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# Subconcussive Preconditioning Modifies Acute Microglial Response and Improves Cognitive Outcomes Post-Concussion

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**Abstract**

Subconcussive impacts are highly prevalent in contact sports and are thought to increase concussion risk. However, the specific conditions under which these subconcussive impacts influence concussion outcomes are uncertain, limiting our understanding of the mechanisms behind repetitive head trauma. Given that subconcussive impacts elicit a microglial response, we examined how subconcussive preconditioning affects microglial morphology and transcriptomic profile, as well as cognitive outcome after concussion. To investigate this question, we developed and validated a scalable, closed-head controlled cortical impact model. Using this approach, we found that although concussion elicited morphological features of hyper-surveillant microglia at 1 day post-injury, they resolved by 9 days post-injury, and subconcussive impacts only produced microglial changes at 9 days post-injury. When subconcussive impacts preceded a concussive impact (i.e., preconditioned concussion), no changes in microglial morphology appeared at either 1 day or 9 days after injury. Subconcussive preconditioning also upregulated microglial genes associated with inhibiting pro-inflammatory pathways and enhancing folate metabolism. Interestingly, subconcussive preconditioning eliminated concussion-associated cognitive deficits in novel object recognition and this cognitive protection was time dependent: preconditioning impacts were only protective if delivered within 2 min of concussion and had no effect if delivered over a 48-h window. These results suggest that some types of subconcussive impacts may offer protection against subsequent concussion and mitigate changes in microglial morphology and inflammatory responses. Understanding this timing window could inform strategies for minimizing cognitive impairments in athletes exposed to repetitive head trauma.

**Keywords:** cognition; microglia; subconcussive preconditioning

## Introduction

An estimated 1.6–3.8 million annual concussions occur due to sports- and recreation-related activities.[1] Subconcussive head impacts, defined as impacts that do not produce immediate neurocognitive deficits, can affect the likelihood of sports-related concussion.[2–7] Although there is a consensus that the hundreds to thousands of subconcussive head impacts that a contact sports player may experience over a season are detrimental to long-term brain health,[8,9] it remains unclear how these repeated exposures affect concussion outcome.[5–7,10,11] One challenge in human studies is uncontrolled variability amongst subjects, including unmeasurable head impact history, genetic predisposition, and psychological factors, which collectively impede our precise characterization of how subconcussive impacts influence concussion likelihood.[12–15] A second

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challenge is that these human studies preclude a mechanistic understanding of the brain's cellular response to repeated exposures, limiting insights that could inform more effective concussion treatments.

Rodent studies, however, afford greater experimental control and can be used to identify relevant impact features and corresponding cellular processes. In particular, rodent studies show that the number and timing of impacts are key modifiers of outcome within the first month post-injury.[16–26] Although mice can sustain a few *subconcussive* impacts and remain cognitively and histologically normal, increasing the number of subconcussive impacts, especially over a long period of time, increases the likelihood of concussion-like outcomes, including microgliosis and white matter degeneration.[16–25] Past studies show that repeated concussions are consistently damaging, regardless of the number of impacts, but increasing the time between first and final impact lessens their cumulative severity, especially with regard to microgliosis, astrogliosis, and axonal damage.[26] Taken together, these studies suggest that a large number of subconcussive impacts, delivered over a long period of time, will likely worsen concussion outcomes, whereas delivering fewer subconcussive impacts over a long period of time prior to concussion may reduce their total burden. Consistent with this notion, Harper and colleagues found that a small number of subconcussive blast injuries delivered over 1–2 days protected against a concussive blast via the kynurenine pathway, which links the nervous and immune systems.[27]

Microglia are often studied as potential mediators of repeated concussive and subconcussive impacts, perhaps because of their ability to rapidly activate several innate immune responses in the brain. Both single and repetitive subconcussive impacts can induce a microglial response,[19,24,28] even in the absence of behavioral deficits.[28] Microglia benefit injury recovery by migrating to the injury site, clearing debris, and coordinating a multicellular injury response through the release of inflammatory molecules (e.g., cytokines). However, persistent microglial activation can be detrimental to injury recovery, leading to neurotoxicity, chronic neuroinflammation, and aberrant synaptic remodeling.[29] Repetitive microglial stimulation is similarly multifaceted: depending on the stimulus nature, microglia can exhibit a stronger or weaker reaction as part of innate immune memory,[30,31] and studies using lipopolysaccharide (LPS) as a stimulus suggest that dose strength, duration, timing, and sequence are critical factors.[30,32] Indeed, preconditioning with LPS can be neuroprotective not only for traumatic brain injury (TBI)[33–35] but also ischemic brain injury[36] and epilepsy.[37–40] Preconditioning using other methods, such as transient ischemia or subthreshold cortical stimulation, can also be neuroprotective against ischemic brain injury and epilepsy and may involve microglia-dependent mechanisms.[41–44]

In the present study, we hypothesized that subconcussive impacts delivered prior to a concussion modify the microglial response and, in parallel, prevent the cognitive deficits that normally occur after a concussive impact. Using a novel closed-head controlled cortical impact model, we investigate whether microglia are sensitive to impact loading conditions, focusing on morphology, phagocytic activity, and RNA expression. We investigate whether brief subconcussive preconditioning desensitizes the microglial response to concussion and whether the timing of subconcussive preconditioning, either delivered over minutes or days, affects preconditioning's ability to modify cognitive outcome postconcussion. This work contributes to our understanding of the complex interplay between subconcussive impacts, microglial activation, and cognitive function, with implications for concussion prevention and treatment in sports and other high-risk activities.

## Methods

### Animals

We used adult male C57BL/6 mice (8–12 weeks old; Charles River Laboratories) housed in an animal care facility with a 12-h light/12-h dark cycle and *ad libitum* access to food and water. Upon arrival, animals were fed Lab Diet 5001 (Animal Specialties and Provisions). All animal procedures were approved by the University of Pennsylvania's Institutional Animal Care and Use Committee (IACUC).

### Experimental design

**Closed-head controlled cortical impact.** To assess how subconcussive impacts affect concussion outcomes, we sought an injury model that would address some of the challenges in interpretability experienced by human studies. Heterogeneity in the impact number, location, and timing, as well as low concussion incidence, have made it difficult to precisely define how subconcussive impacts affect concussion risk in humans.[12] To mimic the head impacts experienced by humans, we sought a closed-head impact model. However, no existing model enables consistent control of the impact site and severity for a lateral closed-head impact. This control is necessary to ensure preconditioning impacts and subsequent injury in the exact same site and to enable comparison between the ipsilateral and contralateral brain hemispheres. These requirements thus exclude traditional controlled cortical impact,[45] weight drop,[46] and closed-head impact model of engineered rotational acceleration.[47]

To overcome the shortcomings of existing models for our study's design, we developed a scalable closed-head impact method to deliver concussive and subconcussive impacts to one hemisphere (Fig. 1A). We outfitted the

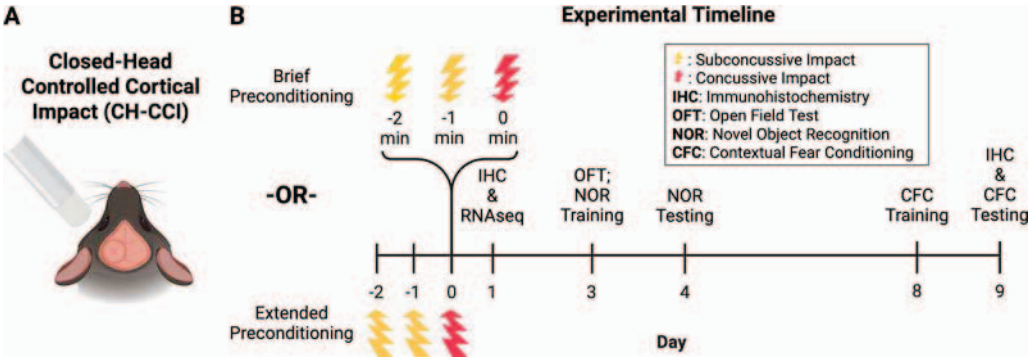



**FIG. 1.** Closed-head controlled cortical impact for subconcussive preconditioning. **(A)** Rubber tip is used to impact the exposed left parietal bone of the animal's skull. **(B)** Experimental timeline for impacts, behavioral assessment, and sample collection. Image created using BioRender.

