

## Research report

# Testosterone treatment restores vestibular function by enhancing neuronal survival in an experimental closed-head repetitive mild traumatic brain injury model

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## ABSTRACT

Repetitive mild traumatic brain injury (rmTBI) results in a myriad of symptoms, including vestibular impairment. The mechanisms underlying vestibular dysfunction in rmTBI patients remain poorly understood. Concomitantly, acute hypogonadism occurs following TBI and can persist chronically in many patients. Using a repetitive mild closed-head animal model of TBI, the role of testosterone on vestibular function was tested. Male Long Evans Hooded rats were randomly divided into sham or rmTBI groups. Significant vestibular deficits were observed both acutely and chronically in the rmTBI groups. Systemic testosterone was administered after the development of chronic vestibular dysfunction. rmTBI animals given testosterone showed improved vestibular function that was sustained for 175 days post-rmTBI. Significant vestibular neuronal cell loss was, however, observed in the rmTBI animals compared to Sham animals at 175 days post-rmTBI and testosterone treatment significantly improved vestibular neuronal survival. Taken together, these data demonstrate a critical restorative role of testosterone in vestibular function following rmTBI. This study has important clinical implications because it identifies testosterone treatment as a viable therapeutic strategy for the long-term recovery of vestibular function following TBI.

## 1. Introduction

Traumatic brain injury (TBI) is a significant public health concern [1]. The Center for Disease Control and Prevention estimates that 3 million people experience TBI in the U.S. annually, accounting for a total annual cost of \$60–80 billion [2]. The World Health Organization

predicts that TBI will surpass many diseases as the leading cause of mortality and disability worldwide and estimates the annual global incidence at 70 million [3]. TBI is the result of an external force onto the head causing an alteration of brain function, which can lead to a variety of symptoms including vestibular, cognitive, emotional, and motor impairment [1,2,4]. TBI consists of two temporal pathological phases

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spanning from the initial impact to multiple secondary injury cascades and progressive neurological deficits [5]. The primary brain injury consists of the immediate damage caused by the physical impact, whiplash force, or shock wave. A secondary brain injury occurs in response to the primary injury and includes inflammation, ischemia, hypoxia-reperfusion injury, raised intracranial and intraocular pressure, hydrocephalus, and infection. Secondary injury occurs in the minutes to days after impact and is diffuse in nature. Little can be done to mitigate primary brain damage once it occurs but the prolonged impact of secondary injury processes may be appropriately mitigated [5,6].

Approximately 80–90 % of TBIs are classified as mild TBI but are largely underestimated because patients either do not seek immediate medical assistance or are difficult to diagnose without self-reported post-traumatic symptoms [1,7–9]. Although many mild TBI patients may recover symptoms spontaneously, a subset (10–40 %) of patients experience post-concussion syndrome, with long-term deficits [10,11]. A single mild TBI may not reliably cause prolonged behavioral deficits but repetitive mild TBI (rmTBI) has been shown to produce compounding damage with an increased likelihood of long-term disability [12–15]. Specific populations are at risk for the cumulative effects of rmTBI and suffering from one mild TBI can exponentially increase susceptibility to additional TBIs [13–17]. These vulnerable risk groups include athletes participating in contact sports, military personnel, victims of domestic abuse, and the infantile and elderly [2]. Individuals suffering from rmTBI have been found to have increased risk of long-term cognitive dysfunction, psychiatric illness, and the occurrence of neurodegenerative disease later in life compared to control and single mild TBI [14,16,18]. Over time, the cumulative effects of many mild TBIs can also lead to neurodegeneration [18–21].

Vestibular symptoms, along with cognitive impairment, are the most common clinical complaints following TBI, but are poorly understood with limited treatment [19,22]. Symptoms manifest as vertigo, dizziness, lightheadedness, and imbalance [19,22,23]. In the U.S. general population, an estimated 16 % of men and 8 % of women live with a TBI, and up to 80 % suffer prolonged vestibular impairment [24]. The prevalence of vestibular deficits resulting from a single mild TBI range from 40 % to 80 % upon admission to the emergency room [1], and repetitive mild TBIs may potentially compound and worsen such effects. The vestibular organs are comprised of the semicircular canals, which detect angular head acceleration, and the otolithic organs (utricle and saccule), which detect linear head acceleration [25]. Vestibular impairment due to TBI may be caused by central or peripheral vestibular system disruption. Central vestibular disruption stems from impaired synaptic connections or neuronal death from neurons within the four vestibular nuclei or their secondary targets to the surrounding brainstem nuclei and cerebellum. Peripheral vestibular disruption stems from physical deterioration of the vestibular organs, impaired temporal bones, or lack of afferent synaptic connections [22,25]. Vestibular impairment is severely debilitating and has a dramatic negative impact on quality of life. Many patients experience some degree of functional recovery from vestibular deficits caused by TBI, but symptoms may often persist for 6 months or longer [22].

Hypogonadism has been well documented in men and women following TBI, leading to suppressed testosterone and estradiol levels [26–28]. Hypogonadism develops in the minutes to days following TBI through a myriad of primary and secondary brain injury processes [29]. On average, long-term hypogonadism occurs in 60 % of patients (a range of 20–80 %) [30–32] and is a risk factor for developing worsening symptoms over time [33–36]. Despite such frequency, sexual hormone dysfunction is not commonly measured or evaluated post-TBI [32]. Hypogonadism occurs due to disruption along the hypothalamic-pituitary-gonadal (HPG) axis [37,38]. Within one month of moderate and severe TBI, low serum testosterone levels were found in 80% of male patients [39], and 46 % of women experienced amenorrhea that persisted up to 5 years post-TBI [27]. Importantly, hypogonadism is also prevalent after mild TBI. A retrospective study found acute

testosterone deficiency in all male mild TBI patients and 93 % persisted throughout the testing period. Comparatively, 43 % of women had at least one low estradiol level [40,41]. The association between TBI and acute hypogonadism suggests a potential correlation between a lack of gonadal steroids and exacerbated secondary injury processes. Long term hypogonadism following TBI thus poses serious health risks for a subset of patients that experience repeated head injuries.

Our lab and others have demonstrated the trophic effects of gonadal steroids on neurons including cell survival, neurotransmitter metabolism, neuronal regeneration, and plasticity [42–48]. Testosterone acts primarily through the androgen receptor, a nuclear transcription factor known to upregulate multiple neural plasticity and regenerative associated genes [49,50]. Both androgen and estrogen receptors are expressed readily throughout the four vestibular nuclei and associated brainstem regions [51–53]. Specifically, administration of testosterone following injury to cranial neurons has been shown to upregulate the expression of neuroprotective genes such as  $\beta$ -II tubulin, 43-kilodalton growth-associated protein, brain-derived neurotrophic factor, post-synaptic density 95, pituitary adenylate cyclase-activating peptide, and neuritin [54–57]. The direct action of these genes, proteins, and associated secondary pathways promotes the integrity and survival of synaptic connections, leading to enhanced neural regeneration. Testosterone also works through non-genomic processes by being further metabolized into dihydrotestosterone and estrogen [58,59]. Further, testosterone administration has been shown to affect the vestibular nuclei by lowering the threshold of activation [49,60]. Prolonged depression of gonadal steroid levels following TBI may impair successful rehabilitation, exacerbating deficits. Hypogonadism following TBI may impair the neuroplasticity required for cellular and molecular neural regeneration.

