ON-LINE APPENDIX

Behavioral Assessment

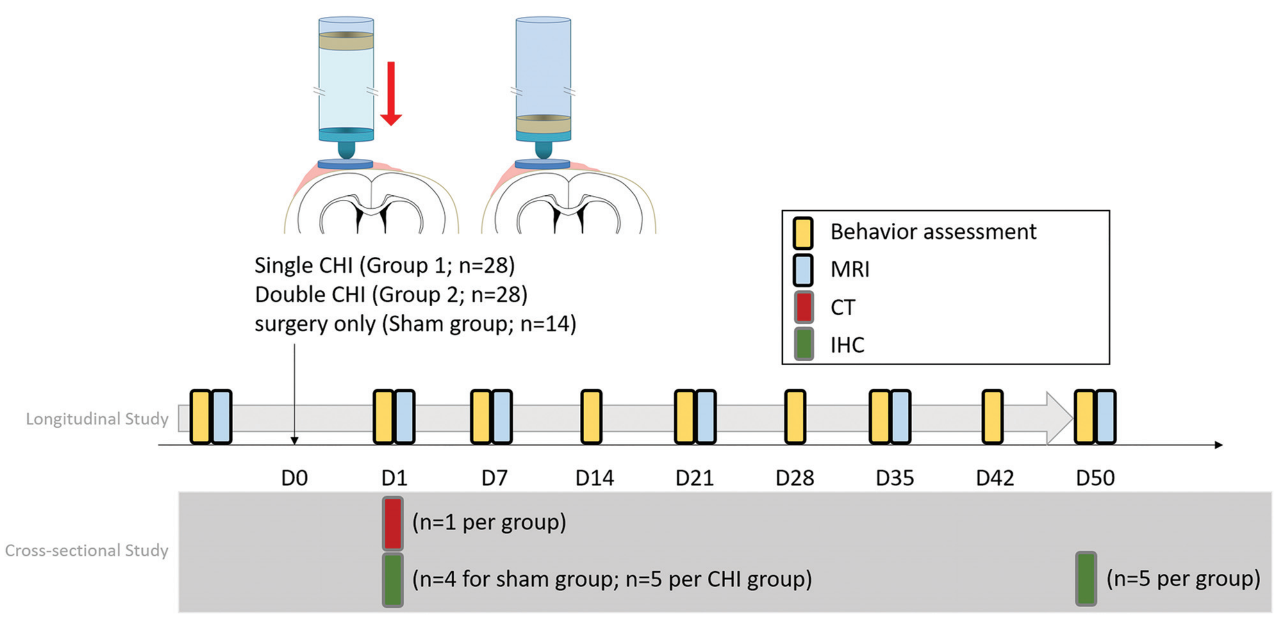
Animal body weight was recorded daily after the operation or CHI before the following assessments. The mNSS, also called Revised Neurobehavioral Severity Score, generally evaluates the motor sensory reflex, muscular status, and balance specifically for rodents after TBI.[1,2] The evaluation is composed of the metrics of behavioral tasks, including balance, landing, tail raise, dragging, righting reflex, ear reflex, eye reflex, sound reflex, tail pinch, and hind paw pinch in a specific sequence. Score points were awarded for the inability to perform the task, the lack of reflex, or abnormal response, which resulted in a total possible score of 20. A higher score indicated greater severity.

The Beam Walk Balance Test assesses the balance and coordinated motor function in rats after CHI or sham surgery.[3] The beam walk apparatus consisted of a beam 80 cm in length, 3.0 cm in width, and 3.0 cm in height elevated 60 cm from the floor. Animals were monitored using a video camera aimed at the beam during the test. The rat was placed at the starting point. The time taken to travel to the opposite end of the beam, make a U-turn, and return to the starting point was recorded. Animals who fell off the beam or did not finish the task received a duration of 180 seconds. Before CHI or sham surgery, the animals had finished the beam walk task within 50 seconds for 3 consecutive days. Two trials were performed for each animal per time point after CHI or sham surgery, and the time of 2 trials was averaged as the duration for the Beam Walk Balance Test.

