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Progression of histopathological and behavioral abnormalities following mild traumatic brain injury in the male ferret

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Abstract

White matter damage is an important consequence of traumatic brain injury (TBI) in humans. Unlike rodents, ferrets have a substantial amount of white matter and a gyrencephalic brain; therefore, they may represent an ideal small mammal model to study human-pertinent consequences of TBI. Here we report immunohistochemical and behavioral results after a controlled cortical impact (CCI) injury to the sensorimotor cortex of adult male ferrets. We assessed inflammation in the neocortex and white matter, and behavior at 1 day post injury and 1, 4, and 16 weeks post injury (WPI). CCI in the ferret produced inflammation that originated in the neocortex near the site of the injury and progressed deep into the white matter with time. The density of microglia and astrocytes increased in the neocortex near the injury, peaking at 4WPI and remaining elevated at 16WPI. Microglial morphology in the neocortex was significantly altered in the first 4 weeks, but showed a return toward normal at 16 weeks. Clusters of microglial cells in the white matter persisted until 16WPI. We assessed motor and cognitive behavior using the open field, novel object recognition, T-maze, and gait tests. A transient deficit in memory occurred at 4WPI, with a reduction of rearing and motor ability at 12 and 16WPI. Behavioral impairments coincide with features of the inflammatory changes in the neocortex revealed by immunohistochemistry. The ferret represents an important animal model to explore ongoing damage in the white matter and cerebral cortex after TBI.

KEYWORDS

astrocytes, astrogliosis, attention, behavior, brain, cerebral cortex, cognitive impairment, GFAP, gray matter, mammals, memory, neuroinflammation, reactive microglia, TBI (traumatic brain injury), white matter

1 | INTRODUCTION

Inflammation, specifically the activation of microglia and astrocytes, continues long after a traumatic brain injury (TBI) event. Human post-mortem studies reveal reactive microglia in the brain up to 18 years post trauma, and rodents exhibit persistent microglial activation up to at least a year after a traumatic injury (Johnson et al., 2013; Loane, Kumar, Stoica, Cabatbat, & Faden, 2014). Rodent brains, however, differ significantly from human brains architecturally, with the rodent brain being lissencephalic, containing little white matter relative to gray matter, and the hippocampus located dorsally. Because it is difficult to

Significance

Traumatic brain injury (TBI) causes chronic motor and cognitive deficits with few effective therapies. The ferret possesses features (a gyrencephalic neocortex and large amount of white matter) that make it an important model to study TBI. We investigated injury progression in a model of cortical contusion. While inflammatory markers appeared in the cortex near the site of injury early, both reactive microglia and astrocytes persisted in the white matter distant to the injury up to 4 months. This animal model represents an important opportunity to explore therapies addressing progressive damage in the white matter of the brain after TBI.

translate basic research to the clinic, an animal model with a brain morphologically similar to that of humans to study the complex pathogenesis of TBI is essential. We recently developed a ferret model of brain injury using the controlled cortical impact (CCI) technique (Schwerin et al., 2017). The ferret has a gyrencephalic brain with a greater ratio of white matter to gray matter than the rodent and the hippocampus located primarily in the temporal lobe, similar to the human. In this study, we investigated the effects of a mild brain injury on inflammatory markers and behavior in the ferret over a period of several months.

Changes in inflammatory makers, specifically the distribution, extent, and morphology of microglia and astrocytes, are key to investigations after brain injury (e.g., Burda, Bernstein, & Sofroniew, 2016; Loane & Kumar 2016). The immune response can be both beneficial and maladaptive and involves components that include morphologic alternations in microglia (e.g., Jassam, Izzy, Whalen, McGavern, & El Khoury, 2017). These cells show dramatic changes in structure and gene expression presumed to correlate with functional profiles. Current ideas suggest that microglia alter their profile after injury along a diverse progression rather than binary changes representing either beneficial or maladaptive responses (Jassam et al., 2017). Up until now, very few studies report the distribution and morphologic changes of microglia and/or astrocytes after TBI in gyrencephalic animals with larger amounts of white matter. To augment our understanding of inflammatory responses to TBI, we followed the morphology and overall distribution of astrocytes and microglia in the cerebral cortex and the white matter both near and deep to the injury site.

The objective of this study was to observe the time course and severity of altered behavior after mild TBI and to observe potential correlations with histologic changes. Several other groups have used ferrets as highly trained animals in behavioral studies. Ferrets are involved in studying discriminative behavior in audition and vision (Gold, Nodal, Peters, King, & Bajo, 2015; Zhou, Yu, Sellers, & Fröhlich, 2016). Our previous report also found them to be excellent animals to participate in behavioral studies after TBI (Schwerin et al., 2017). To address the observation in humans that many individuals have delayed onset of cognitive and motor impairments after TBI, we performed a battery of behavioral tests adapted for the ferret to determine alterations or deficiencies in cognition or motor activity and their progression over a period of 16 weeks after an injury.

2 | MATERIALS AND METHODS

We used 17 adult male ferrets in this study (*Mustela putorius furo*, 1.5–2.0 kg, 6–9 months old, Marshall Bioresources, North Rose, NY). The protocol was approved by the Animal Care and Use Committee at Uniformed Services University of Health Sciences and conducted in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*. Ferrets were housed 2 per cage (modified rabbit cage by Lenderking Caging Products, Millersville, MD), excluding the week after surgery when they were housed 1 per cage, in a temperature- and humidity-controlled room (61°–72°F, 30%–70%) on a 12:12-hr light/dark

cycle. Animals were allowed to habituate for 7 days after arrival. Ferrets had ad libitum access to food and water until behavioral training began. We used food restriction to motivate the animals to perform behavioral tests and monitored their weight, which was not allowed to drop below 90% of the original body weight. All animals received enrichment activity once a week, which involved being put into a playpen with other ferrets and interacting with humans and toys for 10 min. The ferrets were randomly divided into 5 groups with 3 animals per group, including a naïve control group, and 4 TBI groups according to survival time (1 day, 1 week, 4 weeks, 16 weeks). An additional 2 animals survived 1 week and 4 weeks and were used solely in the behavioral experiments. We selected the number of animals based on our previous studies using ferrets for similar assessment.

2.1 | Controlled cortical impact

This model has been described previously (Schwerin et al., 2017). Ferrets were anesthetized in a chamber with 4%–5% isoflurane mixed with 100% oxygen. Each ferret was intubated and transferred to a surgical table, and the anesthesia maintained at 2%–3% isoflurane mixed with 100% oxygen. We monitored each ferret for vital signs and maintained thermoregulation with a Bair Hugger warming system. Subject-specific anatomical magnetic resonance imaging (MRI) coordinates guided the impactor used for CCI to the left primary somatosensory cortex (identified as the dimple in the posterior sigmoid gyrus) using the junction of the supraorbital processes as a landmark. We made a craniotomy, and a 3.0-mm-diameter impactor oriented perpendicular to the cortex was presented at 5 m/s velocity, 100 ms dwell time, and 2 mm depth. After this, we placed an absorbable gelatin sponge (Ethicon, REF 1972, Somerville, NJ) in the craniotomy and sealed the opening with dental acrylic (Duz-All, Coralite Dental Products, Skokie, IL). Ferrets were given cefazolin intramuscularly (IM) (20 mg/kg) at least 10 min prior to surgery and buprenorphine IM (0.01–0.03 mg/kg) immediately after surgery. A second dose of buprenorphine was given IM following behavioral testing at 6 hr post injury. We provided amoxicillin trihydrate/clavulanate potassium (Clavamox, Zoetis, Kalamazoo, MI) orally twice a day for 7 days post surgery (1 ml, 62.5 mg/ml).

2.2 | Magnetic resonance imaging

To consistently select histologic sections from a similar location relative to the CCI lesion, ex vivo MRI scans were performed prior to sectioning, and regions of abnormal diffusion tensor imaging (DTI) values were identified according to previously described imaging findings along with details of image acquisition and processing (Hutchinson et al., 2016; Hutchinson, Schwerin, Avram, Juliano, & Pierpaoli, 2018). The resulting MRI volumes and DTI maps were used to generate montage images of every coronal slice in the brain using ImageJ software (ImageJ 1.46; <https://imagej.nih.gov>). Slices with DTI abnormalities indicative of the CCI site were identified visually. The slice at the central location of the lesion was then used as a guide for histological slice

selection by matching the anatomical features of the MRI and histological slices.

2.3 | Behavioral testing

CCI causes a local injury at the site of impact and can also cause diffuse injury distally (Dixon, Clifton, Lighthall, Yaghmai, & Hayes, 1991); with human behavioral impairments spanning a range of modalities (Ponsford et al., 2014). We therefore decided to run a battery of behavioral tests including sensorimotor and cognitive tests.

All ferrets underwent behavioral testing. Ferrets were euthanized at set time points after injury for histologic analysis: 1 day post injury (1DPI), 1 week post injury (1WPI), 4WPI, and 16WPI. This resulted in a different number of animals at each time point for behavior (control = 3, PRE = 14, 6 hours post injury [6HPI] = 14, 1DPI = 14, 1WPI = 11, 4WPI = 7, 12WPI = 3, 16WPI = 3 ferrets), which was handled by using a mixed-effects model (see Statistical Analysis section below).

Ferrets were acclimated to the behavioral devices prior to the official testing sessions (open field—three 10-min sessions; gait—trained until able to walk the length of the gait paper calmly). We trained the ferrets to alternate arms on the T-maze prior to the official testing (see below). Gerber chicken-flavored baby food motivated the animals to perform the T-maze and gait tests.

Open field, novel object recognition, T-maze, and gait were tested pre injury, 1DPI, 1WPI, 4WPI, 12WPI, and 16 WPI, with open field and gait also tested at 6HPI. Tests were performed in the morning through early afternoon and in the order presented below.