A single mild TBI has been shown to cause chronic vestibular impairment and hypogonadism. [22,33] Therapies to attenuate the increasing severity of vestibular symptoms caused by rmTBI have not, however, been extensively studied. Although the exact mechanisms differentiating rmTBIs from a single mild TBI are not fully understood, it is increasingly clear that repetitive mild TBIs lead to worse clinical outcomes [13,18], greater neurological pathology [13], increased severity of morphological damage in humans and animal TBI models [12–15], and increased susceptibility to additional TBIs [16]. Therefore, a focus of this paper was to examine the vestibular consequences of rmTBIs by developing a clinically relevant rmTBI animal model with detectable chronic vestibular deficiency. Less than 10 % of examined literature attempted to evaluate long-term, chronic deficits of longer than 2 months post-injury in rmTBI animal models [61]. Using modifications to a previously established closed-head rmTBI rodent model eliminated many confounds associated with open-head TBI models and more closely mimicked the kinetic forces associated with clinical TBI [12], we sought to detect chronic vestibular impairment up to 6 months after injury. Current treatment for chronic vestibular dysfunction due to rmTBI has had limited success and mainly involve vestibular rehabilitation [24,62,63]. Therefore, this study also sought to determine the efficacy of testosterone replacement following the development of chronic vestibular impairment due to rmTBI.

## 2. Methods

### 2.1. Animals

All behavioral experiments were performed with male Long-Evans Hooded rats obtained from Envigo Laboratories at 8-weeks old (200–225 g). Animals were housed with controlled temperature and humidity, a 12-h light-dark cycle, and given standard chow and water ad libitum. All experimental animal procedures were approved by the Edward Hines Jr. VA. Institutional Animal Care and Use Committee. Animals were randomly assigned to a rmTBI or sham group. For the first study, both groups were then further randomly separated to receive

castration or sham castration surgery resulting in four groups, sham intact (INT), sham castrated (CAS), rmTBI INT, and rmTBI CAS. For the treatment studies, animals in the rmTBI group were further divided randomly into groups receiving vehicle (rmTBI) or testosterone (rmTBI + T) treatment at 35-days post-rmTBIs.

2.2. Closed-head repetitive mild traumatic brain injury

Animals were anesthetized with 3.5 % isoflurane, the right side of the head was shaved, and sedated animals were carefully positioned on a 5 cm thick foam cushion against a Plexiglas® frame angled 11° from the vertical. A stereotactic frame was used to position a controlled cortical impactor (CCI) device (Leica Biosystems, Inc., Lincolnshire, IL) over the right sensorimotor cortex, and a nose cone delivered maintenance anesthesia. The electromagnetic actuator used has a 5 mm flat steel tip and was angled approximately 20° from vertical. Importantly, minimal readjustments were necessary to ensure the tip of the actuator was perpendicular and flat against the area of impact. Approximate anatomical landmarks were previously defined (0.5 mm anterior and 4.0 mm lateral to bregma) from a study comparing impact positions between open and closed head CCI models [12]. The CCI control box was set to a high pass filter of 1000 kHz and a low pass filter of 1000 Hz. Impact velocity was set to 6.5 m/s at a height of 10.0 mm with a dwell time of 300 ms. These parameters allow for the maximal level of impact for a mild TBI without skull fracture. The anesthetic nose cone was temporarily removed and TBI was then administered. Sham TBI animals were similarly shaved, anesthetized, and properly positioned but didn't receive any impacts. TBI animals received 5 total closed-head rmTBIs, each spaced 48 h apart. All timepoints after the rmTBIs are referred to as days post-injuries (DPI) as depicted in Fig. 1A.

2.3. Castration

A subset of animals was castrated after the first mild TBI or sham injury. While under 3.5 % isoflurane sedation, the scrotum was shaved and a 1 cm incision was made in the midline of the scrotum. The cremaster muscle and tunica vaginalis were opened to expose the testes and part of the spermatic cord. The testes were pulled out and a silk suture was used to ligate the spermatic cord. The testes were removed and the external incision was closed with wound clips. Sham castrated animals underwent the same surgery manipulation but the testes were not ligated.

2.4. Testosterone capsules and serum levels

Crystalline testosterone powder (Sigma-Aldrich, Burlington, MA) was packed into a 1.5 cm length of Silastic tubing, with an inner diameter of 1.57 mm and an outer diameter of 2.41 mm (Dow Corning). Testosterone was packed into 1.0 cm of the tube, and both ends were sealed with wooden pieces, each 0.25 cm. The application of a silicone-based medical adhesive (Factor II, Inc., Lakeside, AZ) ensured a water-proof seal and smoothed out the ends of the capsule. Vehicle capsules were made by sealing empty Silastic tubing in the same manner. Prior to placement, capsules were equilibrated in sterile physiologic saline overnight to allow for the priming of testosterone release. Under isoflurane sedation, testosterone or vehicle capsules were subcutaneously placed at the dorsal nape of the neck. The subcutaneous implantation of testosterone containing capsules produces an average serum testosterone level of 2.12 ± 0.71 ng/ml in TBI INT animals (n = 5) and 3.43 ± 1.77 ng/ml TBI CAS animals (n = 6) after 28 days of implantation. Implants were replaced every 8 weeks for the duration of treatment. Serum testosterone levels were quantified via a testosterone specific competitive ELISA per the manufacturer described methods (Abcam 108666). All samples, controls, and standards were run in triplicate.