Impact One Controlled Cortical Impact Device (Leica Biosystems) with a custom 6-mm diameter rubber tip (Model #E10BP-K6, Pentel). Prior work suggests that impact severity scales with velocity, and helmeted impact velocity scales between humans and rodents.[48] For concussive impacts, we injured the mice using an impact velocity of 3.5 m/sec, which we confirmed in pilot studies to produce behavioral deficits in novel object recognition (NOR) and contextual fear conditioning (CFC; $N = 8$ animals/condition). A 3 m/sec impact velocity produced unreliable behavioral deficits, whereas 4 m/sec increased skull fracture likelihood. Having selected the 3.5 m/sec impact speed for concussion, we then scaled for subconcussion, reducing the impact speed to 1.0 m/sec, consistent with reports of subconcussive impacts occurring in humans at 25–33% of the magnitude of concussive impacts.[49,50]

Prior to surgery, we induced anesthesia with 3.5% isoflurane and maintained it with 1.5–2% isoflurane. We then made an approximately 10-mm incision above the midline of the skull and aligned the impactor over the central aspect of the left parietal bone (Fig. 1A). We set the dwell time to 30 ms and the impact depth to 1 mm to prepare for impact and delivered no impact (sham), a 1-m/sec impact (subconcussion), a 3.5-m/sec impact (concussion), or two 1-m/sec impacts followed by a 3.5-m/sec impact (preconditioned concussion). We sacrificed any mouse that experienced a skull fracture with bleeding.[51] We also examined the skulls at the end-point and excluded any mice with visibly unhealed skull fractures. Among animals that experienced a concussion or preconditioned concussion, ~30% exhibited signs of skull fracture. Subconcussive impact resulted in skull fracture in <5% of impacts. Unconditioned mice (sham, single subconcussion, single concussion) received anesthesia and incision equivalent to the corresponding preconditioned impact timing. Following the impact or sham procedure, we sutured the mouse's scalp closed and placed the mouse in a heated recovery cage until they exhibited normal signs of alertness and ambulation. Following recovery, animals were returned to the colony.

Brief versus extended impact window preconditioning. To determine the effect of the impact window on the cognitive outcome of subconcussive preconditioning, we evaluated two cumulative impact windows: 2 min (brief) and 48 h (extended). Prior works show that impact timing is a key factor influencing the severity of repetitive impacts in both humans and rodents.[6,7,26] We selected 3 total impacts in each window to minimize the detrimental effect of repetitive impacts[26] and to be consistent with prior work examining protective subthreshold preconditioning of concussion.[27] We impacted the mice in the same location each time to ensure a spatially localized effect (Fig. 1A). For brief preconditioning, we administered 2 subconcussive impacts followed by a concussive impact, each of which was spaced a minute apart. For extended preconditioning, we administered the same impact profile but spaced the impacts 24 h apart. For sham and unconditioned concussion for the extended window, we performed sham procedures 24 h and 48 h prior to the final sham or concussive procedure. Experimental Day 0 for both groups was the day of the final impact (Fig. 1B).[26] Animals were sacrificed for RNA sequencing ($N$ = 6/condition [brief]) or histology ($N$ = 3/condition [brief]) at 1-day post-injury (dpi). For behavioral assessment, animals underwent open field test (OFT) 3 dpi ($N$ = 26–31/condition [brief]; $N$ = 15–17/condition [extended]), NOR 3–4 dpi ($N$ = 18–37/condition [brief]; $N$ = 16–19/condition [extended]), and CFC 8–9 dpi ($N$ = 32–57/condition [brief]; $N$ = 16–27/condition [extended]). Following completion of behavioral testing 9 dpi, we sacrificed the animals for histology ($N$ = 3/condition [brief]).

## Neurocognitive assessment

In designing our battery of neurocognitive assessments, we focused on early learning and memory impairment, similar to our previously established timeline.[52] First, mice recovered from injury for 3 days. Then, we used OFT to evaluate for possible motor impairment or anxiety that could interfere with our interpretation of learning and memory deficits.[53,54] To assess learning and memory, we selected two complementary assessments, NOR and CFC, to track acute recovery over time. Because CFC stresses the mice, we administered it last to avoid complicating subsequent behavioral assessment.[55] Although Morris Water Maze is a common learning and memory assessment post-TBI, we did not anticipate a robust deficit in our time frame of interest, given the mildness of our injuries.[19,56,57]

Based on preliminary behavioral studies involving 8 animals/condition, we estimated an expected normalized difference of 0.25 between sham and concussion and coefficient of variation of 25%, necessitating 25 animals/condition for 80% power. However, when we ran the study, the normalized difference was 0.15, and we accordingly increased sample sizes to $\sim$35. For OFT, $N$ = 26–31/condition; for NOR, $N$ = 18–37/condition; and for CFC, $N$ = 32–57/condition. Mice were systematically assigned to an experimental condition. The experimenter conducting behavioral testing was not blind to the experimental condition, but the analyst was blinded to the experimental condition.

### Open field test.

At 3 dpi, we placed individual mice in an empty open field chamber (12" × 15") and allowed them to naturally explore for 15 min. We analyzed how much time the animal spent in the corner, outer, inner, and center areas, as well as total ambulation, using previously described automated methods.[58] OFT allows us to evaluate for motor deficits and anxiety behavior as anxious mice engage in thigmotaxis to avoid open spaces.

### Novel object recognition.

At 3 dpi, we habituated the animals to the open field chamber for 15 min as described in the "Open Field Test" section. We then lightly cleaned the chamber to remove odor cues. Next, during training, we placed two identical objects in the open field and placed the same mouse in the chamber for two 10-min trials to familiarize themselves with the objects, lightly cleaning between trials as before. At 4 dpi, we replaced one of the familiar objects with a novel object in the same position. We placed the animal in the chamber to explore each object for a total of 5 min. We evaluated the time spent interacting with each object using previously described automated methods.[58] Because mice inherently prefer novel objects, increased interaction with the novel object indicates recollection of object familiarity. Novel object preference refers to the time spent interacting with the novel object divided by the total time spent interacting with either object. NOR was performed by the same experimenter during the light cycle under similar temperature and lighting conditions. We excluded mice with an interaction time with either object of less than 10 sec in total.[59]

### Contextual fear conditioning.

At 8 dpi, we placed the mouse in a foot shock chamber with sound attenuation for 3 min before delivering three shocks (0.4 mA) through the grid floor for a 2-sec duration, each 1 min apart. At 9 dpi, we returned mice to the test chamber and recorded their movement over a 3-min span. We removed odor cues with 10% bleach and then with 70% ethanol before each trial. An experimenter blinded to experimental conditions manually scored freezing behavior from the video footage, defining fraction freezing time as the total accumulated freeze time over the total recording time. CFC was performed by the same experimenter for all animals during the light cycle under similar temperature and lighting conditions. We excluded mice noted by the experimenter to exhibit fear and/or anxiety behaviors (including escape behaviors, increased defecation) prior to training.[60]

## Histology

### Perfusion and cryosectioning.

For immunohistochemistry, we euthanized mice (250 mg/kg sodium pentobarbital, i.p.), transcardially perfused with ice-cold 0.9% saline (100 mL) followed by 300 mL of 4% paraformaldehyde (PFA) before collecting brains in 4% PFA. After 24 h, we replaced the 4% PFA with 15–30% sucrose, and once the brain sank (24–48 h later), we replaced it with 30% sucrose again. We froze the brains in optimal cutting temperature compound and sectioned the brain in 20-$\mu$m coronal sections approximately −1.5 to −2 mm from bregma.

### Staining and fluorescence imaging.

We stained 3 nonconsecutive sections per brain for 3 brains per condition. We blocked sections with Fish Serum Blocking Buffer (Thermo Scientific). For primary antibodies, we incubated sections with rabbit anti-Iba1 (1:200; Wako Chemicals) or rabbit anti-amyloid precursor protein (APP) (1:200; Invitrogen) and rat anti-CD68 (1:100; Bio-Rad) for 1 h at room temperature and washed with 1× TBS with 0.1% Tween 20. Iba1 and CD68 are expressed by both microglia and infiltrating macrophages. For secondary antibodies, we incubated Alexa Fluor 488 goat anti-rabbit (1:200; Abcam) with Iba1-stained sections or Alexa Fluor 488 goat anti-rat (1:200; Abcam) and Alexa Fluor 555 goat anti-rabbit (1:200; Abcam) with APP- and CD68-stained sections for 30 min at room temperature and washed with 1× TBS with 0.1% Tween 20. We incubated Iba1-stained sections with NeuroTrace 640/660 Nissl Stain (1:30, Invitrogen), following

manufacturer's instructions to stain neurons. We mounted using ProLong Gold (Invitrogen). We imaged using a BZ-X800 Microscope (Keyence) at 20× magnification. We acquired images in a 10-$\mu$m $z$-stack with a 2-$\mu$m pitch and then stitched together the focused images using BZ-X800 Analyzer Software (Keyence).

