Serum neurofilament light as a biomarker of vulnerability to a second mild traumatic brain injury

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Title: Serum neurofilament light as a biomarker of vulnerability to a second mild traumatic brain injury

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Abstract:

A second mild traumatic brain injury (mTBI) sustained prior to neuropathological recovery can lead to exacerbated effects. Without objective indicators of this neuropathology, individuals may return to activities at risk of mTBI when their brain is still vulnerable. With axonal injury recognised as a neuropathological hallmark of mTBI, we hypothesized that serum levels of neurofilament light (NfL), a highly sensitive biomarker of axonal injury, may be predictive of vulnerability to worse outcomes in the event of a second mTBI. Given this hypothesis is difficult to test clinically, we used a two-hit model of mTBI in rats and staggered inter-injury intervals by 1-, 3-, 7- or 14-days. Repeat-mTBI rats were dichotomised into NfL$^{\text{high}}$ (NfL>median at the time of re-injury) and NfL$^{\text{low}}$ (NfL<median) groups, with behaviour and NfL levels analysed throughout the 28-days, followed by ex vivo diffusion tensor imaging. NfL levels at the time of the second mTBI were found to be predictive of vulnerability to re-injury, with NfL$^{\text{high}}$ rats displaying more neurological signs and a greater potentiation of NfL levels after the second mTBI. Importantly, this potentiation phenomenon remained even when limiting analyses to rats with longer inter-injury intervals, providing evidence that vulnerability to re-injury may not be exclusively dependent on inter-injury interval. Finally, NfL levels correlated with, and were predictive of, the severity of neurological signs following the second mTBI. These findings provide evidence that measurement of NfL during mTBI recovery may be reflective of the vulnerability to a second mTBI, and as such may have utility to assist return to sport, duty and work decisions.
**Introduction:**

Mild traumatic brain injury (mTBI) is a highly prevalent injury in collision sports and in the military[1-4]. There is mounting evidence that a history of mTBI is a major risk factor for suffering a future mTBI, and for experiencing worse symptoms for a longer duration should another mTBI occur[5-7]. The latency between mTBIs appears to be critical, with evidence suggesting that the impact of a previous mTBI on symptom severity and duration lessens as time elapses[8, 9]. The risk of mTBIs in short succession creating a significant cumulative burden has led to the hypothesis that the pathophysiology of mTBI may create a ‘window of increased cerebral vulnerability’ to repeated injury[8-13]. As such, there is a widely recognized need to identify objective tools that can help detect and monitor the heterogeneous neuropathology of mTBI to assist individual clinical decisions, such as when it is safe to return to play, duty or any other at-risk activity after concussion[14].

Diffuse axonal injury is now recognized as a key neuropathological feature of mTBI[15-17]. We and others have recently shown that serum levels of neurofilament light (NfL), a highly sensitive and specific biomarker of neuroaxonal damage, are elevated after mTBI and remain elevated beyond typical symptom resolution[18-23]. While consistently elevated at a group level, the extent and trajectory of NfL changes after mTBI appears to be variable between individuals[18, 19, 23]. We hypothesised that the extent and duration of this neuropathology after mTBI may be a mechanism of a variable vulnerability to repeat mTBI (rmTBI), and as such, serum NfL quantification may have utility to not only identify axonal damage in individuals with suspected mTBI, but also to track neuropathological progression, recovery, and vulnerability to further injury.
This cerebral vulnerability hypothesis is however very difficult to investigate in humans. As such, here we utilised a two-mTBI model in rats, and investigated whether serum NfL levels at the time of a second impact were related to neuropathological and behavioral outcomes. We hypothesised that outcomes would be significantly worse in rats with higher levels of serum NfL at the time of the second mTBI.

Materials and Methods

Animals

Eighty-four male adolescent Sprague-Dawley rats were housed in cages of three on a 12-hour light/dark cycle. Food and water were available ad libitum. All procedures were approved by the Alfred Medical Research and Educational Precinct Animal Ethics Committee (E/2081/2021/M). Animal studies are reported in compliance with the ARRIVE guidelines and the Australian code of practice for the care and use of animals for scientific purposes by the Australian National Health and Medical Research Council.