2.3.1 | Open field

The open field test detects impairments in ambulation as well as anxiety. We used a modified, commercially available rat version of the open field test (100 cm × 100 cm, Stoelting) with the opaque walls extended to 63.5 cm by securing pieces of opaque plastic behind the existing walls. Ferrets were initially placed in a playpen with a tunnel and ball toys for 3 min prior to open field testing to ensure alertness. Within the Anymaze software (Stoelting Co., Wood Dale, IL), we chose the setting to automatically detect the center of the animal (the setting to detect the animal's head did not accurately identify the ferret's head due to differences in head shape from the rodent). For each 10-minute session in the open field, the total distance traveled and the average and maximum speeds were automatically calculated based on the animal center using the Anymaze software and video camera. The number of times the animals reared was indicated by the investigator through a keystroke. The time spent and the distance traveled in the center zone were also measured as an indicator of anxiety. At time points 4WPI, 12WPI, and 16WPI, the animals were given a reminder session in the open field apparatus within a week prior to the test to minimize motor activity related to novelty; this was not needed at the shorter time points because the acclimation and testing periods were in close proximity.

2.3.2 | Novel object recognition

We used the novel object recognition (NOR) test to study short-term memory and investigate attention span and curiosity. This test occurred

immediately after the open field test. We tested NOR in the ferret using vinyl/rubber/plastic dog toys (see Table 1) as described in Schwerin et al. (2017). For the familiarization phase, we secured two identical objects in opposite corners of the open field apparatus and then let the ferret investigate them for 10 min. Each animal then returned to his home cage for 1 hr. In the test phase, a novel object was substituted for one of the original objects. We then recorded the activity of the ferret when returned into the open field apparatus for 10 min using the Anymaze software and video camera, with interest in each object indicated using keystroke by the investigator. Different objects were used for each time point, with the same objects used across animals. The retention index was calculated using data from the test phase at each time point: retention index = time spent investigating the novel object / (time spent investigating the novel object + time spent investigating the original object) * 100.

2.3.3 | T-maze

We used the T-maze to study working memory. The "T"-shaped apparatus consists of 3 segments—the body, sitting perpendicular and between the 2 arms, which are all covered in a metal mesh. The arms have guillotine doors to control access to the arms. The T-maze was located on a platform 74.5 cm above the floor. The end of each arm and the body opens into a plastic crate with guillotine doors. A dividing wall extends from the back of the body (where it attaches to the 2 arms) into the body for 9.5 cm. Ferrets were individually placed in the covered "start" crate with the guillotine door closed. When the door opened, the animal entered the body and chose to go to the left or right of the dividing wall, and then entered the coordinating arm. A food reward was placed in the uncovered "end" crate at the end of the arm. Prior to testing and injury, we trained each ferret to choose the alternate arm following a forced-choice trial (i.e., if the animal is forced to go to the left arm, then in the open choice trial the animal must choose to go down the right arm to receive a reward). Twelve paired trials (forced/choice) were trained per day for 2 to 3 weeks. During training, the "end" crate guillotine door was closed for the incorrect arm, and if the animal chose the incorrect arm, it was allowed to make a correction and complete in the correct arm with the reward. Ferrets were food deprived (to no more than 90% original weight) to motivate them to participate in the training. On testing days, ferrets were given 12 paired trials (with no corrections allowed), and their accuracy was recorded. Ferrets were food restricted 2 to 3 days prior to the testing day. The time between the forced trial and the choice trial was less than a minute.

2.3.4 | Gait

Gait was investigated to detect sensorimotor impairments. Each paw was dipped into a different-colored, water-based, nontoxic tempera poster paint (Palmer Paints), and then the animal walked on a narrow path covered in paper (45.7 cm wide, 2.13 m long). For each testing session, the animal was given 3 trials. Within each trial, 3 samples were measured to determine stride length (measured independently for each paw, from the center of the paw to the center of the next placement

TABLE 1 Objects used during novel object recognition testing following a mild controlled cortical impact in the ferret

	Original	Novel	Color	Shape	Material	Sound	Surface texture	Feature	Length Height (cm)	Width Height (cm)
PRE	 ring	 ball	purple/ blue	ring/ sphere	firm rubber/ thin rubber	none	spikey	hole in middle/ tall	9.5/6.5	9.5/6.5 2/5.5
1DPI	 dog	 rope	purple/ pink-black	long narrow	thin rubber/ rope	squeak/ none	smooth/ rough	none/ strings on ends	15/19	6/5 4/3.5
1WPI	 chicken	 octopus	yellow	long narrow	thin rubber	squeak	smooth	none/ space between tentacles	15/11.5	6.5/5.5 4.5/4.5
4WPI	 cow	 gingerbread	blue/ brown	flat/ spread out	fabric	crinkle	soft	none	16/14	16/12 2.5/5
12WPI	 football	 bones	blue/ multi	3D football/ flat multiple bones	thin rubber/ firm rubber	none	smooth/ bumpy	3D with holes/ multiple bones	8.5/15	6.5/5 4/3
16WPI	 circle mirror	 rectangle mirror	red/ green	complex	hard plastic	none	smooth	Moving parts/ mirror	6/9.5	12/9 7/5.5

DPI = days post injury, WPI = weeks post injury.

of that paw), front/back paw overlap (distance between the center of each paw), and stance width (distance between the center of the left and right paw).

2.4 | Immunohistochemistry

Ferrets were deeply anesthetized with Euthasol (0.22 ml/kg) and transcardially perfused with 1 L ice-cold 0.1 M phosphate-buffered saline (PBS) (pH 7.4) containing 53.1 mg of heparin (Sigma-Aldrich, St. Louis, MO) followed by 1 L of ice-cold 4% paraformaldehyde solution in PBS (Santa Cruz Biotechnology, Santa Cruz, CA). The brains were postfixed in the same solution for 8 to 10 days and then transferred to a storage solution of PBS mixed with 0.03% sodium azide. Coronal sections (50 µm) were cut using a vibratome (Leica VT1000; Leica), and immunohistochemistry was carried out as described previously (Schwerin et al., 2017). Sections located at the injury epicenter (identified by ex vivo MRI imaging) were double

labeled with chicken anti-glial fibrillary acidic protein (GFAP) (1:500; Abcam, ab4674; RRID: AB_304558) and rabbit anti-IBA1 antibody (1:1,000; Wako, 019-19741; RRID: AB_839504) using antigen retrieval (1 × citrate buffer (Thermo Scientific, Waltham, MA) in an 80 °C water bath for 20 min). Label was revealed by incubation with either AlexaFluor 555- or 488-conjugated secondary antibody (1:500) and nuclei counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, D21490, 1:2,000). Sections were mounted with Mowiol 4-88 medium (Polysciences, Warminster, PA). Two ferrets were excluded from the immunohistochemical analysis because of technical issues with the perfusions (1 from the 4WPI and 1 from the 16WPI groups) but were included in the behavioral analyses.

2.4.1 | Imaging and analysis

Coronal brain slices at the injury epicenter were scanned with a digital slide scanner at 10 × magnification using Zen software (Axio Scan.Z1, Carl Zeiss Microscopy, Thornwood, NY). We acquired additional optical

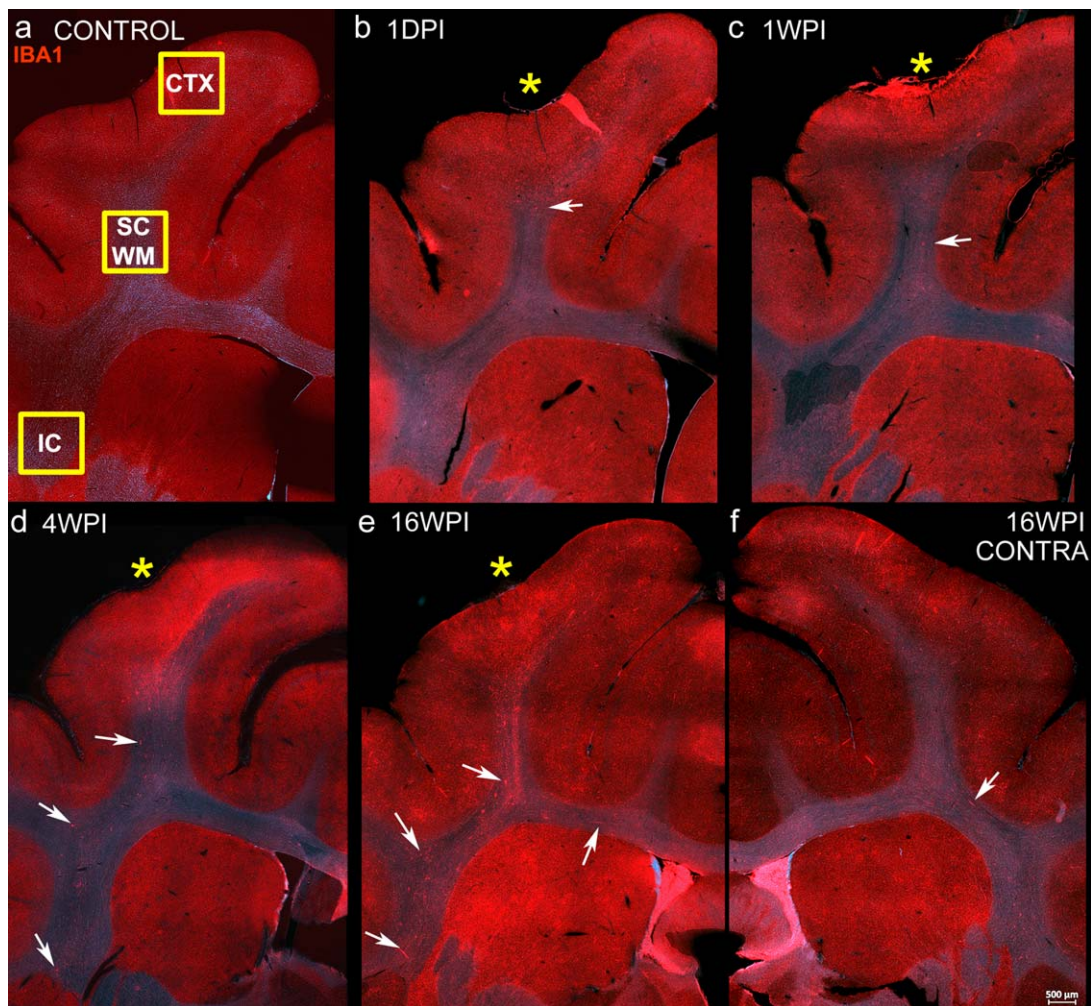


FIGURE 1 Spatiotemporal localization of ionized calcium binding adaptor molecule 1 (IBA1-red)-positive cells in the ferret brain longitudinally following mild controlled cortical impact (CCI) injury of the somatosensory cortex. Representative IBA1 immunostaining in control (a) and in the injured hemisphere of the ferret at different time points following CCI: 1DPI (b), 1WPI (c), 4WPI (d), and 16WPI (e). Nuclei were counterstained with DAPI (blue). Immunoreactivity in the contralateral hemisphere at 16 weeks post injury is shown in panel f. The yellow asterisk indicates the site of CCI. Regions of interest used for density measurements are indicated with yellow boxes: CTX, neocortex; SC-WM, subcortical white matter; IC, deep white matter just superficial to the internal capsule. The white arrows point to microglial clustering. DPI = days post injury, WPI = weeks post injury. Scale = 500 μ m