Image processing and stereology. For each image, we used ImageJ to examine the ipsilateral cortex directly below the impact site (approximately 1.5 $mm^2$) and the ipsilateral hippocampus (Fig. 3M).

To evaluate impact severity, we estimated changes in neuronal density in the cortex and hippocampus at both 1 dpi and 9 dpi. First, we enhanced image contrast by equalizing the contrast histograms to account for variability in staining across sections, sharpened the image, identified regions of interest (ROIs), applied a fixed threshold, and reported the percent area of the ROI above threshold. We systematically excluded sections with high-background or low-staining efficiency in either hemisphere. We examined the fold change in the neuronal density between the ipsilateral and contralateral hemispheres to account for variability in staining across sections. Due to the density of neurons in the hippocampus, we selected this method rather than counting individual neurons.

To complement neuronal density assessment, we also examined axonal damage measured by APP staining at 1 dpi, when APP positivity typically peaks post-injury.[61] Due to the minimal degree of APP staining, we present representative images highlighting APP staining, which was primarily concentrated in the cortex and corpus callosum beneath the injury site.

To understand the morphological changes exhibited by microglia post-injury, we counted and traced microglia in the ipsilateral cortex and hippocampus at 1 dpi and 9 dpi. To prepare images for analysis, we applied a Fast Fourier Transform Bandpass Filter and adjusted image contrast by equalizing the histograms as before. Again, we systematically excluded sections with high background or blurriness, which precluded confident counting and tracing. A scorer blinded to the experimental condition manually counted the microglia in a given ROI. After manual counting, 30 microglia were randomly assigned for tracing from the total identified in a given region. For tracing, the scorer manually outlined the cell soma to assess soma size and traced its processes for Sholl analysis. Based on the traced soma, our software automatically identified the centroid and then drew concentric circles with radii increasing in 5-$\mu$m increments[62] for Sholl Analysis using ImageJ's Sholl Analysis Package.[63]

To complement the microglial morphological assessment, we examined CD68 as a marker of phagocytosis[64] in the cortex and hippocampus at 1 dpi. CD68 positivity can peak as early as 6 h post-injury in response to diffuse mild TBI,[65] and we elected to focus on this acute phagocytic activity. To estimate the density of CD68 staining, as with neuronal density, we enhanced image contrast by equalizing the contrast histograms to account for staining variability, sharpened the image, identified ROIs, applied a fixed threshold, and reported the percent area of the ROI above threshold. We systematically excluded sections with high background or low staining efficiency in either hemisphere. We examined the fold change in the CD68 positivity between the ipsilateral and contralateral hemispheres to account for variability in staining across sections. We selected this method because of the sparse density of CD68 staining and its membrane localization, which made it difficult to confidently associate CD68 positivity with DAPI staining of individual cells.

## CD11b+ RNA sequencing

CD11b+ cell isolation. To assess acute microglial responses to injury, we isolated CD11b+ cells from brain tissue 24 h post-injury from 6 animals/condition. Although CD11b+ cells in the brain are primarily microglia, they also include infiltrating monocytes/macrophages. Mice were lightly anesthetized with 3.5% isoflurane to facilitate handling before euthanizing with 250 mg/kg sodium pentobarbital i.p. Mice were transcardially perfused with ice-cold PBS (15 mL) to remove blood and circulating immune cells from the brain. The brain was quickly removed from the skull, and a 6-mm slice of brain tissue from the ipsilateral hemisphere directly below the site of injury was extracted. Brains were stored at 4°C in tissue storage solution (Miltenyi) for less than 48 h. Brain tissue was dissociated using the Neural Dissociation Kit (P) (Miltenyi) and the gentleMACS Octo Dissociator with Heaters (Miltenyi) according to manufacturer instructions. Myelin was removed using Debris Removal Solution (Miltenyi) according to manufacturer instructions, followed by additional myelin removal using an OptiPrep Gradient of 43.2% iodixanol layered below 9.6% iodixanol.[66] CD11b+ cells were isolated using CD11b Microbeads (Miltenyi), MACS Mini Separator (Miltenyi), and MS Column (Miltenyi) according to manufacturer instructions, with modifications: PB Buffer was prepared with 1% BSA rather than 0.5% BSA to prevent clumping and supplemented with 1 nM EDTA to mitigate RNase activity.[67] Cells were passed through the selection column twice to increase purity. Finally, EDTA was washed out with 1× PBS.[67]

RNA isolation and sequencing. We isolated RNA from CD11b+ cells with the RNeasy Mini Kit (Qiagen) and stored the RNA at −80°C until library preparation using the QIAseq FastSelect RNA Library Kit (Qiagen) for low-input RNA with rRNA removal. We sequenced the library using the NextSeq 1000/2000 P2 XLEAP-SBS

Reagent Kit (200 Cycles) (Illumina; Next-Generation Sequencing Core, University of Pennsylvania) and demultiplexed sequencing data using BCL Convert v4.2.7 (Illumina).

### Statistical analysis

Microglia and neuronal statistical analysis were performed using linear mixed-effects analysis in R v4.4.1 with Tukey's *post hoc* test to account for multiple microglia and histological sections from the same animal.[68] Behavioral statistical analysis was performed using one-way ANOVA (NOR, CFC) or two-way ANOVA (OFT) using Tukey's multiple comparisons method in GraphPad Prism v10. We verified the absence of statistical outliers using robust outlier detection test. Results are expressed as mean ± standard error of the mean of raw data.

Gene expression and canonical pathway analysis were performed using Qiagen's RNA Analysis Portal. We determined differentially expressed genes using the ANOVA-like likelihood ratio test for negative binomial generalized linear models, controlling for multiple comparisons using false discovery rate (FDR). We report protein-coding mRNAs with FDR adjusted $p$ values ≤0.05 and $\log_2$ fold change >2. These significant mRNAs were used to determine canonical pathways; we report up to 10 top pathways with $p$ values ≤0.05. Heat map data represent the $z$-score of the gene counts per million.

## Results

### Head injury model captures severity-dependent cognitive deficits and axonal damage

First, we evaluated the neurobehavioral and neuronal response to our injury model (outlined in Fig. 1). At our concussion impact level (3.5 m/sec, 1-mm depth), there was no change in exploratory behavior or ambulation in the open field at 3 dpi (Fig. 2A; Supplementary Fig. S1A), a significant deficit in NOR at 4 dpi (Fig. 2B), and no CFC deficit at 9 dpi (Fig. 2C). Using scaling principles described in the "Methods" section, our subconcussive impact (1 m/sec, 1-mm depth) led to no significant impairment in open field behavior (Fig. 2A), NOR (Fig. 2B), or CFC (Fig. 2C), consistent with its clinical definition. There was no loss in neuronal density at either 1 dpi or 9 dpi for any impact severity, including preconditioned concussion, in the ipsilateral cortex or hippocampus relative to the contralateral side (Fig. 3A–M). APP pathology indicative of axonal damage was limited at 1 dpi, with no staining for sham or subconcussive animals (Fig. 3N, O) and minor staining in the cortex and corpus callosum for concussion and preconditioned concussion (Fig. 3P, Q).