Experimental groups

Rats were randomly allocated to sustain two mTBI procedures (rmTBI), one sham and one mTBI (single mTBI), or two sham procedures (sham). The rmTBI rats were further broken down into four groups defined by their inter-injury interval: 1-, 3-, 7-, or 14-days. To mitigate the effect of age on injury severity, all groups contained two sub-groups; the first group sustained their first mTBI/sham procedure at the same age, and the second group sustained their second mTBI/sham procedure at the same age. This produced a consistent mean PND (i.e. PND 47) of each mTBI/sham procedure.
Six rats that recorded a score of zero for the video analysis of mTBI-related signs (described below) as well as deemed by an investigator at the time of mTBI procedure to sustain an unsuccessful mTBI were excluded from analyses. One rat was excluded from all analyses due to death immediately following the mTBI procedure, and another due to a cage-associated injury. As such, 76 rats were included in the final analyses: rmTBI-1d (n=13), rmTBI-3d (n=12), rmTBI-7d (n=13), rmTBI-14d (n=13), single-mTBI (n=12) and sham (n=13). To assess the primary question of the association between serum NfL levels at the time of injury and subsequent outcomes, all rmTBI groups (irrespective of inter-injury group) were dichotomised by their NfL level at the time of the second mTBI; the first of which had serum NfL levels less than the median ($NfL_{\text{low}}$), and those that had greater than the median ($NfL_{\text{high}}$). With six 0-day blood samples unable to be collected, final group sizes for the primary analysis were n=23 rats for $NfL_{\text{high}}$ and $NfL_{\text{low}}$ groups. An overview of the study protocol for the primary analysis of $NfL_{\text{high}}$ and $NfL_{\text{low}}$ comparisons is shown in Figure 1.

**Awake closed head injury (ACHI) model of mTBI**

The ACHI model is a well-characterized and clinically relevant model of concussive-like injury[24-26]. It avoids the confounds of anaesthetic and craniotomy present in other models, features clinically relevant biomechanics and induces behavioral deficits that are typically transient. The ACHI impact/sham protocol, and two day habituation protocol was performed as previously described [25]. All behavioural testing described herein was performed at consistent time-points relative to the final sham/mTBI.

**Video quantification of observable neurological signs of mTBI**

By avoiding anaesthetic, the ACHI model of mTBI allows for clinically relevant neurological signs to be observed and quantified immediately after the injury. As such, we developed a scale for quantifying neurological signs in rats that are observable on video. This scale was
developed following the ‘International consensus definitions of video signs of concussion’[27]. This scale quantifies four mTBI-related signs: i) post-impact seizure/tonic posturing; ii) motionlessness; iii) forelimb incoordination whereby one, or both forelimbs were unable to grasp a 2cm beam); and iv) hind limb incoordination whereby the hind limbs slip off the 2cm beam and/or an inability to balance on the beam. The presence of each sign was given a score of one, with a maximum score of four. All videos were analysed by an investigator blinded to the experimental conditions.

**Rotarod**

Sensorimotor function was assessed using the rotarod task[28]. Briefly, rats were placed on a rod accelerating from 4 rpm to 40 rpm over a five-minute period, with latency to fall the primary outcome. Three trials were performed for each session conducted at baseline, and at 1-, 2-, 6-, 13-, and 27-days post the second ACHI/sham.

**Water maze**

Spatial memory was assessed using a water maze previously described[26]. Briefly, a 175cm diameter pool filled with tap water (28 degrees Celsius) with four different visual stimuli suspended above the edge of the pool, and a hidden platform located 3.5 cm below the water surface. Rats were placed at pseudo-random starting locations for ten trials per day (acquisition testing on day 4, reversal on day 5), with the visual stimuli designed to be used as cues to locate the hidden platform. TopScanLite version 2.0 was used to track rats, with latency to find the platform used a measure of spatial memory.

**Elevated plus maze (EPM)**

The EPM is a measure of anxiety-like behavior and was performed 12-days following the second mTBI/sham following protocols previously described[25, 26]. Briefly, a ‘plus-shaped’
maze featuring two 30cm high walls (closed arms) and two arms without walls (open arms) was used, with reduced time spent in the open arms an indicator of anxiety-like behavior.

**Blood and tissue collection:**

Whole blood was collected from the lateral tail vein 1-hour prior to the second mTBI/sham (i.e. 0-day), and 3-, 7-, and 14-days afterwards. Rats were lightly anaesthetised via isoflurane inhalation, a 23” gauge needle was then inserted into the lateral tail vein, and blood slowly collected into BD SST™ microtainer tubes. Blood was centrifuged at 1,500g for 10 minutes, with serum collected and stored at -80 degrees Celsius. At 28-days rats were euthanized as described previously[26], with whole blood collected via cardiac puncture and brains fixed in paraformaldehyde.

