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Research report

Successive bilateral frontal controlled cortical impact injuries show behavioral savings

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HIGHLIGHTS

- The serial lesion effect can be demonstrated in a model of traumatic brain injury.
- Two sequential brain injuries reduce deficits; brain injury is not necessarily additive.
- The serial lesion effect cannot be tied conclusively back to the glial response.

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ABSTRACT

Traumatic brain injuries (TBIs) affect millions of people each year. Research investigating repeated or serial damage in the form of lesions indicates that behavioral deficits are reduced in animals given sequential lesions separated by a sufficient period of recovery. In the lesion literature, this phenomenon is known as the serial lesion effect (SLE). Although the SLE phenomenon is established in the lesion literature, it has not been thoroughly investigated under current models of brain injury. In the current study, a controlled cortical impact of the bilateral frontal cortex was performed in either a single procedure or a serial procedure separated by two weeks. Rats were tested on the Morris water maze, bilateral tactile adhesive removal task, rotarod and Barnes maze task to determine behavioral deficits. Histology was performed to determine lesion size and astrocyte and microglial response. A serial lesion effect was demonstrated across a majority of the behavioral tasks. However, histological analyses did not suggest a clear mechanistic link to the behavioral phenomena. This is the first study to demonstrate the SLE in a model of TBI, suggesting that behavioral deficits may actually be reduced in repeated head injuries, given an adequate time window between injuries.

1. Introduction

Traumatic brain injury (TBI) is a major problem that affects over 1.7 million people in the United States each year, and many more worldwide. These injuries range from mild to very severe. Of the 1.7 million people that visit an emergency room due to TBI, 1.37 million are treated and released, 275,000 are hospitalized and 52,000 die [1]. However, these numbers only account for hospital visits and may inadequately represent the total number of TBIs because a large number of TBIs go unreported [2].

As a result of primary and secondary damage from TBI, many behavioral deficits occur. In humans, sensory and motor impairments have been reported as well as deficits in cognitive abilities such as decision-making, attention and memory [3]. Long-term sequelae can include specific movement disorders, seizures/epilepsy, migraines and increased risk for various dementias [4]. Despite the numerous deficits that occur as a result of brain injuries, there exists a rich literature detailing how both animals and people recover lost function after brain injury [5–11]. This occurs through a variety of mechanisms, both physiological and behavioral. These mechanisms include physiologic processes such as functional reorganization, dendritic branching, collateral sprouting and novel synaptogenesis [8–11] as well as behavioral compensation such as shifts in strategies, novel learning and relearning of behaviors [5,6]. Recent work has focused on how these different processes can be applied to therapeutic rehabilitation and improve recovery of function following injury [7].

However, one question that is still being debated in the field is how the brain responds to multiple instances of trauma. Some studies have found evidence for mild or reduced deficits in repeated

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injuries [12] while others have suggested that multiple brain injuries cause more severe deficits and accelerate other injury-related factors such as β-amyloid and tau accumulation [13,14]. It is also possible that some observed mild deficits after repeated injury might only be mild due to behavioral compensation that occurs after trauma.

In some of the early work studying brain damage and recovery of function, one phenomenon emerged after repeated lesions which became known as the serial lesion effect (SLE). The SLE was first explicitly reported by Adametz [15], when he showed that reticular lesions that resulted in permanent comas were recoverable if produced in a staged operation. The SLE is seen when successive lesions have fewer detrimental effects than lesions of similar size given at a single time-point. The specific properties of this effect were cataloged throughout the 1970s and up into the early 1990s, however it has not been widely studied since then. During that period, several key points were noted about the SLE. Studies found that there is a minimum recovery time between lesions of 7 and 14 days to see behavioral savings [16,17]. Also, the type, extent and location of damage influence whether or not the SLE occurs [18–20]. Other studies noted that the SLE occurs most often when injuries are made in cortical tissue [17,21,22]. Additionally, the type of task appears to influence the degree of SLE observed. Practice-dependent measures such as motor or learning tasks show stronger SLEs [23,24] while basic regulatory functions such as temperature regulation or weight maintenance do not [22,25].

The majority of the work studying the SLE stopped prior to the 1990s, when the major current models for experimental TBI were first developed [26,27]. To date, the SLE has not been examined in a model of TBI that inflicts brain damage in a clinically relevant fashion. Previous studies used electrolytic lesions, ablations or aspirations to produce the damage, which do not mimic the way brain damage occurs outside the laboratory [26]. Though the SLE has been demonstrated many times, the mechanism by which it occurs is still not clear. One possibility that has not been investigated is whether the SLE is related to the glial response after injury. Following brain injury, astrocytes become much more active as they attempt to buffer chemical imbalances in interstitial fluid [28] and microglia begin to proliferate and move to the site of injury in order to initiate immune responses [29]. Early initiation of these responses could potentially prime the brain for subsequent brain injuries in the SLE.

Investigating the consequences of repeated brain injuries could improve the understanding of successive trauma which could lead to the development of therapeutic options for those suffering from TBI. With the high incidence of repeated TBIs, such as those seen in sports, the question of whether or not a SLE can be produced in experimental brain injury is of particular interest. The following experiment was performed to compare a single brain injury to dual (repeated) brain injuries. Based on previous research, the serially injured group was expected to perform better than the bilaterally injured groups following a controlled cortical impact (CCI) frontal brain injury. It was also anticipated that a closer examination of glial activity after injury would yield a mechanistic link to the behavioral phenomenon.

2. Methods

2.1. Subjects

Thirty-two male Long–Evans rats, approximately three months of age (280–300 g) were used in this study. Procedures described in this paper were approved in advance by the Institutional Animal Care and Use Committee. Rats were individually housed in standard cages on a 12 h reverse light cycle and had ad libitum access to food and water. Testing was conducted during the dark cycle. Rats were handled for 10 days prior to surgery to familiarize them with human contact. The same animals were used to conduct both the behavioral and histological portion of this study. Several exclusions were performed in the immunohistochemistry analyses based on the quality of the staining.