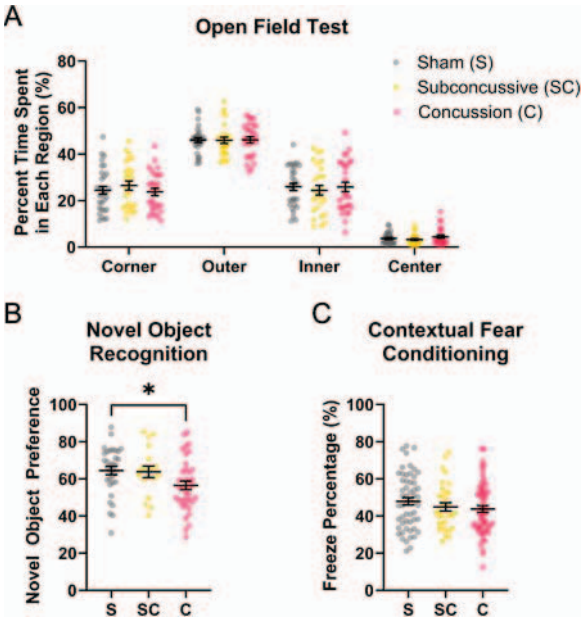



**FIG. 2.** Cognitive impairment scales with injury severity. **(A)** Neither subconcussive nor concussive impacts have a significant deficit in percent time spent in the corner, outer, inner, or center areas of the open field. **(B)** Concussion exhibits a significant deficit in novel object recognition. **(C)** Neither subconcussive nor concussive impacts have a significant effect on freeze percentage. *indicates $p < 0.05$. Statistical testing was performed using two-way ANOVA **(A)** or one-way ANOVA **(B, C)** with Tukey's *post-hoc* test. Data are shown as mean ± SEM. S: sham; SC: subconcussive; C: concussion. SEM, standard error of the mean.

### Impact severity and preconditioning modulate microglial ramification postinjury

Next, we characterized the microglial response to head impact, examining the microglial number, morphology, and expression of the phagocytic marker CD68 at 1 dpi to capture early changes (Fig. 4). At no injury level, there was a change in microglia density or soma size in the cortex or hippocampus (Fig. 4F–I). However, following concussion, microglia exhibited increased ramification (measured by Sholl Analysis) at 1 dpi in the hippocampus, indicating that they assumed features of a hyper-surveillant morphology (Fig. 4K). However, this hyper-ramification in the hippocampus was accompanied by no change in CD68 expression on average (Fig. 4Q), suggesting that the hyper-ramification was not related to phagocytic activity. Interestingly, subconcussive preconditioning had no effect on ramification, suggesting that

subconcussive impacts delivered over a brief 2-min impact window prevented the acute hyper-surveillant morphology observed in unconditioned concussion (Fig. 4K). Preconditioning also had no effect on CD68 expression, similar to single subconcussive and concussive impacts (Fig. 4P, Q).

We further examined microglial number and morphology at 9 dpi to measure more sustained changes (Fig. 5). Unlike 1 dpi, the microglial morphology after concussive impacts was no different from sham with regard to density, soma size, or ramification at 9 dpi (Fig. 5E–J). Preconditioning also had no effect on microglial density, soma size, or ramification at 9 dpi (Fig. 5E–J). Despite no changes in density or soma size, microglia from subconcussed brains exhibited increased ramification in the hippocampus at 9 dpi (Fig. 5E–J). Together, these findings suggest that although concussed mice experienced a transient early increase in microglial ramification in the hippocampus, subconcussive impacts exhibited a more delayed increase in ramification.

### Preconditioning enhances mRNA expression related to folate metabolism and immune suppression in microglia

To investigate the gene expression in microglia associated with acute morphological changes, we isolated CD11b+ cells at 1 dpi directly below the site of injury and performed RNA sequencing. Unexpectedly, among the three impact conditions, single subconcussive impacts significantly affected the largest number of microglial genes relative to sham (Fig. 6A; 1491 genes total) compared with concussion versus sham (Fig. 6B; 1170 genes total) and preconditioned concussion versus sham (Fig. 6C; 765 genes total). Across the three impact conditions, there was considerable overlap in upregulated, and especially downregulated, gene expression relative to sham (Fig. 6G). Indeed, 40% of significantly expressed subconcussive genes were also significantly expressed in microglia from at least one of the other two injury conditions (Supplementary Fig. S2A), with that gene expression overlap increasing to 48% shared for concussion genes (Supplementary Fig. S2B) and 46% for preconditioned concussion genes (Supplementary Fig. S2C).

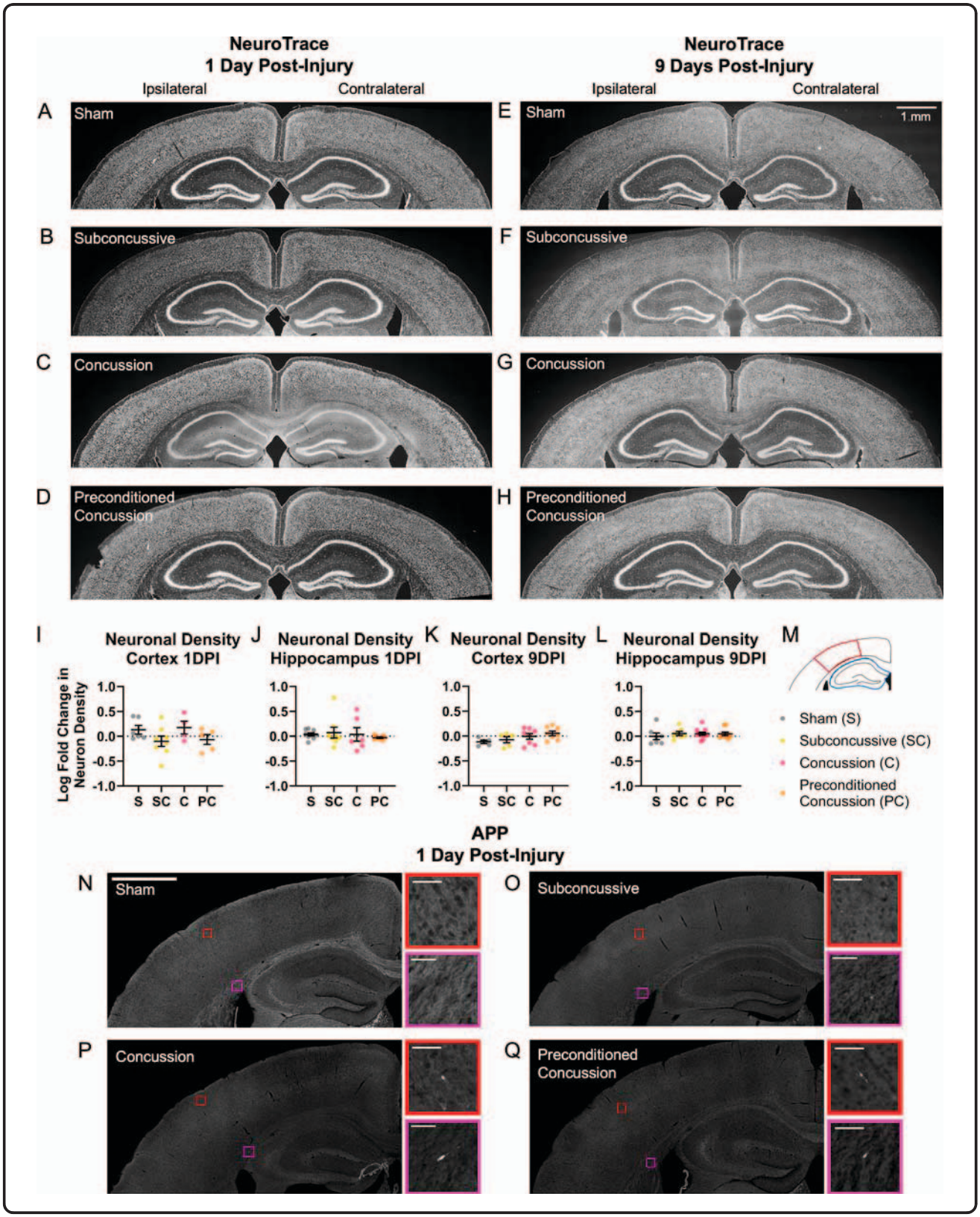
Broadly, all three impact conditions influenced pathways related to mitochondrial function, metabolism, and regulation of gene expression (Fig. 6D–F). In particular, pathway analysis revealed that subconcussive impacts especially affected microglial genes relating to mitochondrial function, metabolism, and gene regulation (Fig. 6D). Among the top 20 differentially expressed genes, several specifically related to mitochondria and metabolism (*Cox17*, *Adck5*, *Fastk*, *Aqp11*) and chromatin/transcription machinery (*Ssrp1*, *Snapc4*, *Rad51ap1*) (Fig. 6M). In contrast, pathway analysis for concussion alone especially implicated inositol triphosphate and NAD metabolism (Fig. 6E) and the top 20 genes related to the cytoskeleton (*Arpc1a*, *Mob2*, *Krt7)* and membrane trafficking (*Plekhj1*, *Tmem106a*, *Gja1*) (Fig. 6M). Preconditioned concussion especially affected pathways relating to cell cycle and development (Fig. 6F), and the top 20 genes included genes relating to mitochondria and metabolism (*Slc25a11*, *Akip1*, *Slc52a3*) (Fig. 6M). In general, this finding broadly aligns with our morphological and immunohistochemical findings: concussion uniquely affects cytoskeletal elements that modify cell morphology, and phagocytosis is not a dominant microglial function at 1 dpi regardless of severity for our closed-head injury model.