**Magnetic Resonance Imaging (MRI)**

DTI was performed with a 9.4T Bruker MRI using a 2-shot echo planar imaging sequence. Diffusion-weighting was performed in 61 directions with $\delta = 4.2$ ms, $\Delta = 12$ ms and b-values $= 2000$ and $4000$ s/mm$^2$. Two $b = 0$ ($b_0$) volumes were also acquired. Other imaging parameters were adjusted to give an isotropic resolution of 250 μm: repetition time = 8 s; echo time = 50 ms; field of view = 3.2 x 2.4 cm$^2$; and 48 axial slices. A subsequent $b_0$ image was acquired with the same imaging parameters and the phase reversed for distortion correction using FSL’s topup [29]. Image processing was performed as previously described using FSL and MRtrix3 software [30, 31]. The mean fractional anisotropy (FA)-value was determined for the ipsilateral and contralateral genu, body and splenium of the corpus callosum.

**Serum NfL quantification**
NFL was quantified on a SIMOA® HD-X Analyzer™ using NFL Advantage kits (Quanterix, Billerica, MA, USA) following manufacturer’s instructions. All samples were above the lower limit of detection (0.038 pg/mL). Two samples were run across all plates with an average inter-plate coefficient of variation (CV) of 6.2%, and the intraplate CV of 4.8%.

Statistics

To investigate differences between the NFL<sub>low</sub> and NFL<sub>high</sub> group on behavioral outcomes a Mann-Whitney U-test was used with an α-value of 0.05 for signs of mTBI and EPM outcomes, and an adjusted α-value of 0.0083 for rotarod and 0.01 for water maze. To assess the relationship between NFL levels pre- and post- the second mTBI, NFL concentrations underwent a natural logarithmic transformation (Ln) and levels across time were assessed with a two-way ANOVA. Post-hoc analyses were performed with Bonferroni’s multiple comparisons. The magnitude of serum NFL change (ΔNFL) was assessed with a Mann-Whitney U-test. MRI outcomes for the three regions of the corpus callosum (genu, body and splenium) were analysed on hemispheres independently with a paired t-test with an adjusted α-value of 0.017.

Spearman’s correlation assessed for the association between observable neurological signs and serum NFL levels. The number of neurological signs in the rmTBI rats was also dichotomized as mild (≤2 signs, n=18), or severe (>2 signs, n=28), with classification ability of serum NFL assessed using an area under the receiver operating characteristic (AUROC) analyses and closest-to-(0,1) corner approach used to determine the optimal cut-off points[32].

To assess the association between inter-injury interval on behavioral and MRI outcomes, a Kruskal-Wallis H test was used to assess for group differences for all outcomes with an α-value of 0.05 for video signs of mTBI and EPM outcomes, and an adjusted α-value of 0.0083
for the rotarod, 0.01 for water maze and 0.017 for MRI outcomes. To assess the relationship between inter-injury interval and subsequent NfL outcomes, NfL concentrations were Ln transformed and a mixed-effects model with the Giesser-Greenhouse correction was performed. Post-hoc analyses were performed with Bonferroni’s multiple comparisons test. An overall group effect of injury on ΔNfL was assessed using a Kruskal-Wallis H test. MRI outcomes were assessed with a Mann-Whitney U-test. All statistical analyses were performed with GraphPad Prism GraphPad Prism version 8.0.2 for Windows (GraphPad Software, CA).

Results:

*NfL*<sup>high</sup> rats had more observable neurological signs and a potentiated serum NfL profile after a second mTBI.