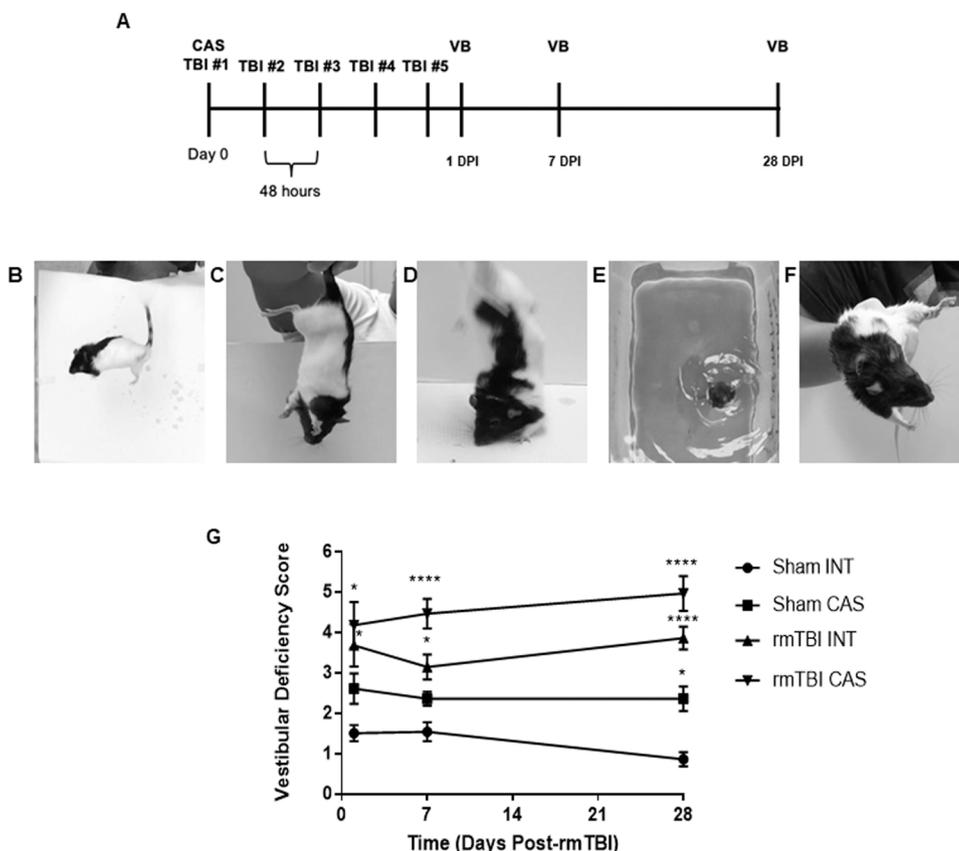


Fig. 1. Repetitive mild TBI produces acute and chronic vestibular behavioral dysfunction. (A) The timeline depicts when the animals underwent a castration (CAS), the 5 TBIs, and the vestibular battery (VB) testing. The vestibular battery of behavioral tests isolates vestibular function with 5 independent tasks including the (B) Air Righting Reflex Test (C) Tail Hanging Reflex Test (D) Forelimb Reach Test (E) Swimming Test (F) Lateral Raise Test. (G) rmTBI elicited significant vestibular deficiency at all timepoints. The Sham CAS group had significantly increased vestibular deficiency scores at 28DPI. (\*p < 0.05, \*\*\*\*p < 0.0001 as compared to the Sham intact (INT)).

## 2.5. Vestibular functional assessment

Using modifications to a previously established vestibular tests [64, 65], a battery of five behavioral tests were used to quantify vestibular dysfunction (see below) and were conducted between 0900 and 1100 h. Vestibular tests were videotaped from multiple angles and scored by two independent reviewers in a blinded manner. Each of the 5 tests may receive a score from 0 to 2, with 0 representing no vestibular dysfunction, 1 representing partial vestibular dysfunction and 2 representing maximum vestibular dysfunction. The medians for each test were summed for respective animals, providing a vestibular dysfunction score with a range of 0–10. All median vestibular deficiency scores within an experimental group were averaged to produce a mean vestibular deficiency score for that respective group and were presented as mean  $\pm$  standard error or the mean. The greater the vestibular deficiency scores, the more severe the vestibular impairment. Vestibular functional assessments were determined for all experimental groups at DPI 1, 7, and 28. Vestibular function was reassessed following testosterone replacement at DPI 42, 63, and 175 to determine the therapeutic effects of testosterone.

### 2.5.1. Air righting reflex test (Fig. 1B)

Rats were held supine and dropped from a height of 30 cm above a foam cushion. Animals with intact vestibular organs are able to rotate completely around the longitudinal axis (score of 0) with symmetry of coordination and successfully land on all four feet at the same time. Animals with vestibular damage exhibit severely abolished or greatly curtailed righting reflexes (score of 2). A score of 1 was given if the rat either had asymmetrical coordination of rotation or was unable to successfully land on all four feet at the same time. The test was conducted over five separate trials on the same day and the median of the scores was utilized for the summation of the total vestibular deficiency score.

### 2.5.2. Tail hanging reflex test (Fig. 1C)

Animals were gently held from the base of the tail and lifted swiftly to a height of 30 cm above the table. Sensory inputs from both otoliths result in a successful orientation, measured by dorsiflexion of the head and neck relative to the ground (score of 0). If the otoliths are damaged, the head will not dorsiflex (score of 2). Beginning when the forelimbs leave contact with the ground, the head and shoulders are observed to measure dorsiflexion. Importantly, animals attempt to twist and escape from the experimenter. Thus, true dorsiflexion was defined as an extension of the forelimbs accompanying the arching of the back muscles. Incidental head raises caused by twisting and turning were not counted as successful orientations. The test was conducted twice, and the average score was utilized for the summation of the total vestibular deficiency score.

### 2.5.3. Forelimb reach test (Fig. 1D)

Animals were gently held from the base of the tail and were lifted to a height of 30 cm. Animals were then rapidly lowered to the ground. Animals with intact semicircular canals can correctly dorsiflex and extend their forelimbs as they approach the ground (score of 0) to avoid hitting their head. Rats with vestibular damage fail to dorsiflex and extend their forelimbs, only doing so when their whiskers or forelimbs come into contact with the ground (score of 2). A score of 1 was given if the rat either fails to dorsiflex or fails to extend their forelimbs. Two trials were conducted. The median of the two scores was utilized for the summation of the total vestibular deficiency score.

### 2.5.4. Swimming test (Fig. 1E)

After being placed in a transparent container, approximately 50 cm  $\times$  50 cm filled with 30 cm of room temperature water, animals underwent a single 1-min testing period. The ability of the animal to successfully swim and remain balanced was scored. Rats with functioning vestibular systems are able to swim comfortably in the

horizontal position and move easily between points. These rats were given a score of 0. A score of 1 was given to animals who had 4 or less, episodes of imbalance during their swim. Episodes of imbalance were characterized as vertical swimming, with the nose at 90° or more, accompanied by frequent spinning and head bobs that appear as if the animal is falling over in the water. A score of 2 was given to animals with 5 or more episodes of imbalance.

### 2.5.5. Lateral raise test (Fig. 1F)

Animals underwent light sedation (haloperidol, 2 mg/kg); a minimum wait time of 30 min was necessary to achieve sedation. Animals were held laterally, with the hindlimbs pressed against the palm. The forelimbs were held with the opposite hand in the same direction the animal was facing. After lifting the animal to 30 cm above the ground, the head and forelimbs were released while holding the hindlimbs held lateral. In this position, only the otoliths on the side facing the ground provide information on head position. Scores were assigned based on the ability of the animal to correct its head positioning relative to the ground. Full correction was given a score of 0, a partial correction was given a score of 1, and no correction was given a score of 2. The test was conducted once on each side and the average score was utilized for the summation of the vestibular deficiency score.