image fluorescent stacks at 20 $\times$ and 63 $\times$ magnification using a Zeiss LSM700 microscope equipped with Zen software (Carl Zeiss Microscopy, Thornwood, NY). Three immunohistochemically labeled sections (no more than 1,000 μ m apart) were analyzed (described below) and averaged for each ferret by an operator blinded to the treatment.

2.4.2 | Percent area labeled analysis

The percent area fluorescently labeled was analyzed at three regions of interest (ROIs) (321 μ m $\times$ 321 μ m) in the injured hemisphere: in the neocortex medial to the impact site (Cortex, Ctx), in the underlying subcortical white matter near the injury site (Subcortical White Matter, SCWM), and deeper in the brain in the white matter just superficial to the internal capsule (Internal Capsule, IC). These ROIs were chosen to investigate inflammation near the injury site in the gray matter and also in the white matter both near and distant from the injury to monitor changes over time. We did not investigate the region immediately under the site of impact because of

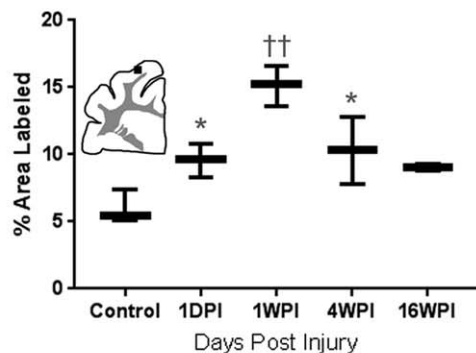
potential scarring. At 20 $\times$ magnification, Z-stacks of 25 to 40 layers were acquired at 0.90- μ m intervals. Images were exported to Fiji (Schindelin et al., 2012), converted to 8-bit, background subtracted (rolling ball radius 50.0 pixels), and autothresholded (Otsu's thresholding method; Otsu, 1979). The percent area labeled was then compared between groups.

2.4.3 | Microglial morphology analysis

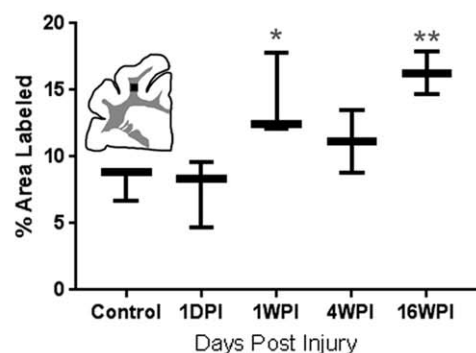
To investigate morphological changes of the IBA1-stained microglia, 63 $\times$ images were collected at the same ROIs (Cortex, Subcortical White Matter, and Internal Capsule). Z-stacks of 30–60 layers were acquired at 0.27- μ m intervals as above. Z-stacks were decreased at the processing stage if individual cells could not be distinguished due to increased cell density. Images were analyzed with Fiji (Schindelin et al., 2012), using the method described by Zanier et al. (Zanier, Fumagalli, Perego, Pischiutta, & De Simoni, 2015). Briefly, the scale was set, brightness/contrast adjusted automatically, and

the image smoothed and converted to binary. The *analyze particles* function was then used with a pixel threshold of 40 microns. Of the particles selected, a cell was analyzed if it colocalized with a single nucleus from the DAPI staining, the majority of the cell body was visible within the image, and the cell could be distinguished from other cells. Crossing processes clearly from other cells were excluded from the measurement by using the drawing tool to “disconnect” them from the cell of interest. The cells that remained were analyzed for the following parameters: area, perimeter, circularity, Feret diameter, aspect ratio, and solidity. Cortical IBA1-reactive cells were sampled from 2 to 3 brains/group, and 2 to 3 slices per brain, to get at least 10 cells per group.

a Microglia - Cortex - Medial to Injury



b Microglia - Subcortical White Matter



c Microglia - White Matter - Internal Capsule

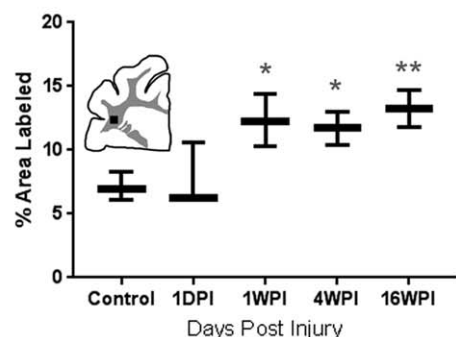


FIGURE 2.

2.4.4 | Microglial cluster analysis

Three coronal slices from the injury epicenter were reviewed at 10 $\times$ and 40 $\times$ magnification, and the number of microglial clusters (greater than 20 μ m across) within the ipsilateral hemisphere white matter was counted across all time points. The number of clusters was averaged across the 3 slices within each animal and compared across each time point.

2.5 | Statistical analysis

Randomization and blinding procedures were employed. Data are presented as mean $\pm$ standard error of the mean. We compared the percent area labeled across groups using a 1-way analysis of variance within each ROI, followed by post hoc pairwise comparisons using Fisher least significant difference test. A mixed-effects model for repeated measures with a random subject effect, condition as a between-subjects factor, and time as a within-subjects factor was used to test significance within the behavioral tests due to the different number of animals at each time point (see Behavioral Testing section). Statistical analyses were performed using SPSS (IBM Corp., Released 2016., IBM SPSS Statistics for Windows, Version 24.0, Armonk, NY) or GraphPad Prism (Version 7.01 for Windows, GraphPad Software, La Jolla, CA; www.graphpad.com). A value of $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Immunohistochemistry

3.1.1 | Activated microglia (IBA1 immunostaining)

Examples of IBA1 immunohistochemistry can be seen in Figure 1. Figure 1a shows a coronal section from a control brain that specifies the regions we examined. Immunoreactive cells are visible in the cerebral cortex near the CCI site (asterisk) and extending throughout the gyrus

FIGURE 2 Region of interest (ROI) density analysis of microglia immunoreactivity. (a) The percent area labeled by IBA1 in an ROI in the cortex medial to the injury. Analysis of variance (ANOVA) showed a significant effect after injury ($F[4,8] = 11.06, p = .0024$). In this region, the 1WPI time point had the greatest percent area labeled, with 1DPI and 4WPI also significantly increased compared with control in post hoc comparisons. (b) In the subcortical white matter, ANOVA also showed significance ($F[4,8] = 5.474, p = .0201$). The 1WPI and 16WPI time points showed significantly greater label than the control group in post hoc comparisons. (c) In the white matter near the internal capsule, ANOVA also showed significance ($F[4,8] = 5.361, p = .0213$). The post hoc analysis showed a significant increase in density starting at 1WPI and continuing to 16WPI compared with control values. Within each graph, the ROI is depicted as the black square in the brain slice illustration (Figure 1). The horizontal bar represents the median across $n = 3$ animals ($n = 2$ for 4WPI and 16WPI), and the whiskers indicate the highest and lowest values. To test significance, a 1-way ANOVA was followed by an uncorrected Fisher least significant difference test. The 1-way ANOVA was calculated on the percent area labeled by the IBA1 antibody in each ROI; the ANOVA was followed by post hoc tests in comparison with the control. DPI = days post injury, WPI = weeks post injury. * $p < .05$, ** $p < .01$, †† $p < .0005$