2.2. Surgery

Surgeries were performed under aseptic conditions according to previous studies [30,31]. Rats were anesthetized with a mixture of ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Rats' heads were shaved and treated with alcohol and betadine. Once pedal withdrawal and tail pinch reflexes were absent, rats were placed in a stereotaxic device. Anesthesia was monitored throughout surgery and additional ketamine given as needed (10 mg/kg). Body temperature was monitored and maintained using a heated pad. After making a midline incision, a 6 mm craniotomy was produced centered on the midline at +3.0 mm from bregma, using a microdrill with trephine while avoiding damage to the dura and meninges. Once the medial prefrontal cortex was exposed, either a CCI injury or sham injury was induced. For the CCI injury, rats were either given a bilateral injury with a stainless steel circular 5.0 mm diameter impact tip or a unilateral injury with a stainless steel semicircular 2.5 mm × 5 mm diameter impact tip. The impactor was pneumatically driven (20 psi) and delivered an impact to a depth of 2.5 mm at a rate of 2.25 m/s for 0.5 s. Following injury procedures, all rats had their incision stapled shut, antibiotic ointment applied and were placed in a heated recovery chamber. For sham rats, a midline incision was made and a craniotomy produced as in the CCI rats. The incision was then stapled shut, antibiotic ointment was applied, and rats were placed in a heated recovery chamber.

Two weeks after the first surgery, all rats received a second surgery. Rats either received a bilateral CCI, unilateral CCI (opposite side of previous, counterbalanced) or a sham procedure. Based on the surgeries, rats were assigned to one of four conditions, bilateral CCI then sham surgery (Bi-Sham, $n = 8$), sham surgery then bilateral CCI (Sham-Bi, $n = 8$), unilateral CCI then unilateral CCI (opposite sides, counterbalanced; Serial, $n = 8$) or sham surgery then sham surgery (Sham, $n = 8$). Following injury procedures, all rats had their incision stapled shut, antibiotic ointment applied and were placed in a heated recovery chamber.

2.3. Morris water maze

Morris water maze (MWM) testing occurred on days 2–11 after the second surgery as previously described [31]. A circular fiberglass tank (1.8 m diameter, 0.75 m height) was filled with water and rendered opaque using non-toxic white tempera paint. Bromide tablets were regularly placed in order to ensure antiseptic conditions. A clear platform was centered in the Northwest corner of the tank at 2 cm under the water level. Rats were placed in the tank at one of four random start locations (North, South, East or West) facing the wall. They were then given 90 s to find the platform. If they were unable to find it, they were guided by hand. Once on the platform, rats were given 30 s before being placed in a heated holding cage. Data, including path length, pattern and latency were recorded via SMART computer software (San Diego Instruments, USA). Rats received 2 trials per day for 10 days with a 15 min intertrial interval (ITI). Start locations were pseudorandomly selected so that rats received one long (South or East) and one short (North or West) trial each day.

2.4. Rotarod task

Rats were tested on the rotarod (Rotor-Rod, San Diego Instruments) task on days 12–16 after the second surgery as previously described [32]. The task consisted of an elevated rotating cylinder that was gradually accelerated to assess gross locomotor performance. The accelerating cylinder had a diameter of 7 cm and was situated approximately 1 m above a foam pad. It was gradually accelerated from 0 to 30 RPMs over the course of 1 min and continued at 30 RPMs for an additional 2 min. Rats were placed on the cylinder against the rotation as it began and the latency till they fell was recorded by breaking infrared beams located below the cylinder. Rats were given four trials per day with an approximately 10 min ITI.

2.5. Bilateral tactile adhesive removal task

The bilateral tactile adhesive removal task was administered on days 17–21 after the second surgery as previously described [33]. Rats had small rectangular adhesive patches (Avery #05412) wrapped around each wrist to form a "bracelet" above the paw. They were then placed back in their home cage and the total latency to remove both patches was recorded. Rats were given two trials per day with an approximately 10 min ITI.

2.6. Barnes maze task

The Barnes maze task (San Diego Instruments) was administered on days 19–24 after the second surgery following procedures adapted from a previous study [34]. The maze consisted of a circular bright open white field (1.25 m diameter, 1.2 m height) with 20 potential escape holes evenly spaced around the edge. Only one box beneath these holes was large enough for the rat to fit into. This maze uses rats' natural aversion to open bright spaces to motivate them to find the escape box. Additionally, the escape box was baited with chocolate milk (2 ml) for additional

reinforcement. Rats were given one adaptation trial in which they were placed in the escape box with the reinforcer. Trials began in a circular cylinder in the center of the maze, which was then lifted to allow the rat to explore. Rats were given four min to locate the escape box. If they had not located it at the end of that time, they were guided by hand to the box. Once in the box, rats were given one min to consume the reinforcer and were then removed from the box and placed in a holding cage. On the fifth day (trial 8), the location of the escape box was reversed to 135° from its original position to assess reversal learning. Data, including path length, pattern and latency were recorded via SMART computer software. Rats were given two trials per day with an approximately 20 min ITI.

2.7. Histology

On the 25th day after the second surgery, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg). Once eye-blink and pedal responses disappeared, rats were transcardially perfused with ice-cold 0.9% phosphate buffered saline (PBS), followed by 10% phosphate buffered formalin (PBF). Brains were removed and placed in PBF for one day. Brains were then processed and embedded in paraffin wax (Tissue Tek III processor, IMEB Inc., San Marcos, CA). After being embedded, brains were sliced at 10 μm on a rotary microtome and mounted onto electrostatically charged slides (Starfrost). Three sections transversing the lesion (+1.0, +2.0 and +3.0 from bregma) were selected to examine lesion size, and one section (+1.0 from bregma) was selected to identify neuronal loss and glial expression. Brains not meeting stringent histological quality standards were not included in the analyses.