## 2.6. Noldus open field vestibular video tracking

Unilateral and bilateral vestibular nuclei lesion studies using Noldus EthoVision XT 15 software assessment of open field exploration have identified behavioral deficits that are associated with vestibular neuronal loss at both acute and chronic timepoints [66,67]. To corroborate our findings from the subjective vestibular battery tests used here, all animals were video analyzed and objectively scored measures of open field with the Noldus EthoVision XT 15 software at 28 DPI and 42 DPI. Rats were placed into the center of a 50  $\times$  50 cm Plexiglass arena and allowed to explore for ten minutes. Videos of recorded activity were analyzed using the software to assess vestibular and motor activity using measurements and data filtering previously described [65,66]. Briefly, the arena was mapped and the animal's center and nose points were identified. Data profiles were set up to analyze data averaged over three frames and were then set to filter data based on specific body points, movement distance, and velocity. Total distance traveled and body speed were based on the animal's center point and were unfiltered, considering all movement states and velocities. Head speeds were also unfiltered but tracked the animal's nose point. Movement states, mobility states, and acceleration analyzed the animal's center point based on when the animal's center point moved more than 0.7 cm at any velocity. Body angle, arena rotations, and meander measurements analyzed the center point when it moved more than 0.7 cm but when the animal was moving only, with a start velocity of 2.00 cm/s and stop velocity as 1.75 cm/s. Arena rotations counted a rotation each time the animal's body moved 30° or more. Axis rotations filtered speed and movement by the same parameters but considered both the animal's nose and center points and counted a single rotation each time the nose-center axis moved 50° or more.

## 2.7. Vestibular neuronal counts

In half of the animals, brains were harvested at 175- days post-rmTBI, flash frozen and stored at -80 C. Fresh frozen brainstems were coronally sectioned at 40  $\mu$ m through the entire medial, inferior, superior, and lateral vestibular nuclei, fixed in 4 % paraformaldehyde and stained with thionin to reveal the neuron cell bodies (Fig. 4C–E). Slides were coded by one investigator and two additional independent investigators counted the vestibular neurons in each section using light microscopy. Vestibular neurons through the entire four vestibular nuclei on both the ipsilateral and contralateral sides were counted using ImagePro software 6.3 (2008). Other cranial nuclei, including the facial

motoneuron, have been shown to have degenerative and regenerative subnuclei populations [68]. Since neuronal regeneration and degeneration in the four vestibular nuclei have not been extensively studied, total vestibular neuronal counts were quantified to ensure there was no bias due to the location of the neurons. The rostral definition of the vestibular nuclei coincides with the termination of the locus coeruleus and the mesencephalic trigeminal nucleus. The superior and medial vestibular nuclei were present at this border. Counts proceeded in the caudal direction. The caudal definition of the vestibular nuclei coincides with the beginning of the subcompact part of the nucleus ambiguus. The inferior and medial vestibular nuclei are present at this border. Using  $100\times$  magnification, vestibular neurons were easily morphologically identified by as nucleated multipolar cell with somatic cell size  $\sim 25\text{--}40\ \mu\text{m}$  and only neurons containing a clear nucleus were counted.

## 2.8. Statistical analyses

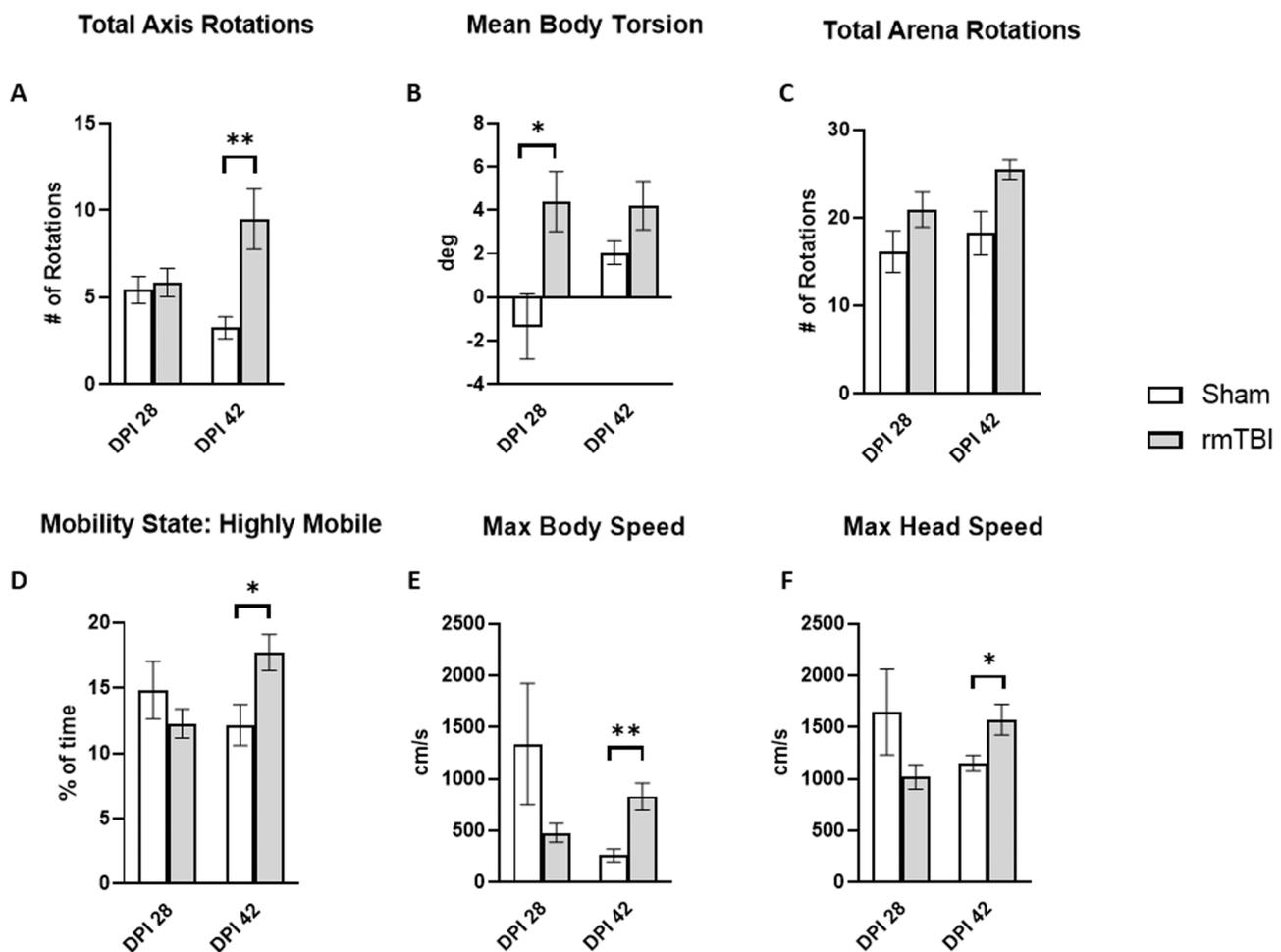
All data are expressed as Mean  $\pm$  standard mean of the error (SEM) and were analyzed with GraphPad Prism 9.2 (San Diego, CA). For the vestibular battery experiment, a three-way ANOVA with repeated measures followed by a Tukey multiple comparisons test was utilized to evaluate the data ( $n = 14$  per groups). Data obtained from each of the individual vestibular battery tests is considered non-parametric because ordinal data is assigned in a ranked order. However, the medians for each of the tests are summed and scores of all animals are averaged in each experimental group, allowing for the use of parametric statistics.

