vs. baseline) on weeks 2 ($p<0.001$), 5 ($p=0.027$) and 8 ($p=0.007$). Nonetheless, there were no group differences at 2 ($p=0.832$), 5 ($p=0.953$) or 8 weeks post-ICH ($p=0.060$). Thus, neither E2 nor EE treatment statistically lessened impairment following ICH.

Fig. 5 – Beam traversing performance at 2 weeks after ICH. Each symbol represents the median beam traversing score of 5 trials. All groups have significantly lower scores (versus baseline), but there were no significant differences among groups. See Results for statistics.

Fig. 6 – The % success on the horizontal ladder walking task for the contralateral-to-stroke forelimb (A), contralateral hind limb (B), ipsilateral forelimb (C) and ipsilateral hind limb (D) during baseline training (BL) and on weeks 2, 5 and 8 post-ICH. All groups had significant impairments with their contralateral forelimbs, which was significantly improved with EE, but not E2 treatment. Use of EE also improved performance with the ipsilateral limbs. See Results for statistics.
2.4.4. Horizontal ladder task
A 3-way ANOVA (Housing and Hormone factors; Time factor: 4 levels) on stepping success with the contralateral forelimb (Fig. 6A) revealed a significant Time effect ($p < 0.001$) with significant impairment evident at 2, 5 and 8 weeks following ICH (within-subjects contrasts: $p < 0.001$ vs. baseline). All interactions in this 3-way ANOVA were non-significant ($p \geq 0.091$) as was the Hormone main effect ($p = 0.808$). However, the Housing main effect was significant ($p < 0.001$). Thus, ICH caused persistent impairment in walking with the contralateral forelimb that was attenuated by EE, but not E2, treatment. As stepping success with the contralateral forelimb is a primary endpoint in this study, and given the variability, the data were also analyzed with 2-way ANOVAs at each time. These analyses revealed that the Housing main effect (favoring EE treatment) was significant at 5 ($p = 0.005$) and 8 weeks ($p = 0.003$), but not at week 2 ($p = 0.118$). The interactions and the Hormone main effect were non-significant ($p \geq 0.102$).

Owing to significant interactions (e.g., Time by Housing by Hormone; $p = 0.008$) in the 3-way ANOVA on the contralateral hind limb data (Fig. 6B), we conducted simpler analyses at each time. First, a 2-way ANOVA on baseline scores with the contralateral hind limb showed no significant differences among groups (Housing main effect: $p = 0.565$; Hormone main effect: $p = 0.389$; interaction: $p = 0.115$). Second, following a significant interaction in the 2-way ANOVA ($p < 0.001$) for week 2 data we conducted a 1-way ANOVA ($p < 0.001$) with post hoc Scheffé tests that showed only the STD-E2 group to be significantly impaired versus the STD-SH ($p = 0.005$) and EE-E2 groups ($p = 0.001$). Third, a 2-way ANOVA showed that there was a significant effect of Housing, favoring EE treatment, at the 5-week test time ($p = 0.014$), whereas the Hormone main effect ($p = 0.369$) and interaction were not significant ($p = 0.510$). Fourth, a 2-way ANOVA on the week 8 data shows that the Housing ($p = 0.051$) and Hormone ($p = 0.594$) main effects and interaction ($p = 0.380$) were not significant.

Owing to a significant Time by Housing interaction ($p = 0.002$) in the 3-way ANOVA, the ipsilateral forelimb data (Fig. 6C) were analyzed with 2-way ANOVAs at each time. Analysis of the baseline scores showed no significant differences among groups (Housing main effect: $p = 0.282$; Hormone main effect: $p = 0.492$; interaction: $p = 0.710$). There was a significant Housing main effect ($p < 0.001$) at week 2 where

Fig. 7 – A diagram of an ICH-induced lesion from a representative animal. (A) The black region represents ventricular space (e.g., ventriculomegaly on the side of the ICH) or dead tissue. The volume of tissue lost at 8 weeks after ICH surgery (B) was not significantly different among groups. See Results for statistics.