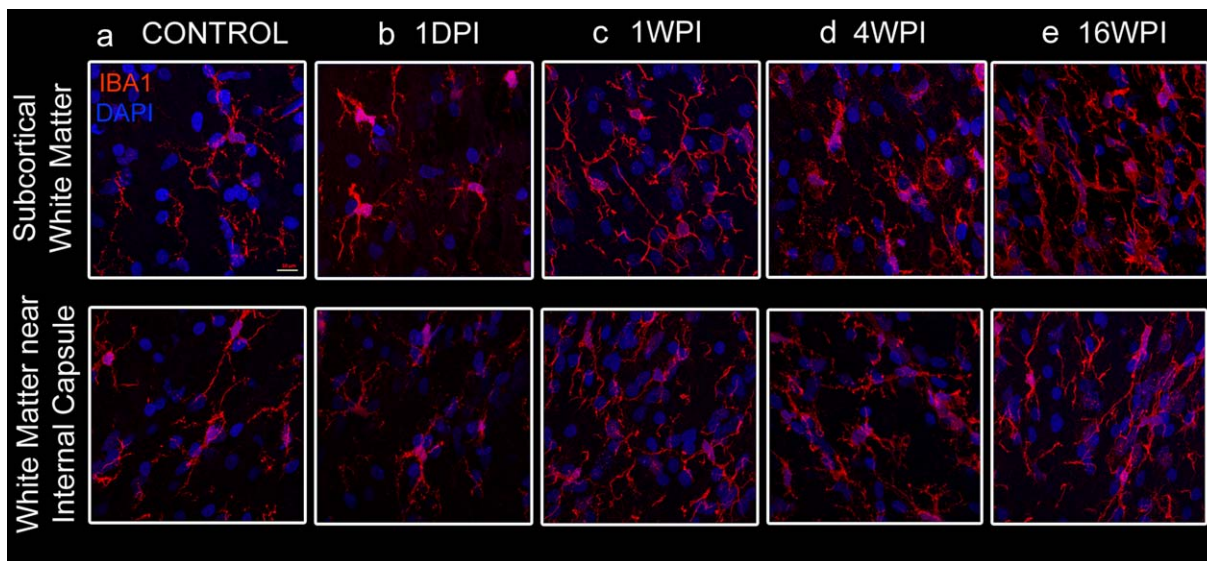


FIGURE 3 Microglial morphology in the white matter of the ferret at different times following a mild controlled cortical impact of the somatosensory cortex. Images are from a coronal section at the epicenter of the injury. The first row is from the subcortical white matter beneath the injury site. The second row is from the white matter near the internal capsule. (a) IBA1-reactive cells (red) in a naïve control with DAPI-stained nuclei (blue). (b–e) IBA1-reactive cells at several time points after injury: 1DPI, 1WPI, 4WPI, and 16WPI. The density of IBA1-reactive cells increased at 1WPI and was maintained throughout 16WPI. Note: images were created with subsets of the Z-stack to more clearly visualize the cells. DPI = days post injury, WPI = weeks post injury. Scale = 10 μ m

that received the injury for all time points examined. We found increases at all the examined time points post injury, although they seemed to peak at 4WPI and then diminish in the neocortex, but remained high and extended into the white matter at all time points. We also observed increases in IBA1 immunoreactivity in the hemisphere contralateral to the injury, especially evident at 16WPI (Figure 1f).

3.1.2 | Percent area labeled - IBA1

To quantify the observations seen in Figure 1, we conducted a density analysis within specific ROIs. These regions included the neocortex medial to the injury site, the subcortical white matter, and the white matter more deeply located and just superficial to the internal capsule (Figure 1 and Figure 2a–c). In the neocortex medial to the injury, the density of microglia increases most dramatically at 1WPI compared with control, but also increases at all time points assessed, and is significantly greater in density at 1DPI, 1WPI and 4WPI; by 16WPI the density attenuates but remains slightly elevated (Figure 2a). In the region of the subcortical white matter, the microglial density increases gradually over time, showing significance compared with control at 1WPI and 16WPI. These changes occur in contrast to the levels of microglial density in the neocortex, which reduce at 16WPI (Figure 2b). Deeper in the white matter, the microglia also show a gradual increase in density over time, revealing significant changes compared with control at 1, 4, and 16WPI (Figure 2c). Overall, these changes reveal a global increase of microglia in the white matter that persists, with a significant increase in neocortical microglia that diminishes over time.

This is further supported by higher-power images in the subcortical white matter and deeper in the corona radiata toward the internal capsule (Figure 3). These images demonstrate the increasing density of IBA1 immunoreactivity and changes in morphology of the

immunoreactive cells over the survival time of 16 weeks. As the microglia increase in density over the 16-week period, their morphology also alters, making it more difficult to distinguish individual cells as they surround structures in the white matter.

3.1.3 | Microglial clusters

We also observe that over a period of weeks, the microglia form clusters, as seen in Figures 1 and 4 (see white arrows/asterisks for examples of clusters). The number of clusters in the white matter of the injured hemisphere increases with time after injury, with the 4WPI and 16WPI groups possessing a significantly greater quantity throughout the white matter compared with the control group. The IBA1-reactive cells also line blood vessels (Figure 4e; yellow arrows point to blood vessels cut longitudinally), and large regions of microglial reactivity appear to surround cells (Figure 4d, green arrows). The microglial clusters continue to extend more deeply into the white matter with longer survival times. Video 1 goes through the Z-stack of a microglial cluster in the white matter.

3.1.4 | Shape parameters for IBA1-reactive cells

Our data regarding the density of microglia indicate that the overall reactivity is strongest in the white matter while it diminishes over time in the cerebral cortex. To further characterize the longitudinal alterations of the microglia, we studied the morphology of the IBA1-immunoreactive cells over the 16-week survival time. We assessed the shape changes of microglia in the neocortex, since their density increases and then diminishes, suggesting demonstration of pathology and a relative return to normal. We also focus specifically on the cerebral cortex because the microglia of the white matter were extremely dense and we found it difficult to distinguish individual cells and

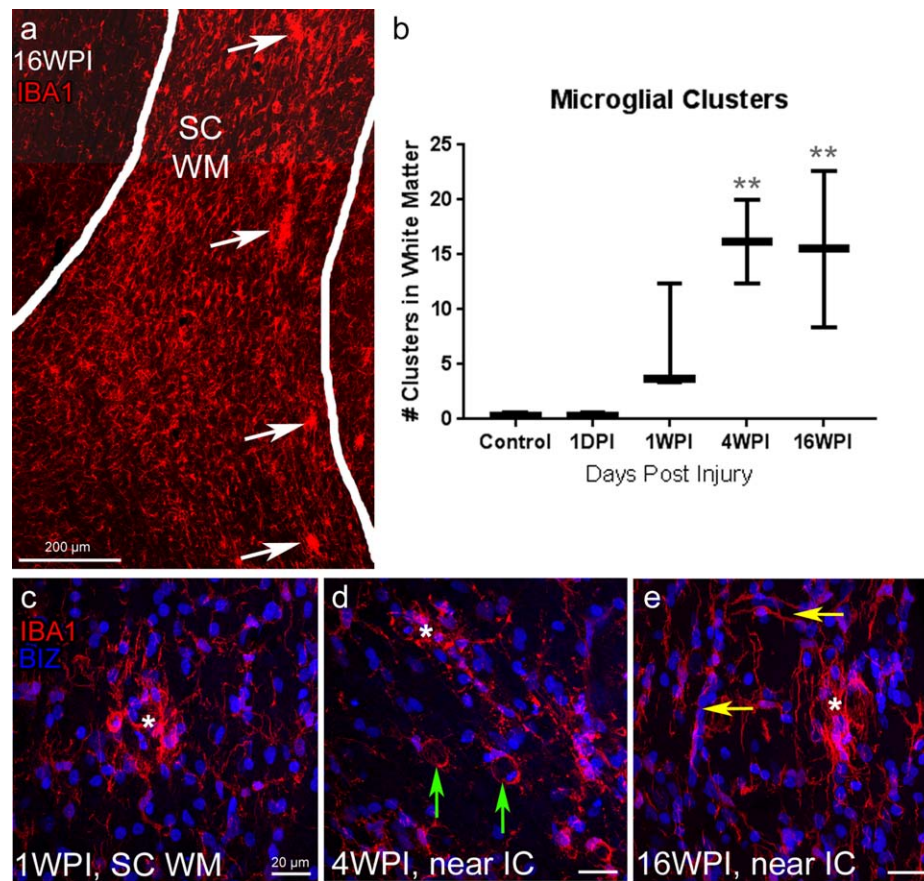


FIGURE 4 Microglial clusters in the white matter of the ferret at different periods of time following a mild controlled cortical impact of the somatosensory cortex. (a) IBA1-reactive microglial clusters indicated by white arrows in the subcortical white matter (SCWM) at 16WPI. (b) In the graph, the horizontal bars represent the median number of microglial clusters counted within the white matter of the injured hemisphere of a coronal brain slice at the injury epicenter in either a naïve control group or at different time points after injury. The whiskers indicate the highest and lowest values of the results. Analysis of variance (ANOVA) indicated significance longitudinally ($F[4,8] = 6.251$, $p = .0139$). The number of clusters was significantly increased at 4WPI and maintained at 16WPI. $N = 3$ for control, 1DPI, and 1WPI and $N = 2$ for 4WPI and 16WPI (with the count across 3 slices averaged per animal). (c–e) High-magnification images of microglial clusters with DAPI-stained nuclei (BIZ) from different regions at different time points after the injury. White asterisks indicate clusters; green arrows point to large regions outlined by IBA1 reactivity; yellow arrows point out IBA1-reactive cells lining blood vessels cut longitudinally. To test significance, a 1-way ANOVA was followed by an uncorrected Fisher least significant difference test. All values are compared with the control value. DPI = days post injury, WPI = weeks post injury, SC WM = subcortical white matter, IC = internal capsule. $**p < .01$. Scale = 200 μm in panel a and 20 μm in panels c–e

processes at time points greater than 1DPI (Figure 3 and Video 1 showing the microglial clusters).

A number of factors define cellular morphology using the analysis of shape parameters described by Zanier et al. (2015). Overall, the morphology of neocortical microglia moves away from the “resting” highly ramified phenotype during the period of increased density and returns to control values as their density returns toward normal, measured in Figure 2. An example of the Fiji-processed images used to measure the morphological changes after injury are shown in Figure 5d–h. Also see Figure 5 for a pictorial description of the terms used to describe the morphologic changes.

Perimeter

After CCI, the mean perimeter (outline of a cell) of IBA1-reactive cells decreases significantly in the neocortex medial and adjacent to the injury site at 1 day, 1 week, and 4 weeks after the injury, but returns to normal at 16 weeks when compared with controls and when the

density measurements return to normal (Figure 5a). This measure likely reduces because of the thickening and shortening of the processes, compared with the numerous multiple fine processes seen in “resting” microglia.

Area

The mean area of IBA1-reactive cells gradually decreases over time so that by 4WPI, this parameter is significantly diminished after CCI compared with controls (Figure 5b). Again, by 16WPI this measure returns to control values.