Data for the video scoring ( $n = 7\text{--}14$  per group in Fig. 2,  $n = 6\text{--}9$  per group in Fig. 3) were analyzed at single timepoints by first assessing normality using the Shapiro-Wilk method. If both groups passed the normality test, the groups were compared with the parametric Welch's t-test. If one or both groups failed the normality test, the groups were compared using the non-parametric Mann-Whitney U test. Serum testosterone levels were analyzed with a one-way ANOVA with repeated measures ( $n = 6$ /group). Data obtained from the experiments studying the effects of testosterone treatment on vestibular function with the vestibular battery tests ( $n = 7$ /group) and the vestibular cell count ( $n = 6$  per group) and were analyzed with a two-way ANOVA with main effects only followed by a Tukey's multiple comparisons tests.  $P < 0.05$  was considered statistically significant.

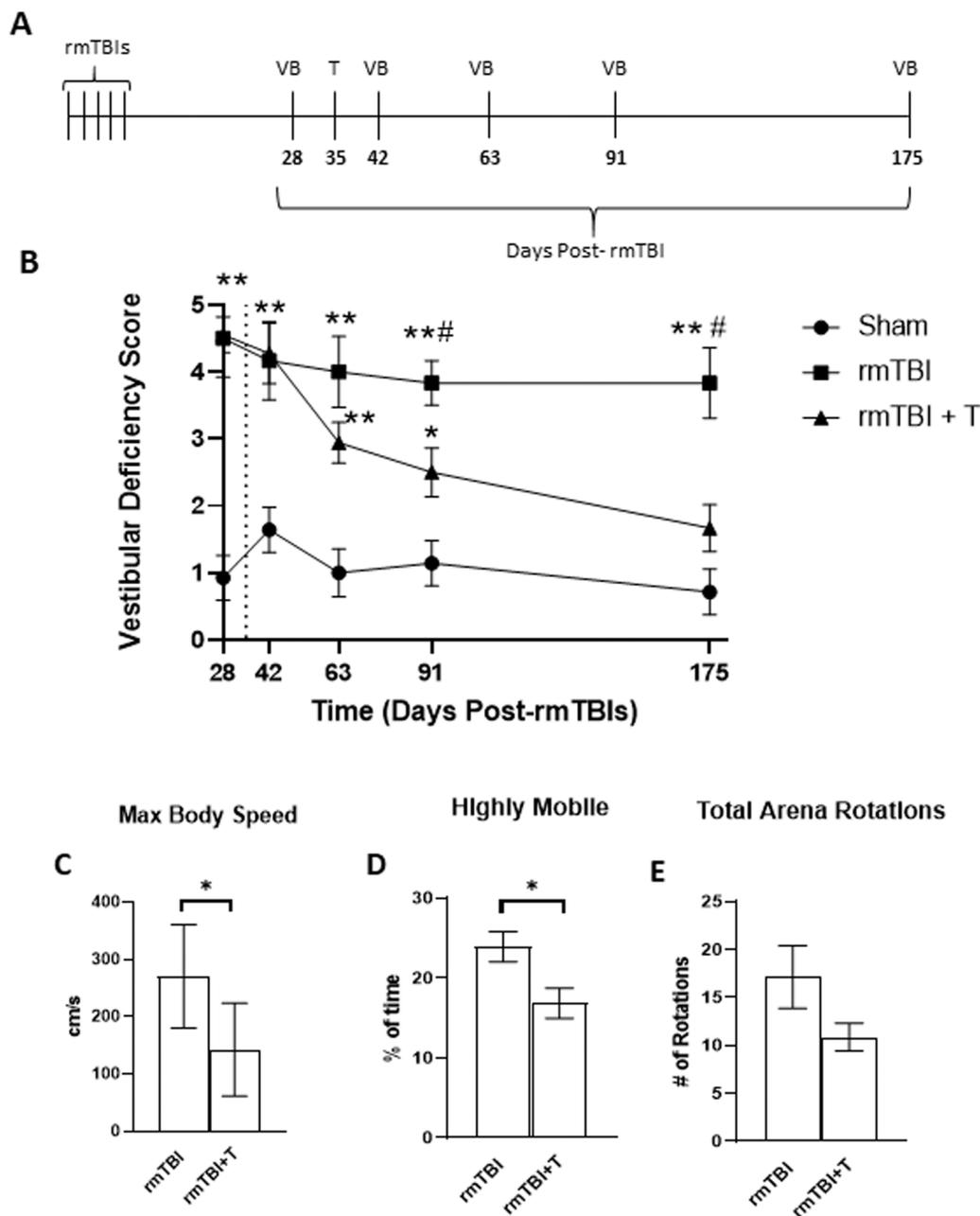
## 3. Results

### 3.1. Repetitive mild TBI elicits sustained vestibular impairment

The Sham intact (Sham INT) group had a vestibular deficiency score of 1.5 or less consistently across all time points. By comparison, a three-way ANOVA with repeated measures revealed a significant effect of injury ( $F(1,52) = 21.55$ ,  $p < 0.0001$ ,  $\eta = 0.56$ ) but there was no significant effect of time. A Tukey multiple comparisons test revealed that both rmTBI intact (rmTBI INT) and rmTBI castrated (rmTBI CAS) had significantly worse vestibular scores as compared to Sham INT at all time points ( $*p < 0.05$ ,  $****p < 0.0001$ ). The rmTBI CAS groups also



**Fig. 2.** Repetitive mild TBI significantly alters open field vestibular behavioral assessments. Rotational behaviors that equate to vestibular function were analyzed with Noldus EthoVision XT 15 at 28 DPI and 42 DPI. Increased (A) total axis rotations (B) mean turn angle (C) total arena rotations (D) highly mobile state (E) maximum body speed and (F) head speed was observed following rmTBI. Data represented as Mean  $\pm$  SEM. ( $*p < 0.05$ ,  $**p < 0.01$  as compared to the Sham).



**Fig. 3. Testosterone treatment restores vestibular function with vestibular battery tests and open field assessments.** (A) A timeline demonstrating that testosterone (T) treatment occurred at 35 DPI and vestibular battery (VB) testing occurred on subsequent days to follow. (B) T treatment significantly reduced vestibular deficiency scores (\*\* $p < 0.01$  as compared to the Sham, # $p < 0.05$  as compared to rmTBI + T). At 175 DPI, the rmTBI + T group was no longer statistically distinct from the Sham group. T treatment significantly reduced (C) mean body speed and (D) highly mobile state at 175 DPI as compared to rmTBI animals (\* $p < 0.05$ ). (E) A reduction trend was observed in the total arena rotations.

had significantly worse vestibular deficiency scores at 1 DPI (\* $p < 0.05$ ), 7 DPI (\*\* $p < 0.01$ ), and 28 DPI (\*\* $p < 0.01$ ) as compared to the Sham castrated (Sham CAS), further demonstrating the effect of injury on vestibular function.