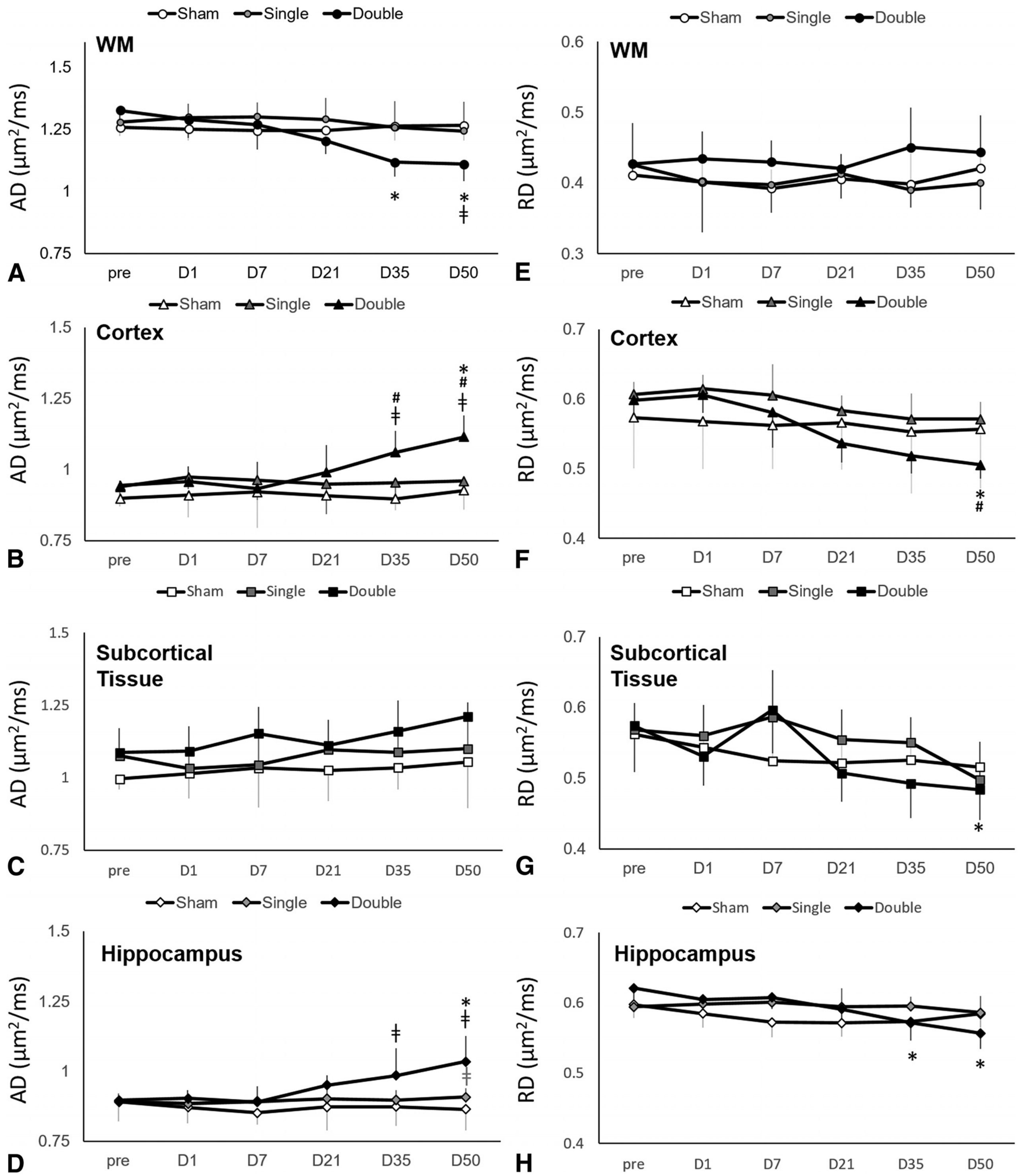
The open-field test was used to assess spontaneous activity and anxiety-like behavior after CHI or sham surgery.[4] Animals were monitored using a video camera in an open arena (50 × 35 cm) for 10 minutes during the test. The inner rectangular zone (40 × 25 cm) at the center of the arena was defined as the center region. The tracking and calculation of the locomotor parameters of each animal during the trial were analyzed using ActualTract software (Actual Analytics).[5] The configuration settings used in the current study were adjusted on the basis of the default parameters described in the ActualTract Manual. The tracking results of ActualTract in each trial were further examined manually to confirm the accuracy. The software automatically quantified the movement duration, total travel distance, center entries, and center time for each trial.