Feret diameter

The Feret diameter, measured as the distance between the farthest points of a cell, evaluates the overall extent of the microglial soma and processes. After CCI, the Feret diameter significantly reduces at 1WPI and 4WPI but returns to normal values by 16WPI (Figure 5c). This

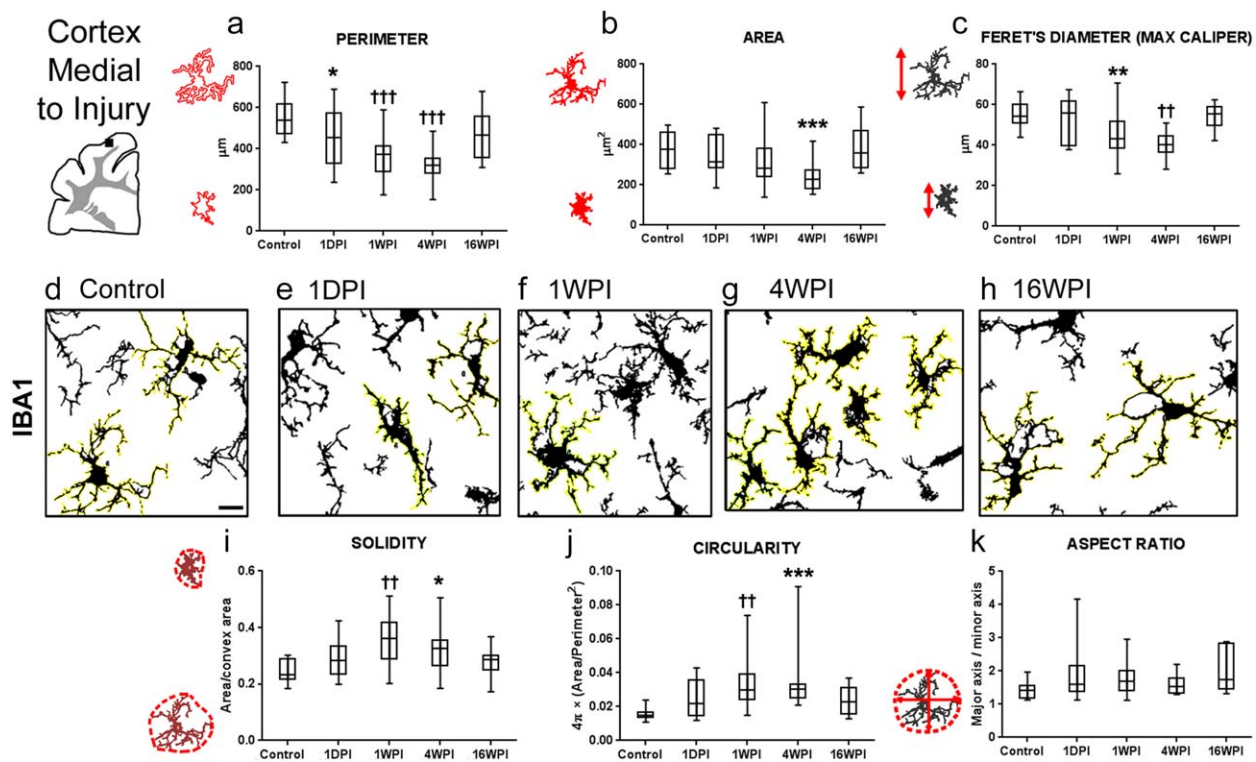


FIGURE 5 Microglial morphology in the cortex adjacent to the injury. (a) The perimeter is the measurement of the outline of the cell. The perimeter was significantly affected by time after injury ($F[4,63] = 9.058$, $p < .0001$), decreasing significantly at 1DPI, 1WPI, and 4WPI compared with control. (b) There was a significant effect of time after injury on the area measurement of microglial cells in the cortex ($F[4,63] = 3.801$, $p = .0079$). The 4WPI time point was significantly smaller than the control value. (c) Feret diameter describes the width or maximum diameter of the microglial cell. There was a significant effect of time after injury ($F[4,63] = 6.607$, $p < .0002$). After CCI, Feret diameter significantly decreased at 1WPI and 4WPI compared with control. (d–h) IBA1-labeled cells from a control (d) and each time point after CCI (e–h). Images taken from Fiji after processing. The cells highlighted in yellow were chosen for morphology analysis (yellow is not the perimeter measurement). Note: images were created using the full Z-stack. (i) The solidity measurement is closer to 1 if the cell is amoeboid shaped and closer to 0 if it is highly ramified and hypertrophic. There was a significant effect of time after injury ($F[4,63] = 4.426$, $p = .0032$). At 1WPI and 4WPI, the microglial cells in the neocortex have larger soma and shorter, thicker processes, making them score significantly higher on the solidity scale than the control. (j) For the circularity measurement, there was also a significant effect of time after injury ($F[4,63] = 4.337$, $p = .0037$). Circularity significantly increased at 1WPI and 4WPI and returned to near control levels by 16WPI. (k) For the aspect ratio measurement, there was not a significant effect of time after injury ($F[4,63] = 1.691$, $p = .1633$). The y axis measurement illustrations were inspired by Zanier et al. (2015), whose analysis we used. The data are displayed as a box and whiskers plot with the horizontal line at the mean and min-to-max values at the whiskers. To test significance, a 1-way analysis of variance was followed by an uncorrected Fisher least significant difference test. All values are compared with the control value. DPI = days post injury, WPI = weeks post injury. * $p < .05$, ** $p < .01$, *** $p < .005$, †† $p < .0005$, ††† $p < .0001$. Scale = 10 μm

suggests that the microglia become less ramified at 1 and 4 weeks but resume their “resting” state at 16WPI.

Solidity

After CCI, IBA1-reactive cells in the cortex display significantly higher solidity (area/convex area) compared with controls at 1WPI and 4WPI (Figure 5i). This alteration in solidity suggests that thickening and consolidation of processes and cell body increase, but return toward normal in the cerebral cortex over time after injury (16WPI).

Circularity

We calculated circularity as $4\pi \times (\text{area}/\text{perimeter}^2)$; it varies from 0 (linear polygon) to 1 (perfect circle). The IBA1-reactive cells show significantly higher circularity after CCI compared with

controls at 1WPI and 4WPI, but return to normal values at 16WPI (Figure 5j).

Aspect ratio

There is no significant effect of time on the aspect ratio measurement (major / minor axis of the best-fitted ellipse) of IBA1-reactive cells (Figure 5k).

Together, these quantitative measurements of changes in morphology demonstrate that microglia in the neocortex experience substantial alterations in shape after a mild CCI, which return to normal by 16WPI. For the most part, the changes reflect an overall thickening of the soma and processes of each cell, which present as significant differences in distinct measurements that include modifications in area, perimeter, Feret diameter, circularity, and solidity.

3.1.5 | Astrogliosis (GFAP Immunostaining)

Staining against GFAP in the same tissue as the microglia shows substantial immunoreactivity in the regions of cerebral cortex adjacent to the injury and also in the subcortical white matter (Figure 6). The staining increases initially in the adjacent regions of cerebral cortex and, over a period of weeks, extends into the subcortical white matter and internal capsule while diminishing only slightly in the neocortex.

3.1.6 | Percent area labeled - GFAP

Our density analysis of GFAP immunoreactivity in the cerebral cortex just medial to the injury reveals that all survival time points have a higher astrocyte density than the control, peaking at 4WPI and remaining significantly increased until 16WPI, the last time point assessed (Figure 7a). In the subcortical white matter, the density of GFAP immunoreactivity increases with time after the injury, with significant differences from control at 4WPI and 16WPI (Figure 7b). Within the white matter near the internal capsule, the

overall density analysis does not show significance, although there is a trend toward increased immunoreactivity over time after injury (Figure 7c).

This analysis shows that astroglial reactivity increases with survival after a mild CCI. In contrast to the microglial reactivity, the increase in astrogliosis remains significantly high in the neocortex and subcortical white matter at 16WPI but does not reach significance in the deeper parts of the white matter.

3.2 | Behavior

3.2.1 | Gait

There was no significant effect of injury on the gait measurements.

3.2.2 | Activity level—total distance traveled

The track plot illustrates that the ferrets maintain activity levels after injury and access all parts of the open field apparatus (Figure 8e). Overall