2.7.1. Hematoxylin and eosin staining

Hematoxylin and eosin was selected to stain cell bodies so that lesion cavities could be visualized and analyzed. Slides were put through sequential washes to remove paraffin, rehydrate, stain, dehydrate and coverslip. To remove paraffin, slides were placed in xylene (3× 5 min), then followed by 100% EtOH (2× 5 min), then 95% EtOH (2× 5 min), 70% EtOH (1× 5 min), then distilled water (1× 5 min) to rehydrate. The staining consisted of hematoxylin (1× 2 min), a rinse in distilled water (5 dips), a brief immersion in bluing solution (1× 5 s), a distilled water rinse (1× 5 min), then 70% EtOH (1× 5 min), and finally eosin (1× 1 min). Slides were then dehydrated in 70% EtOH (1× 5 min), then 95% EtOH (2× 5 min), followed by 100% EtOH (2× 5 min) and clarified in xylene (3× 3 min). Afterwards, slides were coverslipped and prepared for light microscopy.

2.7.2. IBA-1 immunohistochemistry

Slides were put through sequential washes to stain for infiltrating microglia and macrophages. All procedures were done at room temperature unless otherwise specified. Paraffin was removed in xylene (3× 5 min), then rehydrated in 100% EtOH (2× 5 min), 70% EtOH (1× 5 min), then distilled water (1× 5 min). Blocking was performed in peroxidase solution (1× 5 min). Slides were then put through a heat-mediated antigen retrieval step by placing them in a buffer (DV2004, 1:10 dilution, Biocare Medical, Concord, CA) in a decloaker (Biocare) for 30 min at 80 °C. Once completed, slides were placed on a humidified shaker (IQ Kinetic Slide Stainer, Biocare). For further antigen retrieval, a pepsin solution (Carezyme II, Biocare) was applied directly to the slides for 1 min. Slides were then rinsed with 0.9% Tris buffered saline (TBS). Serum blocking was then performed by adding a blocking solution (Rodent Blocker R, Biocare) for 30 min. Following a TBS rinse, rabbit anti-rat IBA-1 primary antibody (CP290, Biocare) was applied for 30 min. Slides were then rinsed with TBS and incubated in rabbit polymer (RMR622, Biocare) for 30 min. After rinsing with TBS, slides were reacted with a 3,3′-diaminobenzidine solution (DAB) for 5 min. Slides were then rinsed in deionized water and counterstained by placing them in hematoxylin for 5 s, then rinsed in deionized water, dipped in bluing solution and placed through sequential washes of 70% EtOH (1× 5 min), 95% EtOH (2× 5 min), 100% EtOH (2× 5 min) and xylene (3× 3 min) to dehydrate and clarify before coverslipping.

2.7.3. GFAP immunohistochemistry

Slides were put through sequential washes to stain for activated astrocytes. All procedures were done at room temperature unless otherwise specified. Paraffin was removed in xylene (3× 5 min), then rehydrated in 100% EtOH (2× 5 min), 70% EtOH (1× 5 min), then distilled water (1× 5 min). Blocking was performed in peroxidase solution (1× 5 min). Slides were then put through a heat-mediated antigen retrieval step as described above. Once completed, slides were placed on a humidified shaker. For further antigen retrieval, a pepsin solution was applied directly to the slides for 5 min at 37 °C. Slides were then rinsed with TBS. Serum blocking was then performed by adding a blocking solution for 30 min. Following a TBS rinse, rabbit anti-rat GFAP primary antibody (CP040, Biocare) was applied for 30 min. Slides were then rinsed with TBS and incubated in rabbit polymer for 30 min. After rinsing with TBS, slides were reacted with DAB for 5 min. Slides were then rinsed in deionized water and counterstained by placing them in hematoxylin for 5 s, then rinsed in deionized water, dipped in bluing solution and placed through sequential washes of 70% EtOH (1× 5 min), 95% EtOH (2× 5 min), 100% EtOH (2× 5 min) and xylene (3× 3 min) to dehydrate and clarify before coverslipping.

2.8. Lesion analysis and cell counts

2.8.1. Lesion analysis

Three sections from each brain through the area of the lesion cavity (+1.0, +2.0, and +3.0 from bregma) were imaged with a digital camera (Olympus DP72) attached to a microscope (Olympus BX-61). Using specially designed software (Visiopharm, Visiomorph, Denmark), the area of the remaining cortex was measured at each depth for both the right and left hemispheres.

2.8.2. Neuronal counts

Neuronal counts were performed in the cortex. Three sites were selected in either hemisphere (six total) of the cortex (+1.8 from bregma) and imaged at 40× (see Fig. 5). Counts for each site were performed using Image-Pro software and then totaled for each region.

2.8.3. IBA-1 cell counts

Analysis of IBA-1$^+$ cells was carried out in the same sites in the cortex. Three sites were selected in either hemisphere (six total) of the cortex (+1.8 from bregma) and imaged at 40× (see Fig. 5). Counts for each site were performed using Image-Pro software and then totaled for each region.

2.8.4. GFAP cell counts

Analysis of GFAP$^+$ cells was carried out in the same sites in the cortex. Three sites were selected in either hemisphere (six total) of the cortex (+1.8 from bregma)) and imaged at 40× (see Fig. 5). Counts for each site were performed using Image-Pro software and then totaled for each region.

2.9. Data analysis

All data were analyzed by an experimenter blinded to group assignments. SPSS (IBM) was used to analyze the data. The mean and standard error of the mean (SEM) were calculated for all data. General Linear Model (GLM) ANOVA with repeated measures was used to examine overall effects of lesion type on behavioral tasks. The Huynh–Feldt correction was used to correct for violations of homogeneity on repeated measures. Fischer's Least Significant Difference (LSD) was used for pairwise post hoc comparisons. Univariate ANOVAs were used to examine differences between groups on histological measures. The LSD post hoc was used on univariate ANOVAs as well. The statistical level of significance was a p-value of less than 0.05.

3. Results

3.1. Morris water maze

The performance on the MWM was evaluated in a 4×10 repeated measures ANOVA (Group [Sham, Bi-Sham, Sham-Bi, Serial] × Day [2–11]). There was no significant group by day interaction, $F(27, 252) = 0.96$, $p = 0.533$. However, there was a main effect of day, showing that rats reduced latencies across the testing period, $F(9, 252) = 36.13$, $p < 0.001$. There was a significant difference between the groups, $F(3, 28) = 7.37$, $p = 0.001$. Specifically, the Serial group performed significantly better than the Bi-Sham group, $LSD(14) = 16.60$, $p = 0.007$, and the Sham-Bi group, $LSD(14) = 12.99$, $p = 0.031$, and was not significantly different than the Sham group, $LSD(14) = 6.94$, $p = 0.237$ (see Fig. 1).