The median serum NfL levels of all rmTBI rats at the time of the second mTBI (i.e., day-0) was found to be 32.0 pg/mL. Rats were then split into two groups (Figure 2A); the first of which had serum NfL levels less than 32.0 pg/mL (*NfL*<sup>low</sup>), and those that had greater than 32.0 pg/mL (*NfL*<sup>high</sup>). Dichotomised by NfL levels at the time of injury, there was an overall effect of group (F(1,44)=44.09, p<0.0001), time (F(2.69,118)=195, p<0.0001) and group X time interaction (F(4,176)=20.8, p<0.0001) on the post second-mTBI profile of Ln serum NfL (Figure 2B). Post-hoc analyses revealed that serum Ln NfL levels were elevated in the *NfL*<sup>high</sup> group at day-3 (95%CI: 1.70-0.663, p<0.0001), day-7 (95%CI: 1.30-0.459, p<0.0001), day-14 (95%CI: 1.04-0.361, p<0.0001) but not day-28 (95%CI:0.562-0.116, p=0.415) compared with the *NfL*<sup>low</sup> group.

To assess the magnitude of serum NfL change after a second mTBI, levels at the time of the second mTBI (i.e., day-0) were subtracted from the serum NfL levels at day-3 (ΔNfL = NfL<sub>3d</sub> - NfL<sub>0</sub>).
– NfL<sub>0d</sub>). NfL<sup>high</sup> rats were found to have a greater ΔNfL than NfL<sup>low</sup> rats (U=173, Median NfL<sup>low</sup>=13.5 pg/mL, Median NfL<sup>high</sup>=44.3 pg/mL, p=0.0448; Figure 2C). The magnitude of ΔNfL was further assessed excluding the shorter inter-injury interval rmTBI groups (i.e., 1-day and 3-day interval), with the rationale being that: i) 7-day and 14-day intervals may be more comparable to typical rmTBI exposure in humans (although days in rats have been compared to weeks in humans), and ii) our previous data with a single injury revealed that NfL levels have plateaued at 7- and 14-days, and therefore ΔNfL values are less impacted by the dynamic changes that occur more acutely. For the rmTBI 7-day and 14-day rats only, the time of second mTBI NfL median was found to be 22.6 pg/mL, with rats subsequently grouped as NfL<sup>high+</sup> (>22.6 pg/mL, n=12) or NfL<sup>low+</sup> (<22.6 pg/mL, n=12). NfL<sup>high+</sup> rats were found to have a greater ΔNfL than NfL<sup>low+</sup> rats (U=28, Median NfL<sup>low*</sup>=21.3 pg/mL, Median NfL<sup>high*</sup>=46.1 pg/mL, p=0.01; Figure 2D).

Finally, NfL<sup>high</sup> rats had a greater number of observable neurological signs of mTBI immediately after the second mTBI when compared to NfL<sup>low</sup> rats (U=143.5, Median NfL<sup>low</sup>= 2, median NfL<sup>high</sup>= 3, p=0.0044; Figure 2E).

**Serum NfL levels at the time of a second mTBI correlate with, and are prognostic of, observable neurological signs**

Spearman’s correlations analyses tested the association between the number of observable neurological signs, and serum NfL levels at the time of second mTBI (day-0), and at 3-, 7-, 14-, and 28-days post-injury. For all rmTBI rats, neurological signs correlated with NfL at the time of injury (Figure 3A; r=0.500, p=0.0004), and at 3- (Figure 3B; r=0.636, p<0.0001), 7- (Figure 3C; r=0.522, p=0.0002) and 14- (Figure 3D; r=0.498, p=0.0004), but not 28-days (Figure 3E; r=0.273, p=0.0662).
The ability of time of injury NfL levels to predict injury severity, as determined by observable neurological signs immediately after the second mTBI (dichotomised by ≤2 or >2 signs) was assessed with the AUROC analysis (Figure 3F). The area under the curve was 0.73 (95% CI: 0.59-0.87, p=0.009) with a sensitivity of 0.64 (95% CI: 0.46-0.79) and specificity of 0.78 (95% CI: 0.55-0.91) at the optimal cut-off of 33.3 pg/mL.

Serum NfL levels at the time of a second mTBI versus sub-acute behavior and chronic white matter integrity.

To assess the relationship between NfL level at the time of injury with behavioral outcomes in the sub-acute stages after the second mTBI, comparisons were made between NfL$_{\text{low}}$ and NfL$_{\text{high}}$ groups for each task. No differences were found in water maze latency to escape for the acquisition (day 4) or reversal testing (day 5). No differences were found for the time spent in the EPM open arm (day 12), nor for rotarod latency to fall at baseline, or 1-, 2-, 6-, 13-, or 27-days post the second mTBI. Sub-acute behavioral data are detailed in Supplementary Figure 2 and Supplementary Table 1.