To differentiate among the impact conditions, we examined how subconcussive impacts and preconditioned concussion changed microglial gene expression relative to concussion, rather than sham. Subconcussive impacts were distinct from concussion primarily based on their effect on genes relating to mitochondrial function (Fig. 6I, K; 1297 genes total), whereas preconditioning affected genes primarily relating to folate metabolism and signaling (Fig. 6J, L; 698 genes total). Interestingly, preconditioning increased gene expression of several negative regulators of inflammatory response (*Tnip2*, *Relt*, *Lag3*) (Fig. 6M), suggesting that preconditioning could prevent concussion-associated inflammatory response.

### Brief preconditioning, but not extended preconditioning, prevents concussion-associated cognitive deficits

We next considered whether the timing of subconcussive exposures would affect the neurological impairments

**FIG. 3.** Head impacts do not affect neuronal density and produce modest axonal damage. Representative images of NeuroTrace staining for **(A–D)** 1 day post-injury (dpi) and **(E–H)** 9 dpi. **(I–L)** No change in ipsilateral versus contralateral neuronal density at 1 dpi in **(I)** cortex or **(J)** hippocampus or **(K, L)** 9 dpi. Statistical testing was performed using a linear mixed effects model followed by Tukey's *post-hoc* test. $\alpha = 0.05$. Raw data shown as mean ± SEM. Left is ipsilateral; right is contralateral. **(M)** Areas of interest are cortex (red) and hippocampus (blue). S: Sham; SC: subconcussive; C: concussion; PC: preconditioned concussion (Brief). **(N–Q)** Representative images of amyloid precursor protein (APP) staining at 1-day post-injury with insets in cortex (red) and corpus callosum (magenta) showing representative varicosities or their absence below the site of injury. Scale bar on full image is 1 mm. Scale bar on insets is 50 μm. SEM, standard error of the mean.




normally occurring in our closed-head impact model. In human studies, there is a clear record of subconcussive impacts preceding an impact causing concussion,[2–7] but their relative effect on cognitive outcomes is unknown. We found that both brief and extended preconditioning, like concussion, had no effect on exploration or ambulation in an open field at 3 dpi (Fig. 7A, D; Supplementary Fig. S1B, C). However, brief preconditioning reversed

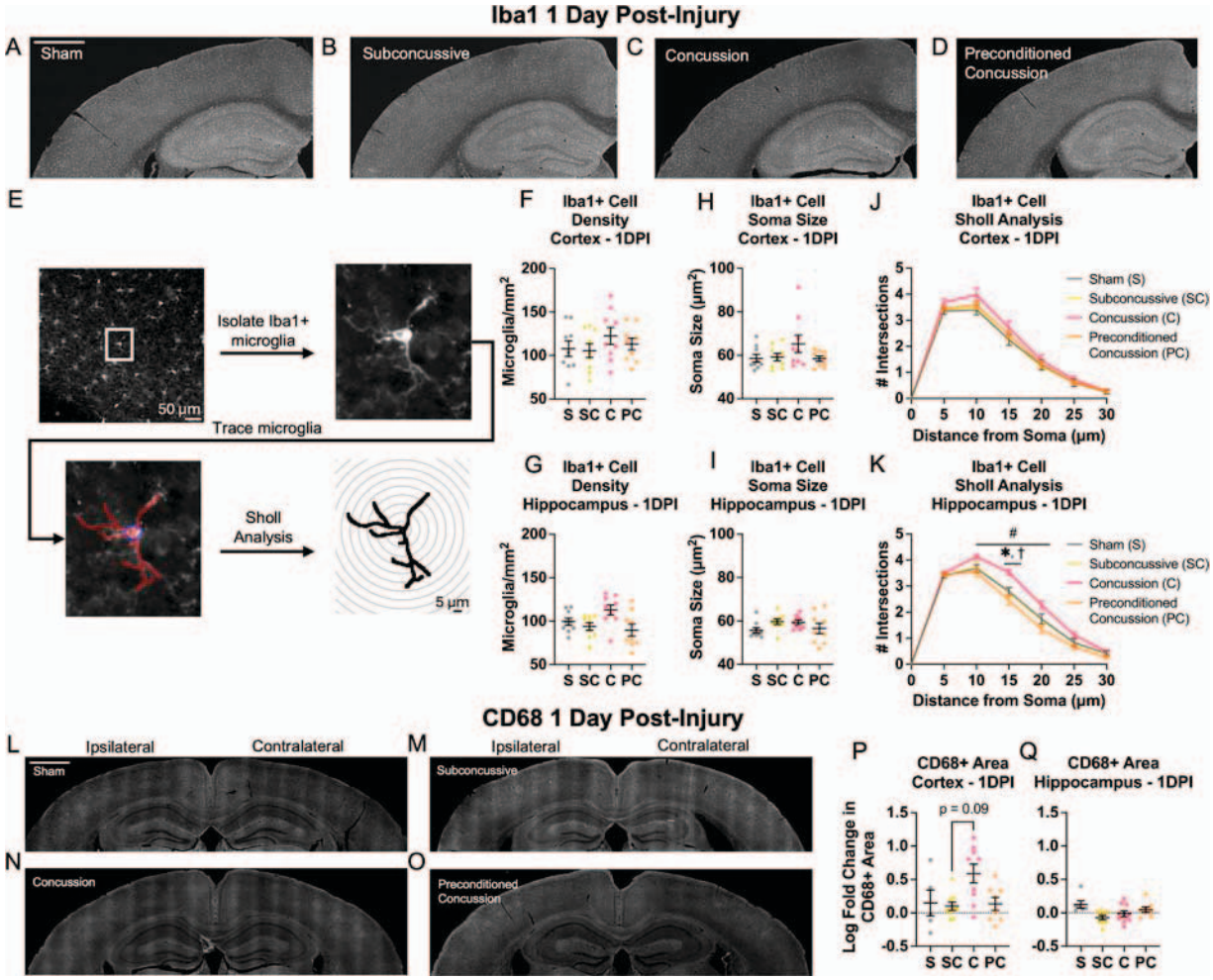



**FIG. 4.** Microglia exhibit acute changes in morphology in the hippocampus following unconditioned concussion. **(A–D)** Representative images of Iba1+ staining in ipsilateral cortex and hippocampus 1-day post-injury (dpi) **(A)** sham, **(B)** subconcussive impact, **(C)** concussion, and **(D)** preconditioned concussion (brief). Scale bar = 1 mm. **(E)** Workflow for tracing microglia soma (blue) and processes (red) for soma size and Sholl analysis. **(F, G)** Microglia density in **(F)** cortex and **(G)** hippocampus, **(H, I)** soma size, and **(J, K)** Sholl Analysis. *indicates $p < 0.05$ for concussion versus sham, †indicates $p < 0.05$ for concussion versus subconcussive impact. **(L–O)** Representative images for CD68+ staining in cortex and hippocampus 1 dpi. Scale bar = 1 mm. **(P, Q)** No change in ipsilateral versus contralateral CD68 staining density at 1 dpi in **(P)** cortex or **(Q)** hippocampus. All comparisons were performed using a linear mixed effects model with Tukey's *post-hoc* test. $\alpha = 0.05$. Raw data shown as mean ± SEM. S: sham; SC: subconcussive; C: concussion; PC: preconditioned concussion (Brief). SEM, standard error of the mean.

concussion-associated deficits in NOR at 3–4 dpi (Fig. 7B) and nearly significantly improved fear response relative to concussion at 8–9 dpi (Fig. 7C). These results suggest that brief preconditioning improved cognitive outcomes across several domains (memory, anxiety) in the acute (4 days) and subacute (9 days) post-injury phase (Fig. 1B). However, the rescue of cognitive deficits from preconditioning was related to the duration of the subconcussive response; extending the same two preconditioning impacts over 2 days, rather than 2 min, did not rescue concussion's NOR deficit (Fig. 7E) or fear conditioning (Fig. 7F).