3.2. Rotarod task

The latencies to fall on the rotarod were evaluated in a 4×5 repeated measures ANOVA (Group [Sham, Bi-Sham, Sham-Bi, Serial] × Day [12–16]). There was a significant day by group interaction, $F(12, 112) = 3.01$, $p = 0.001$. There was a main effect of day, demonstrating increased latencies to fall across testing days, $F(4, 112) = 24.93$, $p < 0.001$. There was a significant difference between the groups, $F(3, 28) = 2.95$, $p = 0.05$. Specifically, the Serial group performed significantly better than the Bi-Sham group, $LSD(14) = 13.91$, $p = 0.021$, but was not different than the Sham-Bi group, $LSD(14) = 4.76$, $p = 0.410$, or Sham group, $LSD(14) = 1.37$, $p = 0.811$ (see Fig. 2).

3.3. Bilateral adhesive removal task

The latency to removal on the bilateral tactile adhesive removal task was analyzed in a 4×5 repeated measures ANOVA (Group

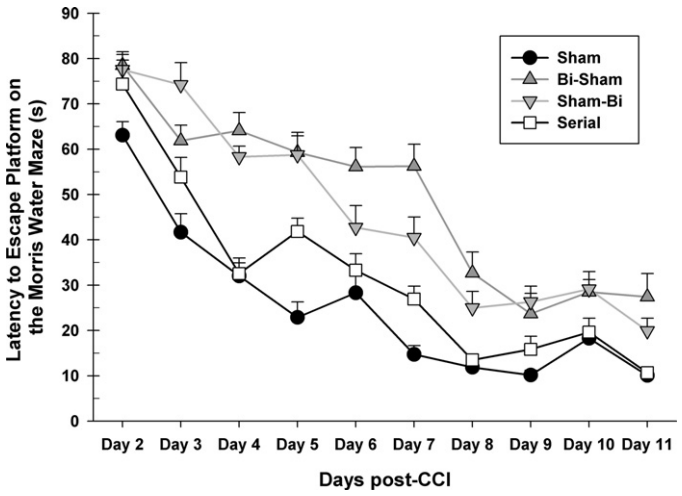

Fig. 1. Latencies to locate the platform in the Morris water maze. Overall, the Serial group took significantly less time to reach the platform compared to the Bi-Sham group ($p = 0.007$) and Sham-Bi group ($p = 0.031$). There was no overall difference between the Serial and Sham groups ($p = 0.237$).

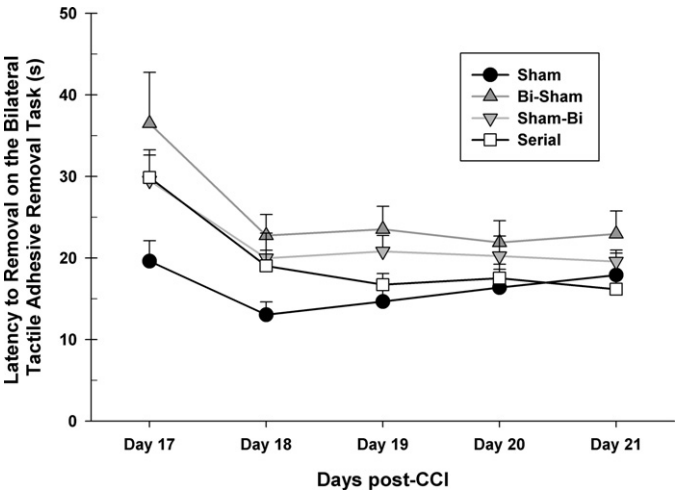

Fig. 3. Patch removal latency on the bilateral tactile adhesive removal task. Overall, the Serial group was significantly faster than the Bi-Sham group ($p = 0.020$). There was no overall difference between the Serial group and the Sham-Bi group ($p = 0.359$) or the Sham group ($p = 0.135$).

[Sham, Bi-Sham, Sham-Bi, Serial] × Day [17–21]). There was no interaction between day and group, $F(12, 112) = 0.88$, $p = 0.569$. However, there was a main effect of day, showing that rats improved over the course of testing, $F(4, 112) = 13.06$, $p < 0.001$. There was also a significant difference between groups, $F(3, 28) = 5.62$, $p = 0.004$. Specifically, the Serial group performed better than the Bi-Sham group, $LSD(14) = 5.67$, $p = 0.020$, but was not significantly different than the Sham-Bi group, $LSD(14) = 2.15$, $p = 0.359$, or the Sham group, $LSD(14) = 3.55$, $p = 0.135$ (see Fig. 3).

3.4. Barnes maze task

Performance in the first phase of the Barnes maze task (reference learning) was analyzed in a 4 × 7 repeated measures ANOVA (Group [Sham, Bi-Sham, Sham-Bi, Serial] × Trial [1–7]). There was an interaction between group and trial, $F(18, 168) = 1.86$, $p = 0.023$. There was a main effect of trial, $F(6, 168) = 43.84$, $p < 0.001$. There was no significant difference between the groups, $F(3, 28) = 2.85$, $p = 0.055$ (see Fig. 4).

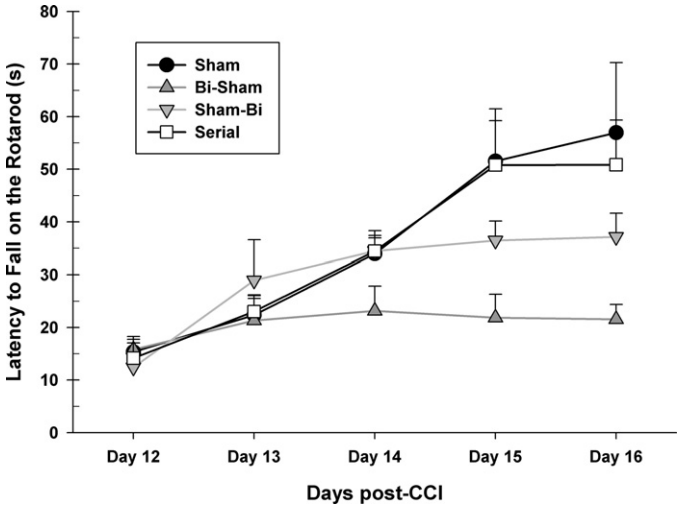

Fig. 2. Latencies to fall from on the rotarod task. Overall, the Serial group stayed on the cylinder significantly longer than the Bi-Sham group ($p = 0.021$). There was no overall difference between the Serial group and the Sham-Bi group ($p = 0.410$) or the Sham group ($p = 0.811$).