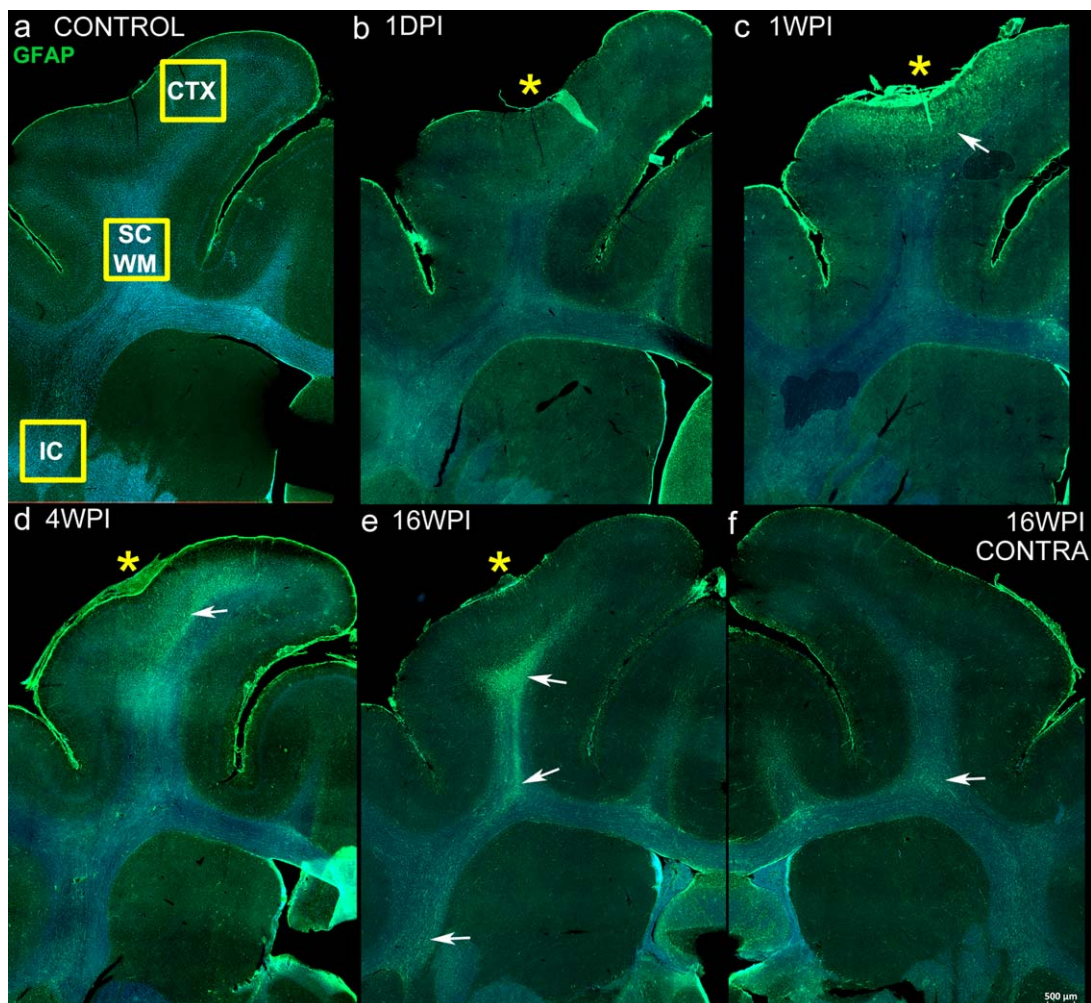


FIGURE 6 Spatiotemporal localization of glial fibrillary acid protein (GFAP-green)-positive cells in the ferret brain following mild controlled cortical impact (CCI) injury of the somatosensory cortex. (a) Representative GFAP immunostaining in a control and in the injured hemisphere of ferrets at different time points following CCI: 1 DPI (b), 1WPI (c), 4WPI (d), and 16WPI (e). Nuclei were counterstained with DAPI (blue). (f) Immunoreactivity in the contralateral hemisphere at 16WPI. The yellow asterisk indicates the site of CCI. The white arrows point to strong astrocytic reactivity. The yellow boxes indicate the regions of interest used for analysis: CTX, neocortex just medial to the injury; SCWM, subcortical white matter; IC, just superficial to the internal capsule. DPI = days post injury, WPI = weeks post injury. Scale = 500 μ m

activity level displays a disparity from normal and is most affected in the injured group at 12WPI compared with controls (Figure 8a).

3.2.3 | Average speed

The injured group shows significantly slower average speed than the control group at 12WPI, but there are no differences at any of the other time points (Figure 8b).

3.2.4 | Maximum speed

There is no difference between any time point and PRE (measurements taken before the CCI) in the control group. No significant differences occur between the injured and control groups at any time point (Figure 8c).

3.2.5 | Rearing normalized to PRE time point

Significantly less rearing activity occurs in the injured group compared with the control group at 12WPI (Figure 8d). Within the injured group, the 12WPI and 16WPI time points are significantly less than the PRE time point (Figure 8d).

3.2.6 | Anxiety

We determined both the time spent and the distance traveled in the center zone as an indicator of anxiety. Track plots in Figure 8e exemplify the ferret's likelihood to traverse the center of the open field apparatus at each of the time points investigated after injury. For distance traveled in the center zone, there are no significant differences between the control and injured groups.

3.2.7 | T-maze—memory

Data in Figure 9a are normalized to the PRE injury condition. All data points in the injured group are less than they were in the PRE condition. There was a significant decrease in accuracy at 1DPI, 1WPI, and 4WPI compared with PRE (Figure 9a,b).

3.2.8 | NOR—memory

Data in Figure 9c are shown normalized to the PRE condition. All data points in the injured group are less than they were in the PRE condition. The retention index measured during the novel object test showed significant memory impairment at 4WPI compared with the PRE time point (Figure 9c,d). The top row in Figure 9d shows the occupancy plots during the familiarization and test phase of the NOR test. The plots showing the TEST phase reveal the animal spending more time near the original object at 4WPI.

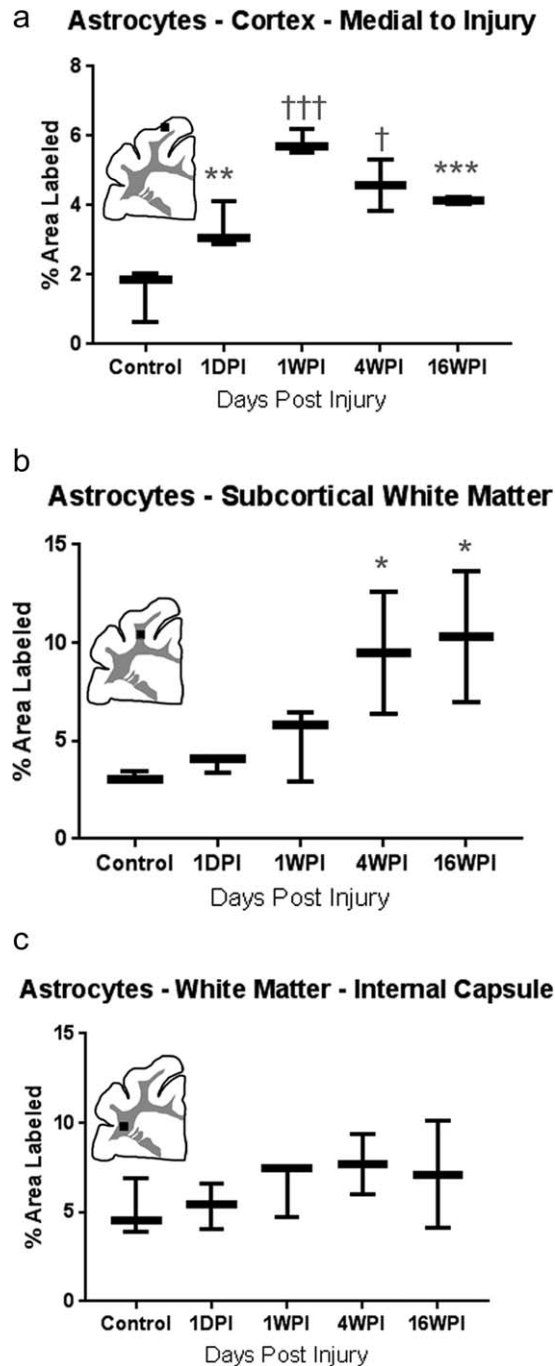


FIGURE 7.

FIGURE 7 Region of interest (ROI) density analysis of astrocytic immunoreactivity longitudinally following mild controlled cortical impact in the somatosensory cortex of the ferret. (a) The percent area labeled by GFAP in an ROI in the neocortex medial to the injury. Analysis of variance (ANOVA) showed significance over time ($F[4,8] = 17.3$, $p = .0005$). The 1DPI, 1WPI, 4WPI, and 16WPI groups all had significantly greater percent area labeled than the naïve control group. (b) In the subcortical white matter, there was a significant effect of time after injury ($F[4,8] = 4.153$, $p = .0413$). The percent area labeled at 4WPI and 16WPI increased significantly when compared with control values. (c) In the white matter just superficial to the internal capsule, there was not a significant effect of time after injury ($F[4,8] = 0.797$, $p = .5594$), although there was a trend toward increased density at all time points. The ROIs are depicted as yellow squares in the brain slice illustration (Figure 6). For each graph, the horizontal bars represent the median across $n = 3$ animals, and the whiskers indicate the highest and lowest values ($n = 2$ for 4WPI and 16WPI; values from 3 slices in each animal were averaged). To test significance, we conducted an ANOVA followed by a Fisher post hoc comparison test. * $p < .05$, ** $p < .01$, *** $p < .005$, † $p < .001$, ††† $p < .0001$

3.2.9 | Curiosity

We measured curiosity as the amount of time spent investigating either object during the familiarization phase of the NOR test (view the occupancy plots on the top row during the familiarization phase in Figure 9d). Over time, the animals show significantly less curiosity in the familiarization phase of the test. This is true, however, for both the control and injured animals and is possibly related to the interest level of the objects (see Table 1), or it could be due to habituation to the task protocol.

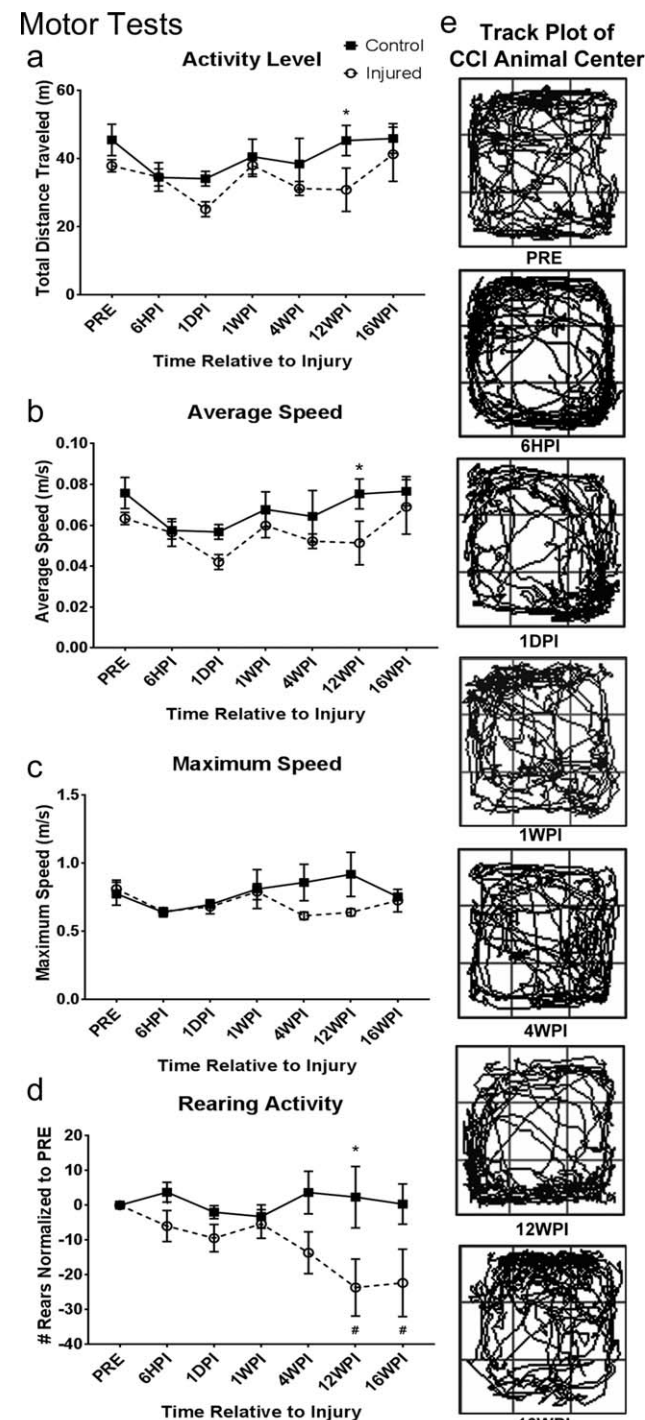


FIGURE 8.