## Discussion

In this study, we examined how microglia increase ramification and modify gene expression in response to different closed-head impacts and, in parallel, how different sequences of head impacts affect cognition. Consistent with past work, microglia display morphological changes

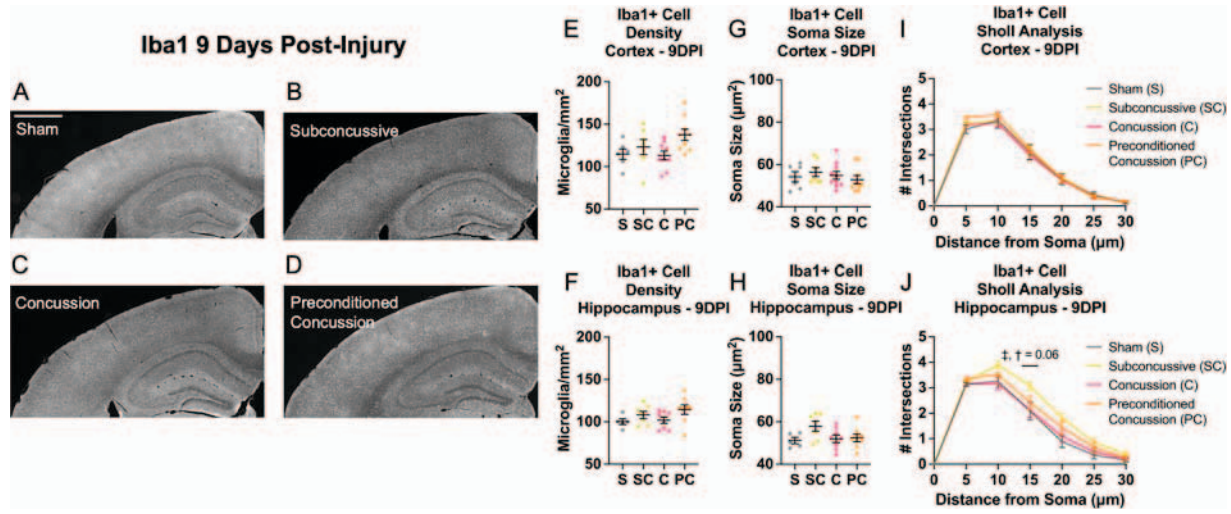



**FIG. 5.** Microglia exhibit delayed changes in morphology in the hippocampus in response to subconcussive impact. **(A–D)** Representative images of Iba1+ staining in ipsilateral cortex and hippocampus 9 days post-injury (dpi) for **(A)** sham, **(B)** subconcussive impact, **(C)** concussion, and **(D)** preconditioned concussion. **(E, F)** Microglia density in **(E)** cortex and **(F)** hippocampus, **(G, H)** soma size, and **(I, J)** Sholl Analysis. ‡indicates subconcussive impact versus sham, †indicates subconcussive impact versus concussion. Comparisons were performed using a linear mixed effects model with Tukey's *post-hoc* test. Raw data shown as mean ± SEM. S: sham; SC: subconcussive; C: concussion; PC: preconditioned concussion (Brief). Scale bar = 1 mm. SEM, standard error of the mean.

and increased gene expression relating to cytoskeleton and mitochondrial metabolism early following concussion. These microglia changes occurred in parallel with impairments in novel object recognition and fear conditioning. In contrast, a single subconcussive impact produced a more delayed increase in ramification with no changes in neurobehavior. Rapidly delivering multiple subconcussive impacts before a concussive impact—one form of preconditioning—not only reduced changes in microglial morphology and pro-inflammatory transcriptional signatures but also erased cognitive deficits associated with concussion. Using subconcussive impacts to reverse the deficits of concussion was time sensitive: extending these subconcussive impacts over days eliminated any protection offered by subconcussive preconditioning.

Previous studies report transient changes in microglia number and morphology following concussive and subconcussive impacts. In the first 72 h post-concussion, microglia increase in number at the injury site[24,69,70] and in soma size,[69,71] with changes often resolving within a week or two.[24,71] Our model produced changes on a similar timeline: the most profound changes in microglial morphology occurred one day post-concussion and resolved by 9 days. However, rather than an amoeboid morphology, we observed an increase in ramification typical of hyper-surveillant or primed morphology,[72] which other groups have also noted in response to closed-head injury.[73] The heterogeneity in microglial response may be attributable to the variety of injury conditions and severities in the literature. Subconcussive impacts' effect on microglia is less precisely characterized in past work. For both single and repetitive subconcussive impacts, some groups report a similar trend to concussion with an acute increase in the number of microglia that resolves within a week.[24,28] Stemper and colleagues observed an increase in number and branching of microglia following highly repetitive subconcussive impacts at 14 dpi, suggesting that sufficient loading can produce persistent microglial pathology, potentially as severe as our acute concussive response.[19] Others have observed rod-like microglia in the acute and subacute response to diffuse TBI,[74] but we did not consistently observe this rare morphology in our ROIs. We also did not observe amoeboid microglia at either time point, but these changes are frequently associated with phagocytosis in areas of necrotic cell death, which was not present in our model.[75] The more subtle changes we observe may be attributable to our less severe subconcussive impact.

Concurrent with acute morphological differences post-injury, we observed changes in microglial mRNA expression consistent with homeostatic adaptation, rather than classical pro-inflammatory activation. Recent work shows that the primary effect of microglia at 1–2 days post-TBI is increased gene expression relating to inflammation

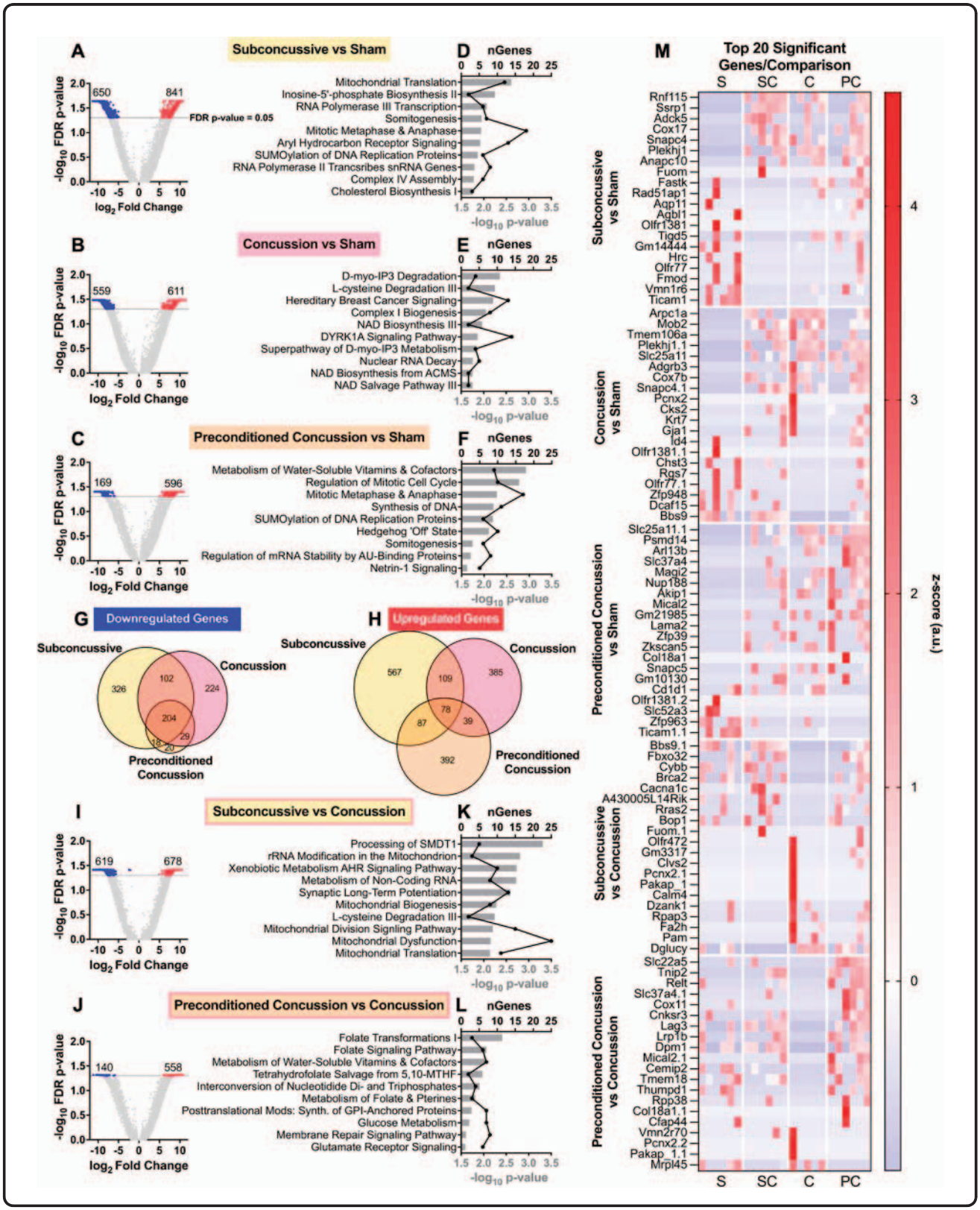
(e.g., *Cd14*, *Cd68*, *Cebpb*) and cytokine/chemokine signaling (e.g., *Ccl12*, *Tnf*, *Cxcl1*, *Il-1b*).[76–78] However, the dynamics and intensity of microglial inflammatory response scale with injury severity,[79–81] and these studies used more severe injury models involving craniectomies. The patterns of gene expression evident in our model may be characteristic of an early, mild response. For example, mitochondrial bioenergetics in microglia respond within hours of injury,[82,83] and factors such as mitochondrial dysfunction, glucose availability, and glycolytic rate can themselves stimulate pro-inflammatory gene transcription.[84] That we observe changes in mitochondrial bioenergetics across all three impact conditions is potentially indicative of ongoing homeostatic adaptation. The genes relating to mitochondrial dysfunction and division signaling pathways upregulated in subconcussive versus concussed mice may portend the delayed hyper-ramification at day 9 post-injury. Directly linking microglial transcriptomic state to heterogeneous morphological states has historically been challenging, especially using bulk sequencing techniques.[85] In our concussed mice, several cytoskeletal genes are upregulated (*Arpc1a*, *Mob2*, *Krt7*); notably, *Arpc1a* is related to actin branching[86] and may contribute to the hyper-ramified morphology in concussed microglia at 1 dpi. More widespread use of emerging spatial transcriptomic techniques will offer a more detailed comparison of transcriptomic and morphological profiles.