A three-way ANOVA with repeated measures also revealed significant effects of gonadal status on vestibular function ( $F(1,52) = 91.12$ ;  $p < 0.0001$ ,  $\eta = 0.23$ ). A Tukey multiple comparisons test revealed the Sham CAS group had significantly worse vestibular scores at 28 DPI ( $2.36 \pm 0.30$ ) compared to Sham INT group ( $0.93 \pm 0.18$ , \* $p < 0.05$ ). Interestingly, there was no effect of gonadal status between the rmTBI groups. This may be due, in part, because the rmTBI INT group had significantly reduced serum testosterone levels as a percentage of their baseline serum levels when standardized to age matched Sham controls ( $17.3 \pm 10.67\%$  at 1 DPI,  $51.8 \pm 20.3\%$  at 7 DPI and  $25.5 \pm 8.5\%$  at 28 DPI;  $p < 0.05$ ;  $n = 6/\text{group}$ ) at each time point. Serum testosterone was reduced to undetectable levels in the Sham CAS and TBI CAS groups ( $n = 6/\text{group}$ ). Since there was no detectable difference on vestibular deficiency scores within the rmTBI groups at all time points determined,

all subsequent studies evaluated rmTBI animals to the Sham INT group.

### 3.2. Vestibular functional deficits following rmTBI confirmed with open field video tracking system

rmTBI significantly altered multiple assessments associated with vestibular function as shown in Fig. 2 and Table 1. When assessing rotational behaviors that equate to vestibular function, rmTBI elicited a significant increase in total axis rotations at 42 DPI (Fig. 2A, \*\* $p < 0.01$ ) and mean turn angle at 28 DPI (Fig. 2B, \* $p < 0.05$ ). Although the total arena rotations were not significant at either time point (Fig. 2C), the directional breakdown of arena rotations in Table 1 showed that ipsilateral arena rotations were increased at 28 DPI (\* $p < 0.05$ ) and 42 DPI (# $p = 0.0568$ ). Mean absolute meander was also significantly increased at 42 DPI (\*\* $p < 0.01$ ).

The vestibular nuclei lesion studies also demonstrated that vestibular neuronal loss is associated with alterations in speed and locomotion [66, 67]. rmTBI elicits significant increases in animals moving in a highly

**Table 1**  
Vestibular behavioral assessments in the open field.

	DPI 28		DPI 42	
	Sham	rmTBI	Sham	rmTBI
<b>Rotational Behavior</b>				
Axis Rotations (number of rotations):				
Ipsilateral	3.43 ± 0.75	2.43 ± 0.49	0.75 ± 0.48	4.75 ± 1.03**
Contralateral	2.00 ± 0.38	3.43 ± 0.55*	2.50 ± 0.29	4.75 ± 0.84 <sup>#</sup>
Arena Rotations (number of rotations):				
Ipsilateral	7.57 ± 0.61	10.29 ± 0.92*	8.50 ± 0.87 11.38 ± 1.0 <sup>#</sup>	
Contralateral	8.57 ± 1.86	10.64 ± 1.50	9.78 ± 2.50 14.13 ± 0.88	
Mean Absolute Meander (°/cm)	61.96 ± 7.85	51.28 ± 3.89	38.63 ± 5.70 77.94 ± 3.43**	
<b>Locomotor Activity</b>				
Total Distance Traveled (cm):				
Body	3318 ± 225	3275 ± 217	3220 ± 298	4199 ± 193*
Head	7244 ± 376	6978 ± 417	7410 ± 510	9030 ± 262*
Mean Speed (cm/s):				
Body	17.08 ± 0.82	17.85 ± 0.75	16.14 ± 1.10	19.37 ± 0.65 <sup>#</sup>
Head	41.53 ± 1.72	43.29 ± 1.41	47.02 ± 2.48	45.76 ± 1.48
Duration Not Moving (% time)	19.40 ± 3.11	31.07 ± 3.09*	23.37 ± 1.62	26.12 ± 1.79
Duration Immobile (% time)	0.72 ± 0.25	6.43 ± 2.50	0.30 ± 0.30	2.36 ± 0.68**

DPI—days post rmTBI, **rmTBI**—repetitive mild TBI (\*  $p < 0.05$ , \*\* $p < 0.01$ , <sup>#</sup> $p < 0.10$ ).

mobile state and increased maximum body and head speed (Fig. 2D–F, \* $p < 0.05$ , \*\* $p < 0.01$ ) at 42 DPI as compared to Sham animals. Additional significant locomotor behaviors were observed to be altered in the rmTBI animals as compared to the Sham and are listed in Table 1 (\* $p < 0.05$ , \*\* $p < 0.01$ ). Collectively, these data agree well with the vestibular deficiency scores and the vestibular battery of tests that our closed-head rmTBI animal model produces marked and significant chronic vestibular deficits.

### 3.3. Testosterone replacement restores vestibular function following repetitive mild TBI

To determine the therapeutic role of testosterone on the restoration of vestibular function after chronic vestibular impairment, animals were implanted with vehicle or testosterone (T) capsules at 35 DPI and subsequently evaluated at 42 DPI, 63 DPI, and 175 DPI as depicted in Fig. 3A. No differences in vestibular function were observed between rmTBI experimental animals prior to their randomization (Fig. 3B). A two-way ANOVA, however, revealed a statistically significant effect between groups ( $F(2,19) = 26.02$ ; \*\*\* $p < 0.0001$ ,  $\eta = 0.75$ ) and time ( $F(3,59) = 9.562$ ; \*\*\* $p < 0.0001$ ,  $\eta = 0.33$ ) on vestibular function. In Fig. 3B, Tukey's multiple comparisons post-hoc test exhibited no significant differences between the rmTBI groups at 42 DPI and 63 DPI. By comparison, both rmTBI experimental groups displayed significant vestibular deficiencies compared to the Sham control group (\*\* $p < 0.01$ ). At 91 DPI and 175 DPI, testosterone treated rmTBI animals demonstrated marked improvement in vestibular function compared to the rmTBI group ( $\# P < 0.05$ ). Importantly, at 175 DPI, the rmTBI + T group was no longer statistically distinct from the Sham control group. When using open field analysis of the parameters deemed

to have vestibular functional components, rmTBI + T animals demonstrated significant improvement in max body speed (Fig. 3C) and highly mobile state (Fig. 3D) at 175 DPI as compared to rmTBI animals (\* $p < 0.05$ ). Although not significant, reduction trends were observed in the total arena rotations (Fig. 3E,  $p = 0.124$ ).