3.2.10 | Attention span

Each time the ferret investigated an object in the familiarization phase of the NOR test, we measured the amount of time spent continuously investigating. The average amount of time spent within those periods is measured as the attention span. Object investigation was frequently stopped to switch to investigating the other object, to explore the testing apparatus, due to external noises. The ferrets would switch between investigating the two objects, and smell along the floor and rear along the walls and noises would distract them. There was no significant difference between the injured and control groups at any time point, although this measure also diminished over time.

4 | DISCUSSION

4.1 | Overview of results

Focal brain injury in the ferret generates persistent changes in microglial and astrocytic immunoreactivity. The density of both markers increase in ROIs compared with control levels (cerebral cortex adjacent to the injury, the subcortical white matter, and the deeper white matter, just superficial to the internal capsule). The astrocytic and microglial reactivity diverge in that the density of the astrocytes remains increased in the cerebral cortex and subcortical white matter up until 16WPI, but does not reach a significant increase in density in the deep white matter, whereas the microglial density remains increased in the deeper white matter but reduced in the neocortex at 16WPI. The number of microglial clusters in the white matter also increase and extend further from the site of injury with time. A transient memory

FIGURE 8 Motor impairment after mild controlled cortical impact (CCI) in the ferret. With a 10-min open field test, we measured activity level (total distance traveled) (a), the average (b) and maximum speed (c), and the number of rears (d) longitudinally after CCI or in control ferrets. We observed a significant decrease in activity level (a) compared with the control group at 12WPI. The average speed was also significantly lower than the control group at 12WPI (b). The maximum speed did not differ between the two groups at any time point. The rearing activity decreased in the injured group compared with the control group at 12 and 16WPI (d). (e) Example of the track plot of the animal's center within the open field at each time point after the injury; center crossing can be observed. Animals were removed from the study at various time points; therefore, the *n* at each time point is different: Control = 3, PRE = 14, 6HPI = 14, 1DPI = 14, 1WPI = 11, 4WPI = 7, 12WPI = 3, 16WPI = 3 ferrets. A mixed-effects model within SPSS was used to test significance. Main effect of time after injury: activity level ($F[6,58] = 3.33, p = .007$), average speed ($F[6,58] = 2.82, p = .018$), maximum speed ($F[6,60] = 1.49, p = .194$), rearing activity ($F[5,44] = 0.84, p = .528$). Main effect of condition: activity level ($F[1,14] = 4.16, p = .060$), average speed ($F[1,13] = 4.84, p = .047$), maximum speed ($F[1,13] = 1.50, p = .242$), rearing activity ($F[1,15] = 5.45, p = .034$). Interaction effect: activity level ($F[6,58] = 1.15, p = .348$), average speed ($F[6,58] = 0.69, p = .655$), maximum speed ($F[6,60] = 1.09, p = .374$), rearing activity ($F[5,44] = 1.12, p = .364$). * $p < .05$, comparison between groups. # $p \leq .05$, comparison with PRE within group

Cognitive Tests

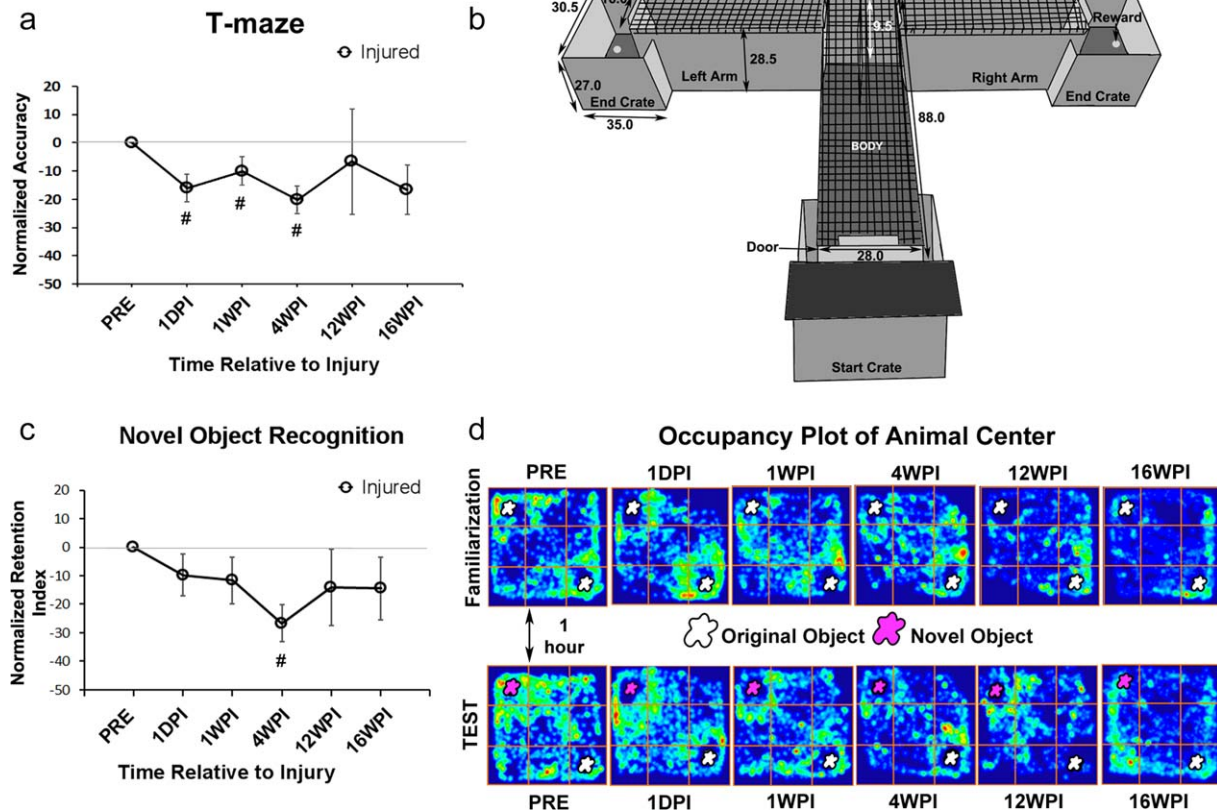


FIGURE 9 Cognitive impairment after mild controlled cortical impact (CCI) in the ferret. For this analysis we compared each ferret with his performance before the CCI (PRE). (a) Within-group analysis showed that T-maze accuracy was impaired at 1DPI, 1WPI, and 4WPI in the injured group compared with PRE. Data are normalized to each animal's PRE time point accuracy. (b) Schematic of the T-maze used for ferrets. Measurements are in centimeters. (c) Within-group comparison showed significant memory impairment on the Novel Object Recognition Test at 4WPI compared with PRE. The retention index = time spent with the novel object / (time spent with the novel + time spent with the original object) * 100. Data are normalized to each animal's PRE time point retention index. (d) Examples of the occupancy plot of the animal's center either during the familiarization phase (2 original objects) or during the test phase at each time point after injury (1 original object and 1 novel object). The warmer colors indicate more time spent in the location. The area with the most activity within a plot is red, but the amount of time spent at the red area varies across plots. Note: this plot indicates the location of the animal center, not the amount of time spent investigating the object; often, the animal would lie next to the object but not investigate it, and therefore, the plot does not directly correspond with the graph in panel c. The location of the novel object varied between the top and bottom positions during testing, but is shown here in the same location for ease of comparison. Animals were removed from the study at various time points; therefore, the *n* at each time point is different for the graphs: Control = 3, PRE = 14, 1DPI = 14, 1WPI = 11, 4WPI = 7, 12WPI = 3, 16WPI = 3 ferrets. A mixed-effects model within SPSS was used to test significance. DPI = days post injury, WPI = weeks post injury. #*p* < .05

impairment occurred at 4 weeks, and rearing activity decreased at 12 and 16 weeks after CCI.

4.2 | Microglial reactivity

We report an increase in microglial density at 1DPI in the cerebral cortex, which correlates with other studies showing substantial inflammatory changes within 24 hr after an injury (e.g., Loane & Kumar, 2016, for review). We also found significant changes in microglial morphology in the cerebral cortex at the 1DPI to 4WPI postinjury time points. With more time, the microglia increase in density in deeper areas of the white matter. In humans, reactive microglia persist up to 17 years after a TBI (Gentleman et al., 2004; Johnson et al., 2013; Ramlackhansingh et al., 2011). In the subacute phase after TBI (2 weeks–1 year), activated microglia with hypertrophic morphology also increase in density

and cluster in the corpus callosum in human pathology (Johnson et al., 2013). Smith et al. (2013) also report an upregulation of microglia after TBI peaking at 3 months, which is most pronounced in the white matter. The current study replicates clinical observations of delayed but persistent microglial activation in the white matter after brain injury.