REFERENCES

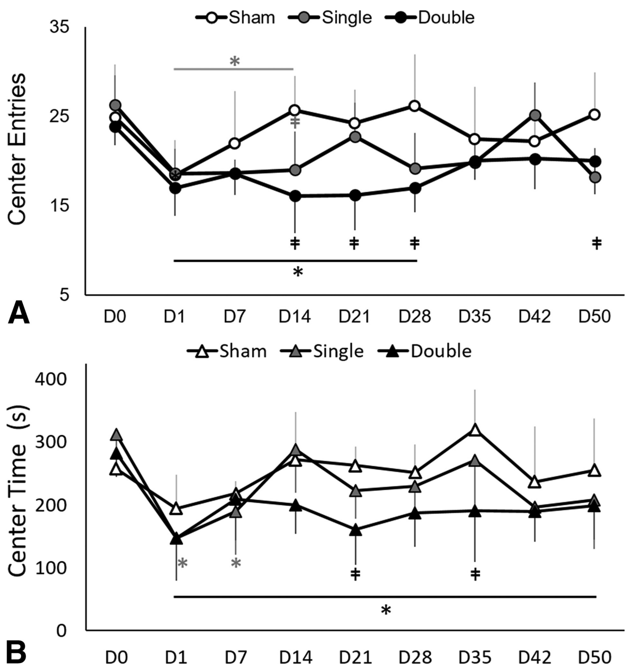
1. Mountney A, Boutte AM, Cartagena CM, et al. **Functional and molecular correlates after single and repeated rat closed-head concussion: indices of vulnerability after brain injury.** *J Neurotrauma* 2017;34: 2768–89 CrossRef Medline
2. Yarnell AM, Barry ES, Mountney A, et al. **The Revised Neurobehavioral Severity Scale (NSS-R) for rodents.** *Curr Protoc Neurosci* 2016; 75:9.52.1–9.52.16 CrossRef Medline
3. Hamm RJ. **Neurobehavioral assessment of outcome following traumatic brain injury in rats: an evaluation of selected measures.** *J Neurotrauma* 2001;18:1207–16 CrossRef Medline
4. Yang LY, Greig NH, Huang YN et al. **Post-traumatic administration of the p53 inactivator pifithrin-α oxygen analog reduces hippocampal neuronal loss and improves cognitive deficits after experimental traumatic brain injury.** *Neurobiol Dis* 2016;96:216–26 CrossRef Medline
5. Green EW, O'Callaghan EK, Hansen CN et al. **Drosophila circadian rhythms in seminatural environments: summer afternoon component is not an artifact and requires TrpA1 channels.** *Proc Natl Acad Sci U S A* 2015;112:8702–07 CrossRef Medline



ON-LINE FIG 1. Animal groups and assessment schedule. Animals were randomly assigned to 3 groups: group 1 ($n = 28$) with single impact, group 2 ($n = 28$) with 2 impacts with a 1-hour interval between impacts, and a sham group ($n = 14$; operation without impact.) Three animals were euthanized at 24 hours ($n = 1$ from group 1, $n = 1$ from group 2, and $n = 1$ from the sham group) for micro-CT imaging to assess the skull for more severe injury. Fourteen animals were euthanized at 24 hours ($n = 5$ from group 1, $n = 5$ from group 2, and $n = 4$ from the sham group), and 15 animals, at 50 days ($n = 5$ from group 1, $n = 5$ from group 2, and $n = 5$ from the sham group) after CHI for histologic examination. The remaining subjects underwent longitudinal MR imaging and behavioral assessment. The inset shows the schematic plot of our proposed CHI model.



ON-LINE FIG 2. Longitudinal changes in AD and RD after CHI. Longitudinal follow-up during the first 50 days shows the evolution of mean AD (*A–D*) and RD values (*E–H*) with time, which differ between white matter and gray matter structures as well as demonstrating important differences between single and double injury. Data are means. ‡ indicates $P < .05$ versus the sham; *asterisk*, $P < .05$ versus baseline (day 0 [D0]); *#*, $P < .05$ versus the single CHI.