We investigated brain white matter integrity at 28-days using DTI. Region of interest was the three regions of the corpus callosum per hemisphere (genu, body and splenium; Figure 4). Despite trends of reduced FA in NfL$_{\text{high}}$ rats, with an α-value of 0.017 (0.05 / 3 regions), no significant differences in FA were found on the left (i.e. ipsilateral side to injury) genu (p=0.0458), body (p=0.269) or splenium (p=0.0450) of the corpus callosum. Similarly, no significant differences were found on the right hemisphere for the genu (p=0.0706), body (p=0.846) or splenium (p=0.293).

The relationship between inter-injury interval and serum NfL outcomes
For the effect of inter-injury interval on serum NfL levels across time (Figure 5A), there was an overall effect of time (F(2.48,170)=195, p<0.0001), group (F(5,70)=14.5, p<0.0001) and time X group interaction (F(20,274)=17.2, p<0.0001). Notably, multiple comparisons revealed that all groups were different to sham at 3- and 7-days. At 14-days, while NfL levels in the rmTBI groups remained higher than sham group, the single mTBI group was no longer different, and at 28-days, only the rmTBI (1-day interval) group was different to sham. See Supplementary Table 2 for a full summary of post-hoc results. No effect of inter-injury interval was found for the ΔNfL ((H(3)=6.77, P=0.0795; Figure 5B).

Inter-injury interval versus sub-acute behavior and chronic white matter integrity.

No differences were found between inter-injury interval groups for the number of observable neurological signs immediately after the second mTBI, EPM time in open arm, latency to find the hidden platform in the water maze, and latency to fall from the rotarod (Supplementary Figure 3 and Supplementary Table 3). For the effect of inter-injury interval on FA in the corpus collosum, no group differences were found (Supplementary Figure 4).

Discussion:

Following an mTBI, the brain can be highly vulnerable to a subsequent mTBI, with this increased vulnerability postulated to be due to pathophysiological changes induced by the first mTBI[8-13]. As such, there is a need for objective biomarkers that can detect the severity of injury and monitor pathophysiological recovery following an mTBI, to ultimately assist decisions surrounding the safe return to sport, duty or other activities at-risk of mTBI[14]. Here, we quantified serum NfL, a clinically applicable circulating biomarker of...
axonal injury, to determine whether levels of this protein at the time of a second mTBI are associated with worse outcomes following rmTBI. Overall, our findings supported the hypothesis that high serum NfL levels at the time of a second mTBI are associated with a greater vulnerability to axonal damage and functional impairments. We found that NfL\textsuperscript{high} rats displayed more observable neurological signs of mTBI immediately after the second impact, and that serum NfL levels at the time of re-injury had a good prognostic ability for the severity of these signs. Moreover, we found that the relative magnitude of NfL increase from time of re-injury to 3-days (\(\Delta\text{NfL}\)) was higher in NfL\textsuperscript{high} rats. Serum NfL levels also correlated with observable neurological signs of injury, and the elevation of serum NfL in rats that had sustained two mTBIs at a shorter inter-injury interval (i.e., 1d) was relatively prolonged.

\textit{Serum NfL levels at the time of a second impact are related to resultant mTBI severity}

We assessed the magnitude of change of serum NfL levels from the time of re-injury to 3-days (\(\Delta\text{NfL}\)) to provide insights into the extent of axonal damage resulting from the second mTBI alone. We found that the \(\Delta\text{NfL}\) values were greater in NfL\textsuperscript{high} rats, indicating that these rats sustained a greater severity of injury from the second impact. It is important to recognise that serum NfL levels at the time of the second impact were not stagnant, with previous and current rat ACHI data indicating a peak of serum NfL at 1-day followed by an exponential decline through to 14-days[24]. It is therefore likely that the rate of decline in NfL was greatest in rats with the shorter inter-injury intervals (i.e., 1- and 3-day intervals). As the NfL\textsuperscript{high} group largely consisted of 1- and 3-day interval rmTBI rats (i.e., 16/23 rats), the finding that the NfL\textsuperscript{high} rats had a greater \(\Delta\text{NfL}\) value, is despite the likely faster clearance of NfL levels at the time of the second mTBI. Furthermore, when removing the 1- and 3-day interval rats from the \(\Delta\text{NfL}\) analysis, the group differences were still present. As such, this
finding provides evidence that NfL levels at the time of second impact may be predictive of the extent of the resultant axonal damage.