Performance in the second phase of the Barnes maze task (reversal learning) was analyzed in a 4 × 4 repeated measures ANOVA (Group [Sham, Bi-Sham, Sham-Bi, Serial] × Trial [8–11]). There was no group by trial interaction, $F(9, 84) = 0.83$, $p = 0.591$. However, there was a main effect of trial, showing that rats improved across trials $F(3, 84) = 24.72$, $p < 0.001$. There was a significant difference between the groups, $F(3, 28) = 5.29$, $p = 0.005$. Specifically, the Serial group performed significantly better than the Sham-Bi group, $LSD(14) = 78.64$, $p = 0.003$, but was not significantly different than the Bi-Sham group, $LSD(14) = 0.46$, $p = 0.985$, or the Sham group, $LSD(14) = 4.42$, $p = 0.854$ (see Fig. 4).

3.5. Lesion analysis and cell counts

3.5.1. Lesion analysis

There were no significant differences between the right and left hemispheres in terms of size of remaining cortex (by group) so the two values were summed for each subject. Each of the three levels selected was analyzed using a one-way ANOVA (Group [Sham, Bi-Sham, Sham-Bi, Serial] × Area). There were significant differences between the groups at all three levels: bregma +1, $F(3, 16) = 6.708$, $p = 0.004$, bregma +2, $F(3, 16) = 9.797$, $p = 0.001$, and bregma +3, $F(3, 16) = 7.711$, $p = 0.002$. For all three levels, a LSD post hoc analysis determined that the only significant differences that existed across groups were observed when comparing the sham prepared animals to any of the three lesion preparations (all $ps < .05$). There were no differences, at any level, when comparing any of the lesion groups to one another.

3.5.2. Neuronal counts

Neuronal cell counts were analyzed in a one-way ANOVA (Group [Sham, Bi-Sham, Sham-Bi, Serial] × Neurons). There was no significant difference between the groups, $F(3, 16) = 2.79$, $p = 0.074$.

3.5.3. IBA-1 cell counts

IBA-1^+ cell counts were analyzed in a one-way ANOVA (Group [Sham, Bi-Sham, Sham-Bi, Serial] × IBA-1^+ Cells). There was no significant difference between the groups, $F(3, 15) = 0.64$, $p = 0.600$ (see Fig. 5).

3.5.4. GFAP cell counts

GFAP$^+$ cell counts were analyzed in a one-way ANOVA (Group [Sham, Bi-Sham, Sham-Bi, Serial] × GFAP$^+$ Cells). There was no

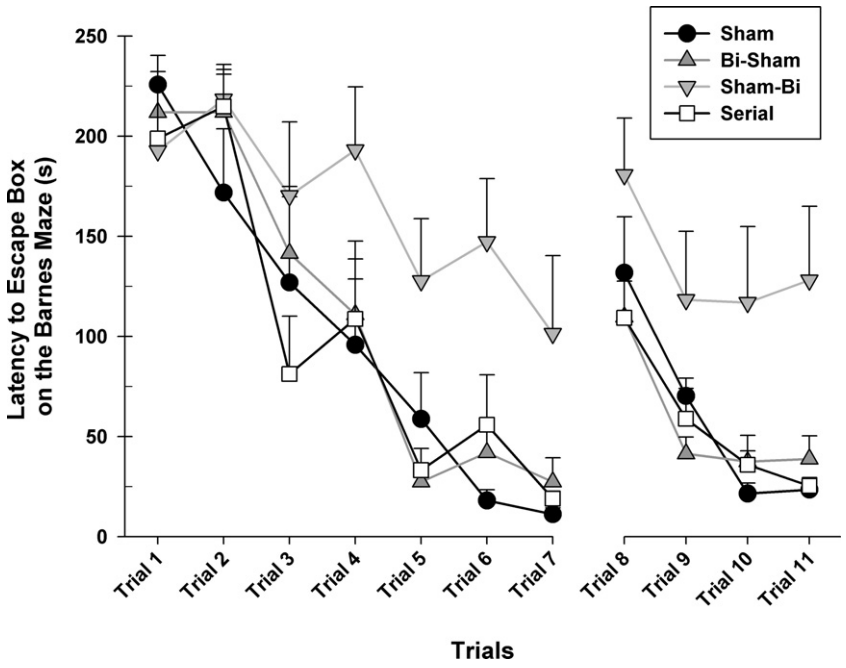

Fig. 4. Latency to locate the escape box in the Barnes maze task for both the reference learning phase and reversal learning phase. Overall, there was no difference between the groups on the reference learning phase ($p = 0.055$). On the reversal learning phase, the Serial group was significantly faster overall than the Sham-Bi ($p = 0.003$). There was no difference between the Serial group and the Bi-Sham group ($p = 0.985$) or Sham group ($p = 0.854$).

significant difference between the groups, $F(3, 15) = 1.934$, $p = 0.165$ (see Fig. 5).

4. Discussion

Prior to this work, serially placed lesions have been shown to demonstrate decreased deficits across a variety of electrolytic lesions, ablations and aspirations compared to single lesions [16,22,23]. This work attempted to determine if the SLE could be reproduced following a TBI induced by CCI. On a majority of behavioral tests, the serially injured group had reduced deficits compared to at least one of the bilaterally injured groups. The exception to this is that on the Barnes maze task no overall effect of group was detected in the reference learning phase, although the level approached significance ($p = 0.055$). However, in the reversal learning phase, the Sham-Bi group had increased deficits compared to the serially injured group. Additionally, the serially injured group was never significantly different from the sham group on any of the behavioral tasks administered. Taken together, this suggests that the SLE can be replicated in the CCI model of TBI, and that the effect shown in this work was robust, spanning almost all aspects of behavior that were tested.