4.3 | Morphologic changes of microglial reactivity in white matter

In a mouse model of concussive brain injury, the microglial reaction is also transient in the cortex, peaking at 1 week but persisting in the white matter up to 7 weeks (Shitaka et al., 2011). The microglial reactivity appears to be closely related to axonal damage as observed with silver staining. Delayed microglia/macrophage reactivity in white matter also appears in relation to vascular damage and blood-brain barrier

breakdown after CCI in rats (Glushakova, Johnson, & Hayes, 2014). We found alterations of microglial morphology in the neocortex that returned toward “resting” morphology by 16 weeks after the injury. In the subcortical and deep white matter, however, microglia remained highly reactive at this late time period. This agrees with several studies of TBI in humans, where microglia do not show immediate increases in the white matter but, instead, demonstrate long-term changes in density persisting from ~2 weeks to a year post injury (Johnson et al., 2013; Smith et al., 2013). The morphology of the microglia did not become amoeboid in the animals we studied here with a mild CCI, although in our previous study that included a more severe injury, the IBA1 immunoreactivity exhibited amoeboid morphology (Schwerin et al., 2017). In the earlier study, we also observed microglia with a rodlike morphology after severe injury. These findings, along with other reports, indicate that the morphological changes observed in microglia after injury differ in response to the character and quality of the injury (Loane & Kumar, 2016).

Chronic microglial activation in white matter may relate to blood-brain barrier breakdown or demyelination post TBI, which would consequently generate myelin debris to be phagocytosed. Microglial phagocytosis occurs in multiple sclerosis, TBI, and other brain injuries (Fu et al., 2014; Huizinga et al., 2012; Lloyd, Davies, & Miron, 2017; Ruying et al., 2014; Sullivan & Armstrong, 2017). It is possible that the dense regions of microglia in our study are in the process of engulfing cells and removing debris (Figures 3 and 4, Video 1).

It is likely that these distinctions in morphology have functional correlates, although our study is limited in this regard. The behavioral results show mild changes in memory and cognition (T-maze and NOR) at 4WPI, a time when both astrocytes and microglia significantly increase their density in the neocortex, and both markers also show increases in the white matter close to the cerebral cortex. This is also the time point when microglia show a high degree of morphological plasticity, which then return toward the “resting” phenotype. It is tempting to consider that the relatively small changes we see in cognitive behavior (i.e., the T-maze and NOR) relate to the presence of reactive microglia, as they both peak at a coincident time point. There are also significant increases in both astrocytes and microglia in the subcortical white matter, which also may influence activity of the cerebral cortex. The timing we observed in the IBA1 immunoreactivity and coinciding behavioral impairments may relate to changes in myelination. Because of the large amount of white matter in the ferret brain compared with rodents, the ferret model of TBI should be integral for continued investigation of the spatiotemporal time course of inflammation as well as the underlying mechanisms.

4.4 | Microglial clusters

Microglial clusters in humans were reported after TBI in 1974 and believed to be related to stretched and damaged axons (Clark, 1974). While axonal swellings related to damage disappear, aggregates of microglia remain scattered throughout the white matter (Whitwell, 2005). Johnson et al. (2013) reported microglial clusters in the corpus callosum of human brain tissue within the first year after TBI. This is similar to the microglial clusters we report at 4 and 16 weeks after the injury that extend distally over time.

Several imaging studies in humans describe a decrease in fractional anisotropy (FA) in the white matter following TBI. This decrease is associated with cognitive impairments and disruptions in white matter integrity after injury (Arenth, Russell, Scanlon, Kessler, & Ricker, 2014; Niogi et al., 2008; Spitz, Maller, O'Sullivan, & Ponsford, 2013). The microglial clusters we observed in this study could be one of the underlying factors affecting FA after TBI. In the white matter, we observed microglia clustering around blood vessels and elsewhere, but have not yet used additional markers to detect vascular damage or axonal damage including demyelination. The microglia may be attracted to invasive cells from the peripheral circulation, reported in injured human brain tissue; however, further investigation is needed to determine whether this is the case (Beschoner et al., 2002).

4.5 | Astrocytic reactivity

In a postmortem study of 104 individuals with closed-head injury surviving up to 30 weeks, a proliferation of GFAP⁺ astroglial cells was measured in the cortex adjacent to the brain injury from 1DPI to 4WPI (Hausmann, Riess, Fiegluth, & Betz, 2000). In the ferret, we also observed a significant increase in astrocyte density in adjacent cortex between 1 day and 4 weeks, which additionally extended to our latest investigated time point of 16 weeks. It is also interesting that the distributions of astrocytes and microglia presented differently. Both astrocytes and microglia increase in density quickly in the cerebral cortex post TBI and then both reduce in intensity by 16WPI, but the astrocytes remain significantly elevated at 16WPI, while the neocortical microglia are reduced. In the subcortical white matter, the microglial increase in density is delayed compared with the cortex ROI, becoming significantly elevated at 1WPI, and the astrocyte density only becomes significant at 4WPI, but both remain significantly elevated at 16WPI. Farther from the injury, in the white matter near the internal capsule, there is a delayed, but significant, increase in microglial density that is sustained until 16WPI, whereas the astrocytic density increases but is not significantly different from control levels. It is also possible that these changes influence the behavior we observed—specifically, rearing—where differences from control values occur later in our observations.

The immune response is different between the sexes and can affect the pathogenesis of diseases (Klein, 2012). One example is that the microglia in females are more activated than those in males; therefore, we might expect differences in the results if we studied female animals (Nissen, 2017). In addition, gender differences in terms of symptoms and disability have been shown between men and women with mild TBI (Styrke, Sojka, Bjoernstig, Bylund, & Stålnacke, 2013). In this study, we chose male ferrets for this study to more closely match the TBI population, which is predominantly male.

4.6 | Behavioral impairments and relation to clinical studies

Humans exhibit memory impairments after mild TBI (Bazarian, Cernak, Noble-Haeusslein, Potolicchio, & Temkin, 2009; Riggio, 2010). In the ferret, we did not detect impairments early after injury in tests that we modified from those commonly used in rodents. Mild impairments in

memory transiently reached significance at 4 weeks and motor ability at 12 weeks. It is possible that the sensitivity of the tests was not great enough to detect further impairment. In both memory tests (T-maze, NOR), greater retention time could magnify the effects. Nevertheless, the time after injury when we observed behavioral impairments coincided with the point at which the greatest immunohistochemical changes for reactive gliosis occurred in the white matter.

Many human survivors develop depression after mild TBI (Bazarian et al., 2009; Riggio, 2010; Scholten et al., 2016). The Patient Health Questionnaire (PHQ-9) is often used to monitor depression severity (Kroenke, Spitzer, & Williams, 2001). One of the questions involves the person's interest or pleasure in doing things, while another ranks the level of difficulty in concentrating. While we did not test depression directly in the ferrets, the curiosity measure can be used as an indicator of interest. In this study, the ferrets with TBI interacted with the objects to a similar degree as the control animals, suggesting no loss of interest. The reduction in rearing in the TBI animals at later time points could be motor related (although the other motor tests were not impaired) or could be related to curiosity (although the animals were not impaired on curiosity compared with controls as measured in the novel object test), or it could be a sign of behavioral despair, in that they will give up more quickly in trying to get out of the open field box. Depressed animals can exhibit futility (e.g., Crawley, 1999; Porsolt, Anton, Blavet, & Jalfre, 1978). Results regarding behavioral despair in mice/rats after TBI using the more aversive swim test or tail suspension test, however, are inconclusive or negative (Malkesman, Tucker, Oz, & McCabe, 2013; Tucker, Burke, Fu, & McCabe, 2017). The open field test would not be called aversive, so the reduction in rearing may be related to something else. Our measurement of attention span during the novel object test is arguably similar to the ability to concentrate, which is measured on the PHQ-9 in people. We did not observe impairment in attention span using this measure after mild brain injury in the ferret.

Humans exhibit anxiety after mild TBI (Bazarian et al., 2009; Riggio, 2010; Vaishnavi, Rao, & Fann, 2009). For an indicator of anxiety, we used the time spent/distance traveled in the center of the open field apparatus. This has commonly been used in rodent studies because rodents do not like bright open spaces (Crawley, 1999). Although not definitive, ferrets are crepuscular and like to burrow in small spaces and tunnels, so it is likely that they do not prefer bright open spaces (Fox, 2014). Also, we used a rodent-sized apparatus, which may not be large enough to test for dislike of the center zone in ferrets. In addition, anxiety measurements in mice/rats after TBI using the open field test are to date inconclusive (Malkesman et al., 2013). Mice decrease their traverse of the center distance traveled in severe, but not mild, injury (Tucker et al., 2017); our animals more likely fall into the mild injury category.

5 | SUMMARY

CCI in the ferret revealed a progression of inflammation that originates in the neocortex near the site of the injury and progresses deep into the white matter with time after injury. Memory impairments coincided

with the greatest degree of inflammation in the cortex, and motor impairments coincided with progression of microglial clustering in the white matter. This gyrencephalic animal model provides opportunities to explore potential therapeutic drugs and to address ongoing damage in the white matter of the brain after TBI.

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CONFLICT OF INTEREST

I have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Conceptualization, S.L.J., S.C.S., C.P. and E.B.H.; Methodology, S.C.S. and S.L.J.; Formal Analysis, S.C.S., S.L.J.; Investigation, S.C.S., M.C., A.O.I., K.L.R. and E.B.H.; Writing - Original Draft, S.C.S. and S.L.J.; Writing - Review & Editing, S.C.S., S.L.J., E.B.H., C.P., and K.R.; Visualization, S.C.S. and S.L.J.; Supervision, S.L.J. and C.P.; Project Administration, S.C.S.; Funding Acquisition, S.L.J. and C.P.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

VIDEO 1 Z-stack video of a microglial cluster in the white matter running between gyri in a coronal slice taken at the injury epicenter, 1 week after mild controlled cortical injury in the somatosensory cortex of the ferret. Red = IBA1 immunofluorescent labeling. Blue = DAPI staining of nuclei. Scale = 10 μ m.

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