Fig. 8 – An illustration (A) of our method to measure cortical thickness (CT) and corpus callosum (CC) area, which was assessed in one coronal section that contained the largest area of injury in each rat. These measurements were done on both the ipsilateral and contralateral-to-ICH hemispheres. Cortical thickness (B) and CC area (C) were significantly smaller in the ICH side, but there were no other treatment effects. See Results for statistics.
the Hormone main effect ($p=0.916$) and interaction ($p=0.425$) were not significant. At week 5, however, both Housing ($p<0.001$) and Hormone ($p=0.020$) main effects were significant; the interaction was not ($p=0.663$). Only the Housing main effect was significant ($p=0.004$) at week 8; the Hormone main effect ($p=0.451$) and interaction ($p=0.343$) were non-significant. Thus, EE treatment consistently improved performance, whereas E2 only improved performance at one time.

Owing to a significant Housing by Hormone interaction ($p=0.044$) in the 3-way ANOVA on the ipsilateral hind limb data (Fig. 6D) it was analyzed with 2-way ANOVAs. For the baseline data (2-way ANOVA) the Housing ($p=0.552$) and Hormone main effects ($p=0.317$) were not significant nor was the interaction ($p=0.293$). However, the Housing main effect, favoring EE treatment, was significant ($p=0.006$) at each post-ICH week, whereas the Hormone main effects ($p=0.065$) and interactions ($p=0.053$) were not. Thus, the ICH did not cause ipsilateral hind limb impairments and EE treatment further improved performance.

### 2.5. Histological outcome

The collagenase-induced ICH caused injury primarily to the striatum and to a lesser extent to surrounding structures such as globus pallidus, thalamus, and corpus callosum. Ventricular enlargement was prominent (Fig. 7A). The total volume of tissue lost (Fig. 7B), which was analyzed by a 2-way ANOVA, was not significantly affected by either Hormone ($p=0.774$) or Housing treatments ($p=0.724$) and the interaction was not significant ($p=0.148$). Two-way ANOVAs showed a significantly smaller cortical thickness (CT; $p<0.001$; Fig. 8B) and area of the corpus callosum (CC; $p<0.001$; Fig. 8C) ipsilateral to the ICH. There was no significant effect on CT of Housing ($p=0.272$) or Hormone treatment ($p=0.369$) and the interactions were not significant ($p=0.484$). Similarly, the CC area data showed no effect of Housing ($p=0.850$) or Hormone ($p=0.092$) treatment and this interaction was not significant ($p=0.068$).

### 3. Discussion

This is the first study to assess whether delayed and chronic E2 treatment influences functional recovery after striatal ICH in rats. A sustained E2 treatment beginning 1 week following collagenase-induced ICH did not notably influence functional recovery or brain injury. Environmental enrichment did facilitate recovery on some tests, but this was mostly unaltered by E2 treatment. These findings, in conjunction with previous work (Auriat et al., 2005; Li et al., 2004b), suggest that E2 treatment improves functional recovery only when it limits injury size (e.g., attenuates bleeding and/or directly reduces cell death), and this effect occurs when E2 is on board around the time of ICH (Auriat et al., 2005; Nakamura et al., 2005).

Our E2 findings mirror those of Farr et al. (2006) who found that delayed E2 treatment did not promote spontaneous functional recovery (a rehabilitation condition was not used) nor increase synaptogenesis in a rat model of focal ischemic stroke. They used a daily dose (1.07 mg pellet, 90-day release; 11.8 μg/day) that was approximately double that we used (6 μg/day) thus arguing against the possibility of finding benefit with a larger E2 dose after ICH. Furthermore, dosages calculated to be between 2 and 24 μg per day lessen injury when given prior to ICH (Auriat et al., 2005). Thus, it would appear that E2 simply does not affect recovery within a natural dose range and with doses that provide considerable neuroprotection in rats when administered prior to ICH. Finally, although we did not measure serum E2 levels, which would have strengthened this study, it was clear that the dose used had a significant effect on body and uterine weights, which are well known to occur, and we had used the same delivery system as in other stroke studies (Auriat et al., 2005; Farr et al., 2006). These E2 pellets, however, may release a large amount of E2 initially (Auriat et al., 2005) and this may be counter-productive. Conversely, it is possible that such a high dose would provide benefit had it been sustained. Thus, alternative dosing regimens (e.g., intermittent), delivery methods, or pharmacologically high doses might influence recovery and warrant further study, especially if they can be administered for a relatively short time and provide benefit while avoiding side effects.