It is important to recognize that serum NfL levels resulting from the first mTBI are not only an indicator of vulnerability to re-injury but also likely reflective of the severity of the initial injury. The variability of serum NfL responses after the first mTBI is likely attributed to some combination of minor inconsistencies in the injury procedure itself and individual factors related to cerebral vulnerability. Such factors may include subtle differences in anatomy (e.g., skull thickness, brain size, neck musculature), physiology (e.g., basal inflammation) and environment (e.g., stressors and socialization) between rats. Combinations of these factors may contribute to a more severe first mTBI, or more prolonged axonal pathology, in some rats when compared to others, with this severity spectrum reflected in serum NfL levels. After the second mTBI, these same factors also likely contribute to outcome, but in this case, our findings indicate that the extent of axonal pathology, as reflected by serum NfL levels at the time of the second mTBI, are key to vulnerability to a subsequent impact. Importantly, the aforementioned ΔNfL findings are suggestive of a greater degree of injury due to the second mTBI alone in NfL\textsuperscript{high} rats. As such, we conclude that serum NfL levels after a single mTBI are likely reflective of injury severity, but also a vulnerability to re-injury that is related to the extent and duration of axonal pathology induced by the initial mTBI. To further understand the contribution of initial injury severity to vulnerability, future research may consider a protocol in which rats are given one of two impact settings (e.g., one 20% greater than the other) for the first mTBI, followed by the same settings for the second mTBI. In addition, studies may consider implementing both serial measures of NfL prior to the second mTBI and longer inter-injury intervals to further understand the association between initial injury severity and ongoing axonal pathology on the duration and potential cessation of axonal vulnerability.
It has been previously shown that observable neurological signs of mTBI (i.e., tonic posturing, motionlessness, motor incoordination, etc.) correlate well to clinical outcomes such as orientation, concentration, and recall[33]. We found that observable neurological signs at the time of the second mTBI where more substantial in NfL<sup>high</sup> when compared with NfL<sup>low</sup> rats. Removing the group dichotomy from analysis, pre-injury NfL levels also correlated well with neurological signs after the second impact. The lack of lasting behavioral deficits in this study limits conclusions on the ability of NfL to predict deficits beyond the acute stages. This finding should however be considered in context, with behavioral testing in rodents often lacking sensitivity to behavioral impairments than can be reported or detected in clinical mTBI[34, 35]. It is possible that alternative tasks or testing at different time-points may have revealed longer lasting deficits. Nonetheless, given that video signs of injury alone have shown some predictive value in clinical mTBI, our finding that serum NfL levels were predictive of the number of neurological signs at the time second mTBI in rats provides preliminary evidence of a potential prognostic utility of this biomarker.

Our DTI analysis focused on FA of the corpus callosum, due to previous ACHI model studies finding FA differences in this region[26]. Although not surviving multiple comparison corrections, 3/6 corpus callosum regions had trends towards lower FA in the NfL<sup>high</sup> rats. It is plausible that the findings of a possible decrease in FA is indicative of a longer-lasting central disturbance to white matter integrity in rats with high NfL levels at the time of injury; however, this trend requires further investigation.

*The effect of inter-injury interval on the profile of serum NfL*

A secondary aim was to investigate the effect of timing between mTBIs alone on subsequent behavioral, structural, and NfL outcomes. While it is well recognised that timing is an important factor to rmTBI outcomes[8, 9], and multiple studies have demonstrated
heightened cumulative effects when mTBIs are separated by 1 to 3 days [11, 13, 36-39], the exact period in which the brain remains susceptible to a second mTBI is not known. The finding that rmTBI with the shortest inter-injury interval (1-day) had the most prolonged profile of serum NfL, may support the hypothesis that the brain is at an increased vulnerability to rmTBI with a shorter inter-injury interval. However, a limitation of this component of the study is the within-group variability in the injury severity. Due to the potential for a discrepancy in age (and hence weight) between the first and last injury (14-days), groups were split in half so that one half of all rats sustained their first mTBI/sham at the same age, and for the second half all rats sustained their second mTBI/sham at the same age. This meant that all groups had a very similar mean age for the two mTBIs. While this allows for most appropriate group level comparisons, it did however, likely contribute to a greater variability within interval groups and may account for the lack of statistical differences between interval groups.