The serially injured group had reduced deficits overall compared to a bilaterally injured group on almost all of the tasks. However, there are some interesting specific details in the behaviors beyond a simple test of differences that should be discussed. In order to control for the two week recovery time between surgeries, the bilaterally injured groups received injuries at different times: one received an injury followed by two weeks of recovery then sham surgery, the other received a sham surgery with two weeks of recovery followed by an injury. However, it appears that the differences between these groups are relatively minor. On two of the tasks (rotarod task, bilateral tactile adhesive removal task), the Bi-Sham group performed significantly worse than the Serial group, while on the reversal learning portion of the Barnes maze task, the Sham-Bi group performed worse than the Serial group. Despite these differential effects, on the MWM both bilaterally lesioned groups were impaired compared to the Serial group. There was also no difference between the two on any histological measure, including lesion size or glial activation. This occurred despite a two-week separation in injury induction, suggesting that the time at which the injury is induced may be less relevant than expected. Additionally, the Barnes maze task data are particularly interesting. The impairment of the Sham-Bi group is markedly higher than that of any other group, despite the similarities between the two bilaterally injured groups on the MWM and the similarities in learning between the MWM and Barnes maze task (see Fig. 4). Though this should not be over interpreted, it may suggest that an injury immediately prior to MWM testing may reduce learning capabilities on a later, similar task.

Though the SLE was demonstrated behaviorally, histological measures failed to illuminate any potential mechanisms by which it is occurring. Previous theories as to why this phenomenon occurs have been inconclusive. Some have suggested a primarily behavioral explanation in which compensations that occur after the first injury continue or prepare for the next injury [23], while others have preferred an anatomically based theory in which the initial injury starts biological processes (e.g. inflammatory response) in motion that reduce the impact of subsequent injuries [18]. While both explanations are plausible, there is likely some combination of both occurring. Additional exploration of the mechanisms at work in the stroke phenomenon of ischemic preconditioning could possibly inform the search for a SLE mechanism. While the stroke mechanisms for preconditioning are still not fully understood, the behavioral and neuroprotective effects resemble those seen in the SLE [35]. The data shown in the current study suggest that the mechanism is not directly related to the glial response that is seen after brain injury. However, the histological data from the current study is limited by the use of a single time point as well as very small group sizes. Future work should expand upon the histology performed here and be directed toward evaluating the time-course of neuroanatomical changes that occur across the recovery period. These could include measures such as acute cell