Interestingly, preconditioned concussion demonstrated early changes to gene expression consistent with negative regulation of pro-inflammatory responses, increasing expression of *Tnip2*, *Relt*, and *Lag3* and folate pathways. *Tnip2* negatively regulates NF-κB signaling, and its upregulation in microglia is protective following spinal cord injury.[87] *Relt*, a tumor necrosis factor (TNF) receptor, is negatively associated with intracranial aneurysms.[88] *Lag3* is an immunosuppressive checkpoint gene that mitigates microglial overactivation,[89] especially in white matter[90]; however, *Lag3* is also implicated in α-synucleinopathies,[91] suggesting that its careful balance is required for successful neuroprotection. Preconditioned concussion also stimulated pathways relating to folate metabolism. Folic acid has broad effects on neuroinflammation, prompting anti-inflammatory gene expression through NF-κB, JNK, and p38 MAPK pathways,[92,93] regulates gene expression via epigenetic mechanisms,[94] and is neuroprotective against neurological diseases,[95] including TBI.[96] Taken together, these findings suggest that the broad effect of the acute microglial response to preconditioning is to reduce inflammation, which may contribute to later improvements in cognitive function.

Microglia can play a key role in learning and memory in both healthy and diseased states.[97] For this reason, microglia are candidate mediators for the reversal of cognitive deficits we observed after subconcussive preconditioning. In healthy brains, for example, microglial production of TNF-α is required for sustaining fear memory,[98] and microglial phagocytosis and synapse elimination are required for eliminating fear memory.[99] Indeed, microglial activation during sensitive periods of development can impair neuron–microglia communication and synaptic modulation to produce learning deficits that only emerge days after the activation.[100] In TBI, depleting microglia prior to and immediately following TBI can reverse cognitive deficits associated with TBI, suggesting that preventing acute inflammation may be an effective strategy for improving outcomes.[76,101] Furthermore, cognitive deficits are associated with reactive microglial morphologies in TBI[101,102] and beyond.[103–105] Taken together, our microglial analysis and neurocognitive analysis suggest that early increases in microglia with hyper-surveillant morphology are associated with worse cognitive outcomes.

To our knowledge, there is no precedent for examining the microglial response to subconcussive preconditioning in a model of TBI. Studies examining repetitive subconcussive impacts largely focus on neuronal damage as a mechanism.[16,18,21] Furthermore, these studies do not examine the interplay between subconcussive impacts and concussion outcomes. However, there are examples in similar neurological diseases, including ischemic stroke and epilepsy, which examine subthreshold preconditioning and microglial tolerance. In ischemic stroke,

**FIG. 6.** Injury broadly upregulates genes relating to mitochondria, metabolism, and transcriptional regulation; preconditioning uniquely affects gene expression relating to folate metabolism and suppression of pro-inflammatory programs. **(A–C)** Volcano plots highlighting significantly up- (red) and down- (blue) regulated genes relative to sham ($Log_2$ Fold Change > 2.0; FDR $p$ value < 0.05). **(D–F)** Pathway analysis showing up to 10 pathways significantly affected based on significant gene expression relative to sham ($p < 0.05$). **(G)** Overlap in up- and **(H)** down-regulated genes relative to sham across impact conditions. **(I–J)** Volcano plots highlighting significantly up- and downregulated genes relative to concussion ($Log_2$ Fold Change > 2.0; FDR $p$ value < 0.05). **(K–L)** Pathway analysis showing up to 10 pathways significantly affected based on significant gene expression relative to concussion ($p < 0.05$). **(M)** Heat map showing the $z$-score for the top 20 differentially expressed genes for each comparison. S: sham; SC: subconcussive; C: concussion; PC: preconditioned concussion (Brief). FDR, false discovery rate.




brief ischemia can be used to precondition against more prolonged ischemic events and is associated with distinct microglia morphological changes and extent, much like what we observe with subconcussive impacts and preconditioning. Alternatively, kindling, a form of repetitive low-level seizure simulation, can be used to both lower the seizure threshold and induce spontaneous seizures[106] and, less commonly, precondition the brain