The EE treatment significantly facilitated recovery after ICH, which is in line with studies examining traumatic and ischemic brain injury (Biernaskie and Corbett, 2001; Johansson, 2003; Will et al., 2004). However, improvements were not found on all four behavioral tests and they were not large. For example, compared to STD-treated animals, who obtained an average success of ~74% with the contralateral forelimb in the ladder test, EE improved post-ICH performance to ~82%, but this was still substantially below baseline performance (93% success). Moreover, the only significant effect in the tray test occurred at 2 weeks post-ICH, and no effect was found in the cylinder and beam tasks. In order to explain the failure to broadly and more completely improve performance, one might argue that striatal ICH is simply more difficult to treat than motor cortex injury where EE treatment appears to be more efficacious. This appears to be the case with forced running exercise, which often facilitates recovery after ischemic injury (Wang et al., 2001), but not after ICH-induced striatal damage (Auriat et al., 2006). Similarly, amphetamine has been repeatedly, but not always, shown to improve recovery after ischemic injury whereas it provides no benefit for striatal ICH (Auriat et al., 2008). In contrast, the comparable EE treatment protocol used in that study partially facilitated recovery after ICH as we presently observed. Another possibility is that some EE studies may overestimate the effects of enrichment by comparing those animals with the ones singly housed. In this and our previous work we group housed our control rats, which should then diminish the ‘effect size’ with EE treatment.

It is also possible that the behavioral tests we used were unable to detect small treatment effects. Although, given that EE improved performance, it is hard to use such an explanation to explain the failure of E2 to improve recovery. Furthermore, we used four behavioral tests, which have all been previously shown to detect ICH-induced striatal injury to varying extents (MacLellan et al., 2006), which we presently showed. Nonetheless, while many behavioral tests are effective lesion detectors, they are sometimes unable to effectively distinguish among treatment groups including...
those with markedly different ICH-induced lesion volumes (Auriat et al., 2005; MacLellan et al., 2006). This potential weakness (test insensitivity) may be especially concerning with the beam task in which only a subset of animals showed impairment. The analysis of reaching success in the tray task was also compromised by a loss of statistical power. Here many rats switched limbs from the initially preferred forelimb to the ipsilateral-to-stroke forelimb following ICH. Thus, significant benefit may have been observed with EE at 5 and 8 weeks had many rats not switched preference, although it is also possible that no effects would have been observed with greater group sizes (i.e., transient benefit). Interestingly, the substantial change in limb preference observed in the tray task was persistent and unaffected by EE or E2 treatment, which concurs with the data from the cylinder task. Cumulatively, these data suggest that a rat’s ability to use a limb may be improved by rehabilitation (EE) while its preference to use it, if given the choice, may not be altered. Perhaps this effect stems from the failure of conventional therapies to effectively deal with learned non-use (Taub and Uswatte, 2003). Indeed, rats are free to use whichever limb they choose in EE cages; thus, we did not specifically target the impaired limb with this treatment. Therefore, EE treatment may be improved by adding task-specific training (Biernaskie and Corbett, 2001), such as skilled reaching with the impaired limb, in order to counteract any over-reliance upon the ipsilateral-to-stroke limb that may be promoted by EE treatment. Indeed, constraint-induced movement therapy, focusing on skilled reaching, effectively promotes recovery after striatal ICH in rats (DeBow et al., 2003). This is why we did not force rats to use their impaired limb (e.g., restricting use of the normal limb) in the tray task as it might have rehabilitated all groups.