ON-LINE FIG 3. Anxiety-related behavior after CHI. Center entries (*A*) and center time (*B*) after single and double CHI compared with a sham operation. Both single-and double-injury groups showed a decreased number of center entries compared with baseline, beginning at 24 hours after CHI. While center entries in animals with a single injury showed recovery after 14 days, center entries in animals with double injury remained significantly lower compared with baseline through day 28. Both single- and double-injury groups showed decreased center time compared with baseline beginning at 24 hours after CHI. While center time in animals with a single injury recovered after 7 days, center time in animals with double injury remained significantly lower compared with baseline and did not resolve by day 50. Data are means. ‡ indicates $P < .05$ versus the sham; *asterisk*, $P < .05$ versus baseline (day 0 [D0]).

On-line Table 1: Summary of MD in different groups[a]

MD (μm^2/ms)	Baseline	Day 1	Day 7	Day 21	Day 35	Day 50
Sham group						
WM	0.72 ± 0.02	0.72 ± 0.02	0.71 ± 0.02	0.71 ± 0.02	0.71 ± 0.03	0.71 ± 0.02
Cortex	0.71 ± 0.01	0.70 ± 0.02	0.70 ± 0.02	0.71 ± 0.04	0.68 ± 0.03	0.68 ± 0.04
Subcortical region	0.79 ± 0.03	0.78 ± 0.04	0.78 ± 0.06	0.79 ± 0.02	0.79 ± 0.03	0.78 ± 0.07
Single CHI						
WM	0.72 ± 0.02	0.73 ± 0.02	0.73 ± 0.02	0.72 ± 0.04	0.71 ± 0.03	0.70 ± 0.04
Cortex	0.74 ± 0.04	0.73 ± 0.03	0.74 ± 0.06	0.71 ± 0.05	0.71 ± 0.05	0.70 ± 0.04
Subcortical region	0.84 ± 0.06	0.83 ± 0.06	0.85 ± 0.09	0.81 ± 0.05	0.87 ± 0.01	0.79 ± 0.08
Double CHI						
WM	0.73 ± 0.01	0.72 ± 0.02	0.74 ± 0.04	0.72 ± 0.03	0.71 ± 0.05	0.72 ± 0.04
Cortex	0.72 ± 0.01	0.72 ± 0.03	0.71 ± 0.06	0.75 ± 0.05	0.70 ± 0.05	0.70 ± 0.05
Subcortical region	0.83 ± 0.03	0.78 ± 0.05	0.80 ± 0.04	0.78 ± 0.07	0.75 ± 0.08	0.78 ± 0.02

[a] Data are means.

On-line Table 2: Summary of FA in different groups[a]

FA	Baseline	Day 1	Day 7	Day 21	Day 35	Day 50
Sham group						
WM	0.64 ± 0.02	0.65 ± 0.03	0.65 ± 0.03	0.64 ± 0.02	0.65 ± 0.03	0.64 ± 0.02
Cortex	0.30 ± 0.01	0.31 ± 0.02	0.31 ± 0.03	0.31 ± 0.01	0.31 ± 0.02	0.31 ± 0.02
Subcortical region	0.43 ± 0.04	0.43 ± 0.03	0.43 ± 0.02	0.45 ± 0.03	0.44 ± 0.02	0.44 ± 0.03
Single CHI						
WM	0.64 ± 0.01	0.63 ± 0.03	0.64 ± 0.03	0.63 ± 0.03	0.63 ± 0.02	0.62 ± 0.02
Cortex	0.30 ± 0.03	0.30 ± 0.02	0.32 ± 0.02	0.32 ± 0.02	0.33 ± 0.02	0.31 ± 0.02
Subcortical region	0.45 ± 0.02	0.47 ± 0.03	0.46 ± 0.04	0.47 ± 0.03	0.48 ± 0.01[b]	0.50 ± 0.02[b]
Double CHI						
WM	0.64 ± 0.02	0.64 ± 0.03	0.63 ± 0.03	0.62 ± 0.01[c]	0.61 ± 0.01[b,c]	0.61 ± 0.02[b,c]
Cortex	0.29 ± 0.01	0.30 ± 0.02	0.31 ± 0.02	0.32 ± 0.02	0.33 ± 0.01[c]	0.34 ± 0.01[b,c]
Subcortical region	0.45 ± 0.02	0.47 ± 0.04	0.46 ± 0.03	0.49 ± 0.03[c]	0.51 ± 0.01[b,c]	0.51 ± 0.02[b,c]

[a] Data are means.

[b] Statistically significant difference of $P < .05$ between the CHI and sham groups.

[c] Statistically significant difference of $P < .05$ compared with baseline.