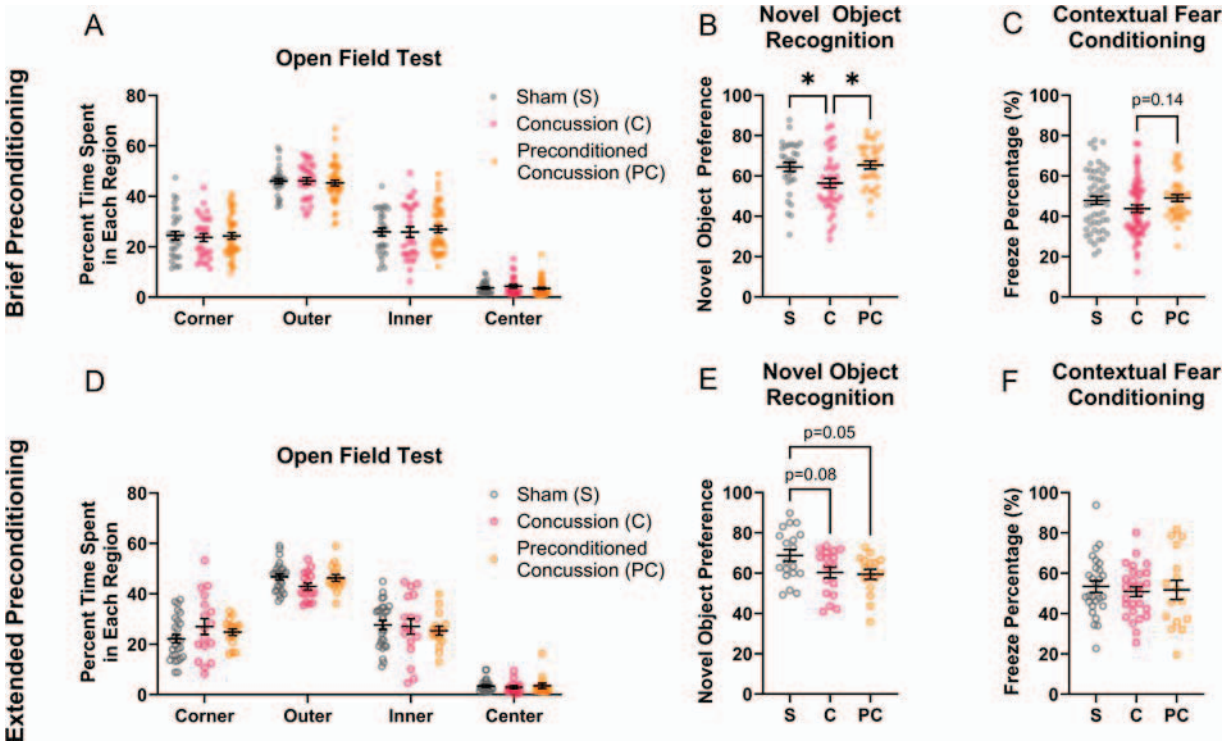



**FIG. 7.** Brief, but not extended, preconditioning improves cognitive outcomes. **(A)** Brief preconditioning (2-minute window) had no effect on percent time spent in the corner, outer, inner, or center areas of the open field. **(B)** Brief preconditioning significantly improves novel object preference compared with concussion. **(C)** Brief preconditioning improves fear memory relative to concussion, albeit non-significantly ($p = 0.14$). Sham and concussion data in A–C are a reproduction of Figure 2. **(D)** Extended preconditioning (48-h time window) had no effect on percent time exploring the open field. **(E)** Extended preconditioning produces the same near-significant deficit in novel object preference as concussion. **(F)** Neither concussion nor extended preconditioning significantly affects fear memory. *indicates $p < 0.05$. Statistical testing was performed using two-way ANOVA **(A, B)** or one-way ANOVA **(C–F)** with Tukey's *post-hoc* test. Data are shown as mean ± SEM. S: sham; SC: subconcussive; C: concussion; PC: preconditioned concussion. SEM, standard error of the mean.

against seizures.[42,43,107] Indeed, microglia have a beneficial role in secondary seizures and reduce the burden of kindled seizures,[108] and kindling preconditioning regulates microglial gene expression.[107] There is also significant overlap between these neurological diseases, for example, ischemic preconditioning is effective in TBI,[109–111] and TBI can induce post-traumatic epilepsy[112] and make the brain more susceptible to kindling[38] via neuroimmune mechanisms. Beyond subthreshold neurological stimulation, systemic methods such as LPS administration, exercise, heat, and anesthesia can confer neuroprotection against acute brain injury by modulating the brain's inflammatory response.[113] Most common among these is LPS preconditioning, which directly and systemically stimulates the body's innate immune response.[114] At low doses, LPS protects against subsequent TBI, reducing cell death and neurodegeneration, improving neurocognitive function, reducing pro-inflammatory cytokines, and reducing phagocytic microglia.[33–35] However, LPS use is complicated by side effects, including acute weight loss[33] and locomotor impairments.[34] Together, this past work linking the response of microglia to the neurological outcome has led to a broad rationale for identifying brain-specific immunological therapeutic strategies.

In addition to desensitizing the brain before a more severe injury, microglia can play several roles in the brain during recovery. Microglia play a key role in synaptic remodeling,[115] and hyper-ramified microglia in particular are hypothesized to mediate dendritic spine loss.[116] One way microglia modify synapses is by remodeling the extracellular matrix,[117] including the

perineuronal nets, which stabilize synapses[118] and may underpin long-term memory storage.[119] Microglia also secrete factors, including cytokines and other neurotrophic factors, that can affect inflammatory cascades and synaptic plasticity.[120] In preliminary studies, we did not measure long-lasting changes in a panel of serum cytokines (data not shown), but that does not rule out earlier changes. Microglia also remove cellular debris following injury.[120] However, given the absence of neuronal injury or tissue loss in our injury model as well as the absence of amoeboid microglia often associated with phagocytosis,[72] we do not expect this to be a significant microglial role affected by our model. Indeed, we saw no increase in CD68 immunohistological staining or CD68 gene expression canonically associated with phagocytosis. It is more likely that preconditioning affects microglia-mediated synaptic plasticity to improve cognitive function, perhaps through the attenuated or delayed appearance of the hyper-ramified, pro-inflammatory microglia after subconcussive preconditioning.

To enable pre-clinical characterization of subconcussive preconditioning, we made several methodological decisions with trade-offs for clinical interpretation. First, we injured the mice in the same location for repetitive impacts, controlling for spatial distribution while focusing on impact timing's role in microglial activity. Whether the suppression of pro-inflammatory responses should be local (as in the present study), distant (as in remote ischemic preconditioning), or global (as in LPS preconditioning) to the site of injury is unclear, but may contribute to variability in clinical concussion diagnosis and outcomes. Second, our relatively minor concussion model likely contributed to the modest benefit of subconcussive preconditioning. In more severe injury models with more pronounced pro-inflammatory effects, there is likely a ceiling where subconcussive preconditioning exacerbates pro-inflammatory responses. Despite these limitations, our study points to acute inflammation as a key factor influencing concussion outcomes and indicates that prophylactically or acutely reducing the pro-inflammatory immune response, potentially by drugs such as minocycline[121] or by folic acid supplementation, may minimize concussion symptoms. Moreover, it suggests that preconcussion inflammatory state may influence concussion incidence and diagnosis. Notably, however, our study did not examine the long-term consequences of preconditioning following initial neuroprotection.

In summary, our work shows that rapidly delivered subconcussive impacts can precondition the brain against the deficits normally caused by concussion. We find that changes in microglial morphology and transcriptional profiles differ between subconcussive and concussive impacts and differ yet again with preconditioning. In general, the alterations in microglial morphology resemble similar changes reported in other models of preconditioning, and alterations in gene expression reflect the mildness of our impacts. As evident through past work in preconditioning, our initial observations provide the groundwork for exploring more directly the role of microglia and, in the longer term, revealing the mechanism(s) that affect the timing and duration of this phenomenon. Together, these findings can provide critical insights into expanding the role of microglia from surveillance and maintenance in the injured brain into providing temporary protection of the brain from damaging insults.

## Transparency, Rigor, and Reproducibility Statement

All surgical procedures were performed in accordance with the IACUC at the University of Pennsylvania. Mice were systematically assigned to experimental conditions. A sample size of 3 mice/experimental condition with 3 sections/brain was planned for histology based on prior work.[122] For microglia analysis, an experimenter blinded to the experimental condition counted all microglia in an ROI and then randomly assigned 30 microglia for morphological analysis, similar to prior studies.[62] A mixed-effects model was selected for analysis of microglial soma size and Sholl analysis to account for variability in sections from the same brain.[62,68] A sample size of 6 animals/condition was planned for mRNA analysis based on prior work.[76] Multiple comparisons testing was performed using FDR. A sample size of 25 mice/experimental condition was planned for behavioral studies to detect significant differences with $\alpha = 0.05$ and 80% power, which was increased to 35 mice/condition based on a lower-than-anticipated normalized difference observed in our new TBI model. Exclusion criteria can be found in the "Methods" section. NOR and CFC were performed during the light cycle by the same experimenter under similar conditions (e.g., time of day, temperature, lighting). Behavioral analysis was conducted by an experimenter blinded to the experimental condition. Multiple comparisons testing was performed using Tukey's method.

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## Authors' Contributions

E.D.A.: Conceptualization, methodology, software, formal analysis, investigation, writing—original draft preparation, writing—review and editing, visualization, validation, and data curation. K.K.: Formal analysis,

investigation, software, validation, and writing—review and editing. A.P.G.: Methodology, formal analysis, validation, and project administration. A.N.: Methodology, formal analysis, and software. E.G.: Methodology, formal analysis, software, validation, and data curation. D.V.A.: Methodology. D.F.M.: Supervision, conceptualization, methodology, funding acquisition, and writing—review and editing.

## Author Disclosure Statement

The authors have no competing interests to disclose.

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## Supplementary Material

Supplementary Figure S1
Supplementary Figure S2

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