Our study has several practical implications for assessing outcome after collagenase-induced striatal ICH in rats. First, while the beam task is able to detect long-term deficits, it seems relatively insensitive to treatment effects because many animals show little to no impairment. Of course, this will depend upon the insult severity and location. Second, while the cylinder task was sensitive to ICH, it appears to assess a behavior (spontaneous limb usage) that is resistant to therapy. Thus, the cylinder should not be the only behavioral test used, but we do recommend its use with other tests, especially considering the brief time required to conduct testing. Third, the tray task, which is designed to assess skilled reaching, is also persistently sensitive to ICH-induced striatal damage, but the possibility of rats switching limb preference must be considered. MacLellan et al. also reported that animals switch limb preference in the single-pellet reaching task and this depended upon the ICH lesion size (MacLellan et al., 2006). Thus, these tests can determine whether limb preference is affected by therapy. In either case, we do not recommend forcing rats to use their contralateral-to-stroke limb, such as by bandaging their ipsilateral limb, as it may rehabilitate the rat, thereby confounding or masking treatment effects. A similar problem would occur with providing extensive testing (to increase sample size). Either way, this also adds to the time required to complete testing, which is considerable with such tests. Fourth, of the tests used, the horizontal ladder test seemed most sensitive to ICH and treatment effects, and it is quick to conduct and easy to analyze. Thus, we recommend its use in future studies with the caveat that there may be no ipsilateral limb impairment, as is commonly seen (DeBow et al., 2003; MacLellan et al., 2006), and that the contralateral hind limb data are generally more variable.

The EE treatment did not lessen the total volume of brain tissue lost at 8 weeks after ICH. This contrasts with a study using constraint-induced movement therapy after ICH wherein rehabilitation lessen tissue loss when administered after a 1-week delay (DeBow et al., 2003). In addition to rapid tissue destruction at the time of ICH, the collagenase model of ICH also causes some loss of tissue over weeks, likely due to continuing atrophy such as of white matter tracts (MacLellan et al., 2007). Thus, it is possible for delayed rehabilitation treatments to reduce injury volume. The E2 treatment also did not reduce injury, but this is not surprising given that it was administered after a delay of 1 week and previous studies found benefit with pre-ICH treatment. Nonetheless, it remains possible that the EE and E2 treatments had a transient effect on cell loss or atrophy that dissipated over the lengthy survival time used in this study. Indeed, weak neuroprotectants have a history of providing only transient benefit whereas more potent therapies provide lasting benefit (Recommendations for standards regarding preclinical neuroprotective and restorative drug development, 1999). This may be the case with rehabilitation treatments as well.

In summary, E2 treatment failed to notably influence either spontaneous or rehabilitation (EE) facilitated recovery in the collagenase rat model of ICH. In contrast, EE treatment provided significant benefits, albeit not on all tests nor to a great extent. Accordingly, E2 treatment appears to improve sensory/motor recovery from striatal injury only when administered around the time of ICH. The same situation appears to be the case with ischemic injury to the motor system. While these findings indicate that E2 will not be an effective therapy when given later after a stroke, they do show that E2 will not harm recovery from motor system injury. Thus, studies that administer E2 early to limit stroke damage should not have to worry about subsequently impeding recovery by continuing the treatment over an extended time. This is an important clinical issue as stroke patients may conceivably receive chronic hormone therapy.

4. Experimental procedures

4.1. Animals

Seventy-two female Sprague–Dawley rats were entered into this study. They were obtained from the Biosciences breeding colony at the University of Alberta and were cared for in accordance with the Canadian Council on Animal Care guidelines. Procedures were also approved by the Biosciences Animal Care and Use Committee at the University of Alberta.

Rats were housed in groups of 3 to 4 in standard polycarbonate cages (38 cm width × 49 cm length × 20 cm height) unless otherwise stated, and on a 12 h light cycle. They had free access to food and water, except during

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behavioral testing when they were kept at 90% of their free-feeding weight taking into account natural gains with age. They were handled for 15 min per day for 5 days prior to the start of the experiment. Rats were randomly assigned to treatment conditions: STD-SH (n=17), STD-E2 (n=12), EE-SH (n=17) and EE-E2 (n=19). Seven other rats died during surgery prior to treatment assignment. Fig. 1 illustrates the time line of experimental procedures.

4.2. Body weight measurements

Body weight (g) was measured at the time of OVX and ICH surgeries and at 1, 5 and 8 weeks post-ICH.