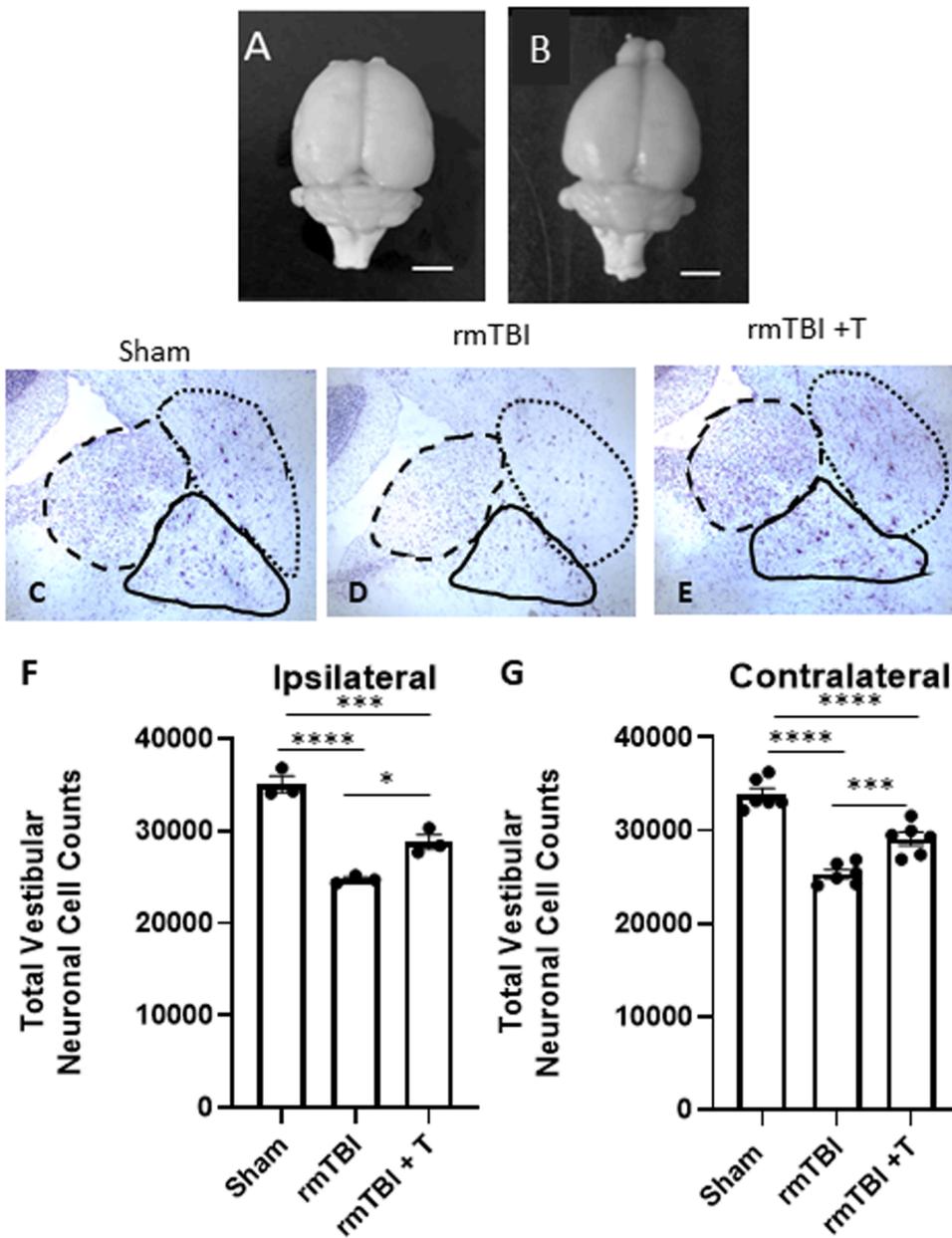
### 3.4. Repetitive mild TBI results in chronic neuronal cell loss

In contrast to open head TBI models, our closed-head rmTBI model did not produce any detectable gross histopathological alteration in brain architecture at 175 DPI consistent with most clinical TBI injuries (Fig. 4A and B). However, quantitative changes in neuronal cell counts were observed in the thionin-stained vestibular nuclei. Fig. 4C–E displays sample sections of the thionin-stained vestibular nuclei from the Sham control, rmTBI and rmTBI + T experimental animals, respectively. The total number of thionin-stained vestibular neurons in the entire vestibular subnuclei was summed for both the ipsilateral (Fig. 4F) and contralateral sides (Fig. 4G). Two-way ANOVA analysis detected a significant effect of both injury and treatment for the ipsilateral (\*\*\* $p < 0.0001$ , Injury  $F(1,6) = 109.8$ ,  $\eta = 0.95$ ; Treatment \* $p < 0.01$ ,  $F(1,6) = 17.02$   $\eta = 0.74$ ) and contralateral (\*\* $p < 0.001$ ,  $F(1,6) = 64.40$ ,  $\eta = 0.91$ ; Treatment \* $p < 0.01$   $F(1,6) = 15.16$ ,  $\eta = 0.72$ ) vestibular nuclei. Total vestibular neuronal counts revealed significant decrease in neuronal cell counts in both the ipsilateral ( $24,822 \pm 1083$  neurons, \*\*\* $p < 0.0001$ ) and contralateral ( $25,355 \pm 463$  neurons; \*\*\* $p < 0.0001$ ) vestibular nuclei of the rmTBI animals as compared to the sham control animals ( $34,537 \pm 567$  neurons,  $33,876 \pm 663$  neurons, respectively). This equates to a 28.1 % and 25.2 % cell loss in the ipsilateral and contralateral vestibular nuclei, respectively. Testosterone treatment significantly improved neuronal cell counts in both the ipsilateral ( $28,407 \pm 770$  neurons, \* $p < 0.05$ ) and the contralateral ( $29,123 \pm 701$  neurons, \*\*\* $p < 0.001$ ) nuclei, equating to a 10.4 % and 11.2 % increase in neuronal survival, respectively. Interestingly, there was no differences of neuronal vestibular counts between the ipsilateral and contralateral sections of the rmTBI animals, suggesting that the neuronal loss from rmTBI is due to a diffuse secondary injury.

## 4. Discussion

Vestibular dysfunction is one of the leading causes of disability following mild TBI, and such impairment is exacerbated with repetitive mild TBIs [18,69]. Many patients demonstrate some recovery from deficits, but a subset develops long-term vestibular impairment in the broader scope of post-concussion syndrome [17,70]. Utilizing a clinically relevant closed-head rmTBI animal model, our modified vestibular battery of tests was able to reliably detect both acute and chronic vestibular impairment following five successive injuries consecutively spaced 48 h apart. Strikingly, administration of testosterone after the development of established chronic vestibular dysfunction elicited marked improvement in vestibular function possibly due to improved neuronal survival. To our knowledge, this is the first study to quantify the effects of testosterone on restoration of vestibular function in a clinically relevant closed-head model of TBI.