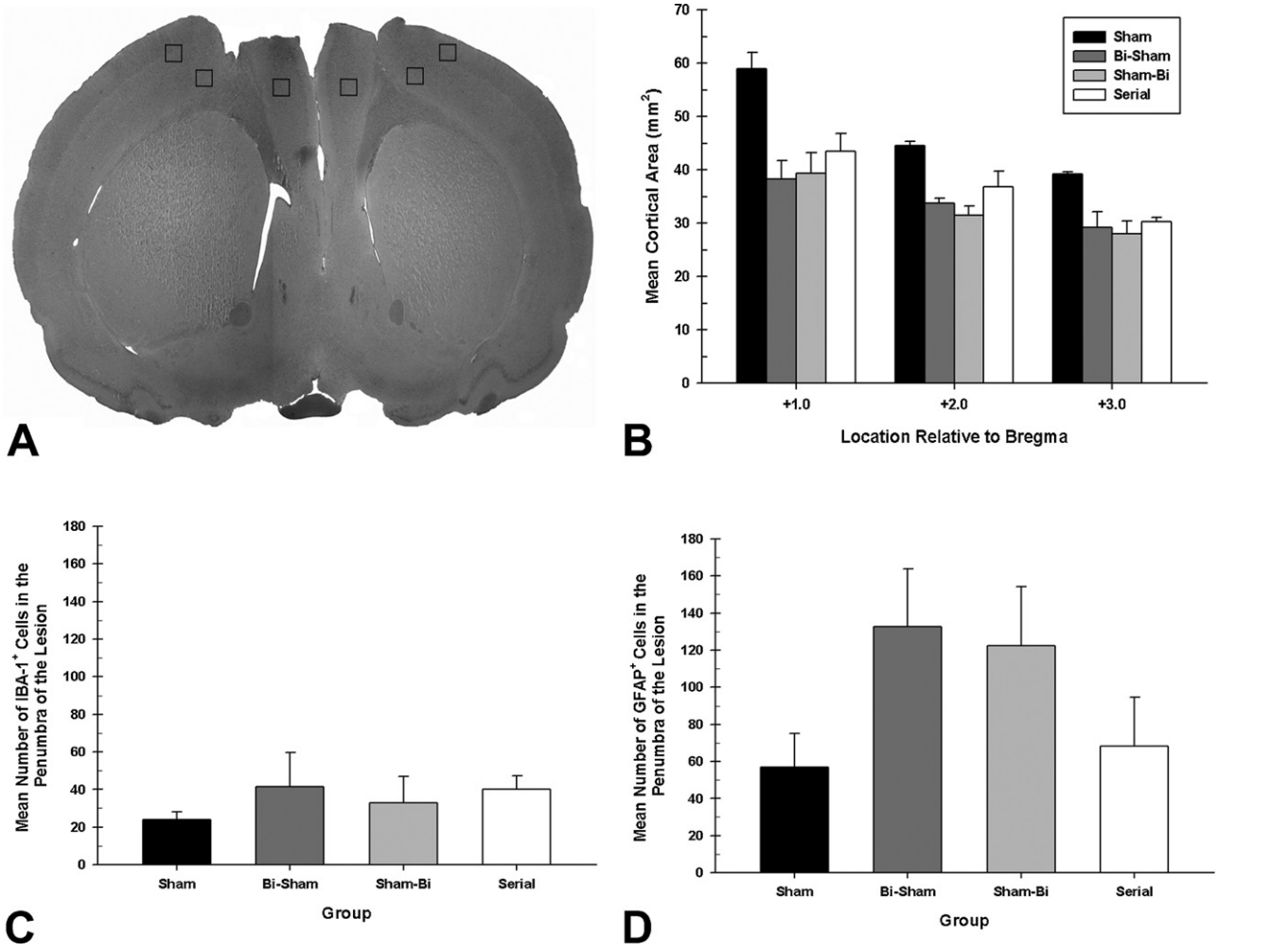

Fig. 5. Histological findings. Panel A shows an image of a serially lesioned brain. Boxes indicate locations for images taken at 40× in the cortex that were used to count neurons, IBA-1⁺ cells and GFAP⁺ cells. Panel B shows a graph comparing the remaining cortical tissue as an estimate of lesion size. Despite an effect of injury on lesion size, there was no difference between the Serial group and either bilaterally injured group. Panel C shows a comparison of IBA-1⁺ immunoreactivity in the cortex across the different groups. There was no significant difference between the groups. Panel D shows a comparison of GFAP⁺ immunoreactivity in the cortex across the different groups. There was no significant difference between the groups.

death, distribution of gliosis as well as the glial response and span the intra-lesion interval and post-lesion time points.

The SLE shown in the current work was likely the culmination of several factors. The design of the study was intended to maximize the likelihood of observing a SLE. This follows from several years of work in the area that highlight the conditions under which an SLE would be observed [17,18,22,23]. The time in between the two injuries was set at 14 days in order to maximize any recovery processes that occur in the acute time-window after injury [17]. The area injured was primarily cortical with some underlying subcortical damage [18,22]. The behaviors chosen are all practice-dependent, making it more likely to be able to see an SLE [23]. One additional factor that may have influenced the reduced deficits could be tied to the anesthetic that was utilized as well as the number of times it was administered (twice). Ketamine is a non-competitive *N*-methyl-D-aspartate receptor antagonist, which has been shown to have neuroprotective effects [36]. These optimal parameters could potentially be considered a limitation of the current study, but they provide a starting point for any further investigation concerning the SLE in TBI. Given the increasing interest in the effects of repeated TBI, it will be very important to identify parameters under which recovery can be maximized.

Ideally, these findings can be used to help drive further research into both why the SLE occurs and what processes are occurring in the case of repeated injuries, such as those seen in sports. Interest in sports-related brain injuries has received a large amount of interest from the popular media as well as within the scientific community [37–40]. Recent reports have noted the prevalence of dementia pugilistica (punch-drunk syndrome) in boxers [37,39] as well as elevated levels of β-amyloid protein plaques and elevated tau protein, markers of Alzheimer's disease [40]. In football players, repeated concussions have been shown to increase recovery times [38]. In general, the interpretation from the medical community following these studies is that the consequences of repeated TBIs are cumulative or possibly even multiplicative. However, in the experimental field the effects of repeated brain injuries are debated. Much of the current literature examining repeated TBI focuses on short inter-injury intervals of around 24 h and frequently observes increased impairment [13,41,42]. However, some evidence is starting to emerge from the repeated brain injury literature showing effects similar to what was seen in this study, using only a three day inter-injury interval, suggesting that the relationship may not be so simple [12]. Based on what has been seen in this study and the lesion literature, further investigations into longer inter-injury intervals may be warranted. Further research regarding TBI outcomes may lead to better functional recovery for those suffering from TBI-related deficits. This is especially important when considering sports injuries, in which an individual frequently returns to

the sport and may sustain additional injuries. By determining optimal windows and mechanisms of recovery, physicians may be able to reduce additional damages to athletes who are most likely to receive repeated brain injuries.

Conflict of interest

The authors have no financial interests in the outcome of this study.