4.3. Behavioral training and testing

We used a battery of tests to assess skilled reaching (tray task), spontaneous forelimb usage (cylinder task) and walking ability (elevated beam, horizontal ladder). Tests were chosen because they have been previously shown to be sensitive to motor system injury including that caused with the collagenase model of striatal ICH (MacLellan et al., 2006).

4.3.1. Tray reaching task

The tray reaching box (Whishaw, 2000) measures 26 cm high × 28 cm deep × 19 cm wide and is made of Plexiglas with 2 mm vertical steel bars interspaced 9 mm in the front. Rats learn to reach through the bars to retrieve food pellets (17% Layer Prostock Feed; Masterfeeds, Edmonton, Alberta) placed in a shallow tray (4 cm wide × 0.5 cm deep) just outside the bars. Following food deprivation to 90% of baseline weight, rats were trained over 10 consecutive days (1 h per day). On days −1, 14, 35, and 56 (relative to ICH), rats were video recorded for 10 min, which was subsequently analyzed as % successful reaches (/successful reaches/total reaches) × 100. A successful reach was one in which the rat successfully reached through the bars with its initially preferred (dominant) forelimb and retrieved and ate the food.

4.3.2. Forelimb use asymmetry (cylinder) test

Rats were placed in a transparent cylinder (20 cm diameter, 45 cm high) for 10 min while being videotaped from below. Spontaneous movements to explore the walls were categorized as an independent wall contact with either the ipsilateral (to ICH lesion) or contralateral paw or co-usage (Schallert et al., 2000; Shanina et al., 2006). At least 5 independent wall touches were needed to be considered a reliable measure of forelimb use and rats that did not reach this cutoff were excluded from this analysis. From videotape analysis we calculated an asymmetry score defined as the (number of contacts with contralateral forelimb + ½ both)/ (ipsilateral forelimb use + contralateral forelimb use + both) × 100 (MacLellan et al., 2006; Shanina et al., 2006). With this measure normal animals score near 50% whereas those with motor system damage, such as a striatal ICH (Auriat et al., 2005; MacLellan et al., 2006; Shanina et al., 2006), have smaller scores indicating diminished usage of independent contralateral paw movements relative to the ipsilateral paw and co-usage. Rats were assessed the day prior to ICH and on days 14, 35 and 56 following ICH.

4.3.3. Beam task

On the last day of tray reaching task training, rats were trained to cross an elevated horizontal beam (1.10 m long; 3.20 cm wide). This was achieved by initially placing them on the beam at increasing distances from the goal box, located at the end of the beam, until they easily crossed the entire beam. Baseline performance was measured on the day prior to ICH, whereas testing occurred on days 14, 35 and 56 post-ICH. We used a modified version of Feeney et al.’s (1982) rating scale to score videotaped beam task sessions (Auriat et al., 2008; MacLellan et al., 2006). Briefly, a rating scale (0 to 7) was used to assess each cross (5 per test day) on the beam walking test with 0 being the worst case and 7 being the best performance (Auriat et al., 2008; MacLellan et al., 2006). The median score of the 5 sessions was analyzed as was the summed score of the first two sessions.

4.3.4. Horizontal ladder walking task

On the last day of the tray reaching task training, rats were trained to cross a 1 m long horizontal ladder with variably spaced rungs (3–5 cm; 4 crosses). On behavioral testing days (14, 35 and 56 days post-ICH), rats were videotaped crossing the middle 0.5 m segment of the horizontal ladder (Metz and Whishaw, 2002). The total number of steps and slips made with each limb was recorded for 4 crosses per behavioral test day. A slip was counted when the limb slips completely through the bars. The success rate for each limb was calculated as follows: (number of successful steps/total number of steps) × 100. Performance on this task is affected by striatal ICH, at least for the contralateral forelimb, which was our primary endpoint (DeBow et al., 2003; MacLellan et al., 2006).

4.4. Major surgical procedures

Aseptic surgical technique was used (e.g., autoclaved or hot-bead sterilized instruments, autoclaved surgical drapes). The OVX surgery was done 2 weeks prior to ICH and 3 weeks prior to E2 treatment. All rats were subjected to OVX and ICH surgeries.