Previous studies have sought to detect chronic vestibular impairment utilizing the rotarod or the balance beam walk [71,72]. Importantly, these tests can be confounded by stimulation of motor components. The rotarod has been used in many studies to specifically isolate the motor [13,73] and vestibular system [74]. Vestibular impairment in this study was detected by a battery of tests developed to detect partial dysfunction and minimize potential confounds with other sensory components. Thus, a modified vestibular battery of tests was utilized to rigorously isolate vestibular function in this paper. While the vestibular and motor sensory components are experimentally inseparable, the battery of vestibular tests used here significantly reduced interference of motor function allowing for an improved approximation of isolated vestibular



**Fig. 4. Significant vestibular neuronal cell loss with repetitive mild TBI that is improved with testosterone.** (A) Sham and (B) rmTBI brains harvested at 175 DPI do not show any detectable gross histopathological alteration in brain architecture. Representative pictures of thionin-stained sections of the (C) Sham, (D) rmTBI, and (E) rmTBI + T groups show the medial magnocellular vestibular nucleus depicted within the solid line, the medial parvocellular vestibular nucleus within the dashed lines, and superior vestibular nucleus within the dotted lines. Total vestibular neuronal cell counts throughout the entire vestibular nucleus were quantified in the (F) ipsilateral and (G) contralateral vestibular nuclei. rmTBI resulted in significant reduction in neuronal cell counts (\*\*\*\* $p < 0.0001$  and \*\*\* $p < 0.001$  as compared to the Sham group) that was markedly improved in the rmTBI + T group (\* $p < 0.05$ , \*\*\* $p < 0.001$  as compared to the rmTBI group).

function. Video analysis using open field parameters established in vestibular nuclei lesions studies was further used in this study to provide objective confirmation of chronic vestibular behavioral deficits [66,67]. Using the open field analysis, the chronic vestibular deficit in the rmTBI animals took longer to observe compared to the vestibular lesion studies, likely due to the fact that the rmTBI injury is not as severe of an injury as compared to the immediate vestibular neuronal loss in a lesion study. However, this study has demonstrated that the open field video analysis is sensitive enough to detect vestibular deficit in less severe injury models.

A stepwise increase of vestibular deficiency score averages for experimental groups was observed immediately following rmTBIs whereby the Sham INT control group exhibited the lowest deficiency scores followed by increasing scores in the Sham CAS, TBI INT, and TBI CAS. Castration elicited significant vestibular deficit indicating a critical role of testosterone for proper vestibular function. rmTBI significantly increased the vestibular deficiency scores at all time point compared to Sham INT, indicative of effect of injury on vestibular function. Although rmTBI CAS consistently had higher vestibular deficiency scores

compared to rmTBI INT, no significant difference was observed between these groups. One possible explanation for the lack of significant difference observed between the two experimental rmTBI groups is that both groups have lower testosterone levels as a result of either injury or castration.

Many of the current pharmacological studies following mild TBI or rmTBI focus on therapies administered in the acute phase following injury. However, patients with mild TBI or rmTBI are not often treated in the acute phase because many show improvement without intervention or they simply do not seek treatment. The most common clinical scenario is only that rmTBI patients seek treatment after the symptoms become chronic. Therefore, it is of high clinical importance to development therapeutic modalities that can improve function following persistent dysfunction. The current study treated the rmTBI animals with testosterone at 35 DPI, after the confirmation of established chronic vestibular dysfunction. Testosterone treatment significantly improved vestibular function even when administered after the development of established chronic vestibular impairment. It is important to note that the improvement in vestibular function seen with testosterone treatment

was not immediate. In fact, it took approximately 2 months to observe a significant restoration of vestibular function. As noted previously, testosterone is not only necessary for reproductive function, but also inhibits pro-inflammatory cytokine secretion and stimulates neuroprotective secondary messenger cascades promoting neuroplasticity, regeneration, and synaptic potentiation [42,43,54]. Additionally, long-term reduction in testosterone levels may also cause cumulative cellular damage and reactive oxygen species formation, delaying the effects of testosterone. This is the first study to demonstrate testosterone can enhance vestibular functional recovery even when given after the establishment of chronic vestibular dysfunction, suggesting a potential therapeutic role for testosterone following rmTBI.

The mechanism by which testosterone may help restore vestibular function following rmTBI may involve improved neuronal survival. Significant vestibular neuronal death was observed in the rmTBI animals. Interestingly, testosterone-treated rmTBI animals displayed significantly more thionin stained vestibular neurons compared to vehicle control rmTBI animals. Although testosterone treatment significantly improved vestibular neuronal survival, the rmTBI + T group still demonstrated less neuronal survival compared to the Sham control group. The initial damage from rmTBIs that existed for 35 days prior to testosterone replacement therapy may have caused a baseline amount of neuronal death that was not recoverable. We speculate that testosterone treatment at 35 DPI prevented further neuronal cell death by resolving the neuroinflammatory environment and possibly initiating cellular and synaptic recovery. Interestingly, there did not seem to be large differences of neuronal vestibular counts between the ipsilateral and contralateral sides, indicating that the injury to the vestibular system in our closed-head rmTBI model is due to a diffuse secondary injury that elicits injury to both sides of brainstem equally.

## 5. Conclusion

This study examined the effects of repetitive mild traumatic brain injury on vestibular function and is the first to demonstrate that testosterone treatment, even when given after the onset of established chronic vestibular deficiency, enhanced restoration of vestibular function and improved vestibular neuronal cell counts. The vestibular battery of tests provided a reliable method for examining acute and chronic vestibular specific deficits and may be used for other injury and disease courses. This study also identified that the open field video analysis is sensitive enough to detect vestibular deficits even in less severe vestibular injury models. Future studies will be necessary to determine the time course and mechanism underlying the vestibular dysfunction and neuronal cell loss from rmTBI. Since women commonly have greater rates of vestibular deficits and more severe symptoms following TBI [75], future studies will be directed toward rmTBI in females to determine why such sex differences exist and what role gonadal steroid might play in the recovery of vestibular function.

## CRedit authorship contribution statement

**Eileen Foecking:** Conceptualization, Writing – original draft, Writing – review & editing, Resources, Formal analysis, Supervision. **Arthur Segismundo:** Investigation, Project administration, Writing – original draft, Data curation, Formal analysis. **Krista Lotesto:** Investigation, Formal analysis, Validation. **Edward Westfall:** Investigation. **Alyssa Bolduan:** Investigation. **Tony Peter:** Investigation. **Douglas Wallace:** Methodology, Writing – review & editing. **Dorothy A. Kozlowski:** Methodology, Writing – review & editing. **Evan Stubbs:** Resources, Writing – review & editing. **Sam Marzo:** Conceptualization. **Susanna Byram:** Data curation, Writing – review & editing.

## Author Disclosure Statement

No competing financial interests exist.

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