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References

[1] Center for Disease Control. Injury prevention and control: traumatic brain injury; 2010 http://www.cdc.gov/TraumaticBrainInjury/index.html
[2] Bazarian JJ, McClung J, Shah MN, Cheng YT, Flesher W, Kraus J. Mild traumatic brain injury in the United States, 1998–2000. Brain Injury 2005;19:85–91.
[3] Ashman TA, Gordon WA, Cantor JB, Hibbard MR. Neurobehavioral consequences of traumatic brain injury. The Mount Sinai Journal of Medicine 2006;73:999–1005.
[4] N.I.H. Consensus Development Panel on Rehabilitation of Persons With Traumatic Brain Injury. Rehabilitation of Persons With Traumatic Brain Injury. The Journal of the American Medical Association 1999;282:974–83.
[5] Brody DL, Holtzman DM. Morris water maze search strategy analysis in PDAPP mice before and after experimental traumatic brain injury. Experimental Neurology 2006;197:330–40.
[6] Bury SD, Jones TA. Unilateral sensorimotor cortex lesions in adult rats facilitate motor skill learning with the unaffected forelimb and training-induced dendritic structural plasticity in the motor cortex. The Journal of Neuroscience 2002;22:8597–606.
[7] Kleim JA, Jones TA. Principles of experience-dependent neural plasticity: implications for rehabilitation after brain damage. Journal of Speech, Language, and Hearing Research 2008;51:S225–39.
[8] Kolb B, Gibb R. Sparing of function after neonatal frontal lesions correlates with increased cortical dendritic branching: a possible mechanism for the Kennard effect. Behavioural Brain Research 1991;43:51–6.
[9] Sabel BA, Dunbar GL, Stein DG. Gangliosides minimize behavioral deficits and enhance structural repair after brain injury. Journal of Neuroscience Research 1984;12:429–43.
[10] Schallert T, Leasure JL, Kolb B. Experience-associated structural events, subependymal cellular proliferative activity, and functional recovery after injury to the central nervous system. Journal of Cerebral Blood Flow and Metabolism 2000;20:1513–28.
[11] Scheff S, Price DA, Hicks RR, Baldwin SA, Robinson S, Brackney C. Synaptogenesis in the hippocampal CA1 field following traumatic brain injury. Journal of Neurotrauma 2005;22:719–32.
[12] Allen GV, Gerami D, Esser MJ. Conditioning effects of repetitive mild neurotrauma on motor function in an animal model of focal brain injury. The Journal of Neuroscience 2000;99:93–105.
[13] Laurer H, Bareyre FM, Lee VMYC, Trojanowski JQ, Longhi L, Hoover R, et al. Mild head injury increasing the brain's vulnerability to a second concussive impact. Journal of Neurosurgery 2001;95:859–70.
[14] Uryu K, Laurer H, McIntosh T, Pratico D, Martinez D, Leight S, et al. Repetitive mild brain trauma accelerates Aβ deposition, lipid peroxidation, and cognitive impairment in a transgenic mouse model of Alzheimer amyloidosis. The Journal of Neuroscience 2002;22:446–54.
[15] Adametz JH. Rate of recovery of functioning in cats with rostral reticular lesions: an experimental study. Journal of Neurosurgery 1959;16:85–98.
[16] Walbran BB. Age and serial ablations of somatosensory cortex in the rat. Physiology & Behavior 1975;17:13–7.
[17] Spear PD, Barbas H. Recovery of pattern discrimination ability in rats receiving serial or one-stage visual cortex lesions. Brain Research 1975;94:337–46.
[18] Dru D, Walker JB, Walker JP. Recovery of pattern vision following serial lesions of striate cortex in rats. Brain Research 1975;88:353–6.
[19] Finger S, Marshak RA, Cohen M, Scheff S, Trace R, Niemand D. Effects of successive and simultaneous lesions of somatosensory cortex on tactile discrimination in the rat. Journal of Comparative and Physiological Psychology 1971;77:221–7.
[20] Fass B, Wrege K, Greenough WT, Stein DG. Behavioral symptoms following serial or simultaneous septal-forebrain lesions: similar syndromes. Physiology & Behavior 1980;25:683–90.
[21] Reyes R, Finger S, Frye J. Serial thalamic lesions and tactile discrimination in the rat. Behavioral Biology 1973;8:807–13.
[22] Nonneman AJ, Kolb B. Functional recovery after serial ablation of prefrontal cortex in the rat. Physiology & Behavior 1979;22:895–901.
[23] De Castro JM, Zrull MC. Recovery of sensorimotor function after frontal cortex damage in rats: evidence that the serial lesion effect is due to serial recovery. Behavioral Neuroscience 1988;102:843–51.
[24] Glendenning RL. Effects of training between two unilateral lesions of visual cortex upon ultimate retention of black-white discrimination habits by rats. Journal of Comparative and Physiological Psychology 1972;80:216–29.
[25] Bell J, Gruenthal M, Finger S, Mangold R. Effects of one- and two-stage lesions of the posterior hypothalamus on temperature regulation in the rat. Brain Research 1981;219:451–5.
[26] Lighthall JW. Controlled cortical impact: a new experimental brain injury model. Journal of Neurotrauma 1988;5:1–15.
[27] Dixon CE, Lyeth BG, Povlishock JT, Findling RL, Hamm RJ, Marmarou A, et al. A fluid percussion model of experimental brain injury in the rat. Journal of Neurosurgery 1987;67:110–9.
[28] Myer DJ, Gurkoff GG, Lee SM, Hovda DA, Sofroniew MV. Essential protective roles of reactive astrocytes in traumatic brain injury. Brain 2006;129:2761–72.
[29] Arvin B, Neville LF, Barone FC, Feuerstein GZ. The role of inflammation and cytokines in brain injury. Neuroscience and Biobehavioral Reviews 1996;20:445–52.
[30] Oyesiku NM, Evans C-O, Houston S, Darrell RS, Smith JS, Fulop ZL, et al. Regional changes in the expression of neurotrophic factors and their receptors following acute traumatic brain injury in the adult rat brain. Brain Research 1999;833:161–72.
[31] Smith JS, Fulop Z, Levinsohn SA, Darrell RS, Stein DG. Effects of the novel NMDA receptor antagonist gacyclidine on recovery from medial frontal cortex contusion injury in rats. Neural Plasticity 2000;7:73–91.
[32] Hamm RJ, Pike BR, O'Dell DM, Lyeth BG, Jenkins LW. The rotarod test: an evaluation of its effectiveness in assessing motor deficits following traumatic brain injury. Journal of Neurotrauma 1994;11:187–96.
[33] Vonder Haar C, Anderson GD, Hoane MR. Continuous nicotinamide administration improves behavioral recovery and reduces lesion size following bilateral frontal controlled cortical impact injury. Behavioural Brain Research 2011;224:311–7.
[34] Barnes CA. Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat. Journal of Comparative and Physiological Psychology 1979;93:74–104.
[35] Dirnagl U, Simon RP, Hallenbeck JM. Ischemic tolerance and endogenous neuroprotection. Trends in Neurosciences 2003;26:248–54.
[36] Hirota K, Ketamine Lambert DG. Its mechanism(s) of action and unusual clinical uses. British Journal of Anaesthesia 1996;77:441–4.
[37] Echemendia R, Julian L. Mild traumatic brain injury in sports: neuropsychology's contribution to a developing field. Neuropsychology Review 2001;11:69–88.
[38] Guskiewicz KM, McCrea M, Marshall SW, Cantu RC, Randolph C, Barr W, et al. Cumulative effects associated with recurrent concussion in collegiate football players. The Journal of the American Medical Association 2003;290:2549–55.
[39] Roberts GW, Allsop D, Bruton C. The occult aftermath of boxing. Journal of Neurology, Neurosurgery, and Psychiatry 1990;53:373–8.
[40] Tokuda T, Ikeda S, Yanagisawa N, Ihara Y, Glenner GG. Re-examination of ex-boxers' brains using immunohistochemistry with antibodies to amyloid β-protein and tau protein. Acta Neuropathologica 1991;82:280–5.
[41] Creeley CE, Wozniak DF, Bayly PV, Olney JW, Lewis LM. Multiple episodes of mild traumatic brain injury result in impaired cognitive performance in mice. Academic Emergency Medicine 2004;11:809–19.
[42] DeFord SM, Wilson MS, Rice AC, Clausen T, Rice LK, Barabnova A, et al. Repeated mild brain injuries result in cognitive impairment in B6C3F1 mice. Journal of Neurotrauma 2004;19:427–38.