4.4.1. OVX surgery

Rats were anesthetized with isoflurane (4% induction, 2% maintenance in 70% N2O and 30% O2). An approximately 1 cm long incision was made in the skin and muscle on each side demarcated by the caudal end of the ribs. Both ovaries were identified, tied off with suture, and excised. The incision was infiltrated with Marcaine (Sanofi Canada, Markham, ON, Canada) and sutured closed.

4.4.2. ICH surgery

Rats were anesthetized under isoflurane as for the OVX surgery. They were then placed in a stereotaxic frame while body temperature was measured with a rectal thermocouple probe and maintained at normothermia (36.5–37.5 °C) with a heating pad. A midline scalp incision was made and the skull was leveled between Bregma and Lambda. A small burr hole was made 3 mm lateral to Bregma, on the side contralateral to the preferred limb (as determined by the tray reaching task). A 26-gauge needle (Hamilton syringe part # 80308, Hamilton, Reno, NV, USA) was lowered 6.0 mm below the surface of the
skull and 0.7 μL of sterile saline containing 0.14 U bacterial collagenase (Type IV — S; Sigma, Oakville, ON, Canada) was infused into the striatum over 5 min to create an ICH (DeBow et al., 2003; MacLellan et al., 2006; Rosenberg et al., 1993). The needle remained in place for another 10 min to prevent reflux. The burr hole was sealed with a metal screw (model MX-080-2; Small Parts, Miami Lakes, FL, USA) then the incision was infiltrated with Marcaine and stapled closed.

4.5. Treatment conditions

Groups of 3–4 rats were randomly assigned to Housing and Hormone treatment conditions at 1 week after ICH. Groups of animals were assigned to either the same standard housing (STD) or environmental enrichment (EE) as changing cage mates would have caused undue stress. Rats were randomly assigned to receive either estrogen pellet implantation (E2) or sham procedure (SH).

4.5.1. Estrogen treatment

One week following ICH, rats were quickly anesthetized with isoflurane anesthesia for a small incision on the back of the neck. A 17β-estradiol (E2) pellet (0.36 mg; 60-day release; Innovative Research of America Inc., Sarasota, FL, USA), which continuously releases estrogen, was then implanted subcutaneously (E2 treatment) or no pellet was given (SH treatment). The wound was sutured closed.

4.5.2. Housing treatment

Immediately following E2 or SH treatment the rats were either returned to STD housing or placed in an EE cage that measured 77 cm long × 77 cm high × 37 cm wide. These cages, which had 3 levels connected by ramps, allowed rats to access a 30 cm diameter running wheel adjacent to the EE cage. Each week new "toys" (e.g., beams, plastic children’s toys, etc.) were introduced to replace existing ones. As well, the location of the food and water inside the cages was also changed weekly to encourage exploration. Animals remained in their assigned housing until the end of the experiment.

4.6. Histopathology

Rats were euthanized the day following the last test session (~2 months post-ICH) by an overdose with sodium pentobarbital (80 mg/kg, i.p.; Somnotol, MTC Pharmaceuticals, Cambridge, ON, Canada). Following clamping of the descending aorta, the rat’s upper body was transcardially perfused with 0.9% saline followed by 10% neutral buffered formalin. Uteri, aorta, the rat’s upper body was transcardially perfused with

Volume of tissue in a hemisphere = average (area of the complete coronal section of the hemisphere – area of ventricle – area of tissue damage) × interval between sections × number of sections.

The cortical thickness for each hemisphere was measured at maximal lesion site (Fig. 7A) using Scion Image as previously done (MacLellan et al., 2007). Similarly, corpus callosum injury was quantified by measuring its area in each hemisphere at the maximal lesion site (MacLellan et al., 2007).

4.7. Statistics

All behavioral and histological analyses were performed in a blinded fashion. Data were analyzed with SPSS (v. 15, SPSS Inc, Chicago, IL) using ANOVA for parametric data (e.g., cylinder scores) and non-parametric statistics for beam test scores. A p value of <0.05 was considered statistically significant.

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