Our primary analysis of NfL[^] high versus NfL[^] low rats does however shed some light on the duration of vulnerability. Firstly, when removing the 1- and 3-day interval rats from the ΔNfL analysis, the NfL[^] high versus NfL[^] low group differences were still present in 7- and 14-day interval rats, indicating a degree of axonal vulnerability that may exist beyond the commonly reported 3-day window in rats. Moreover, in the full data set analysis (i.e., all rmTBI irrespective of inter-injury) interval, it is notable that NfL[^] high rats were not exclusively derived from those given a shorter recovery period (i.e., 7/23 NfL[^] high rats had recovery periods of 7- or 14-days). These findings provide some evidence that axonal vulnerability in the event of a second mTBI is unlikely to be solely dependent on time since the initial mTBI, and that monitoring serum NfL levels during recovery at an individual level may be necessary to inform decisions such as when it is safe return to play decisions after sport-related concussion.
**Limitations:**

It is important to consider the limitations of the study. Firstly, separating rmTBI rats by the median NfL level was an *a priori* hypothesis of the study; it is however, an arbitrary threshold and may not represent the most appropriate threshold to predict vulnerability to re-injury. Nonetheless, this median NfL level (32.0 pg/mL) was very similar to the optimal cut-off concentration in AUROC analysis (i.e., 33.3 pg/mL). While serum NfL is a widely recognised and highly sensitive biomarker of axonal injury, this study did not directly assess central measures of axonal damage at the time of injury. In addition, due to differences in group sizes as well as a high within group variability for the inter-injury groups, it is not possible to determine whether monitoring timing or serum NfL levels may have more utility to mitigate the cumulative effects of rmTBI. Notably, although serum NfL reflects the extent of axonal pathology, it is not possible to determine if this or another accompanying and potentially correlated pathology, such as glial, metabolic, vascular or inflammatory alterations, is key to cerebral vulnerability. Related to this, it is important to note that we have not compared the utility of NfL to other promising biomarkers of mTBI, such as GFAP and S100B, and that literature to date indicates that a panel of markers that reflect different aspects of pathology and have different kinetics, are likely to have greatest utility in the diagnosis and management of mTBI. Finally, this study investigated young male rodents only; future studies are required to determine the influence of age and biological sex.

**Conclusion:**
Overall, this study provides novel evidence that serum NfL levels during recovery from an mTBI are reflective of vulnerability to poorer outcomes in the event of a subsequent mTBI. We found that serum NfL levels at the time of the second impact were predictive of mTBI severity, with rats with high NfL displaying more observable neurological signs, and a greater rise in subsequent NfL levels when compared to rats with low NfL. Notably, the potentiated NfL increase phenomenon remained when the analysis was limited to rats with longer recovery periods (i.e. 7- and 14-days). While it is difficult to translate biological timeframes between rodents and humans, it is accepted that biological processes in rodents are relatively accelerated. As such, these findings indicate that this vulnerability may be present for several weeks or months after clinical concussion. Considered alongside emerging clinical evidence that serum NfL rises are a common but heterogeneous feature of mTBI and sport-related concussion, these novel findings suggest that monitoring NfL levels during recovery may be informative for individual return to duty, work or sport decisions.

Commentary:

Background: Axonal injury is a prominent and potentially ongoing pathophysiological feature of mTBI. Serum NfL levels have been shown to be substantially but variably increased after mTBI. The potential for repeated mTBIs in short succession having cumulative effects is widely recognized but the mechanism is poorly understood.

Translational significance: This study shows for the first time that serum NfL levels measured after mTBI might indicate the extent of cerebral vulnerability to a second mTBI. This finding provides strong initial evidence that NfL might be useful to help inform the safe to return to sport or duty after mTBI.
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References:


Figure Legends:

Figure 1: Overview of study protocol.

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Figure 2: NfL levels at the time of a second mTBI are indicative of the resultant mTBI severity.

(A) Box plots of serum NfL levels at the time of injury for each of the four rmTBI inter-injury groups. The four rmTBI inter-injury groups were collapsed to create an all rmTBI group. The median NfL level for all rmTBI rats was 32.0 pg/mL. Rats with NfL levels lower than the group median were allocated to the NfL\textsuperscript{low} group, and rats with levels greater than the group median were allocated to the NfL\textsuperscript{high} group. (B) Group level plots (median, IQR) show the temporal profile of NfL at the time of second injury, and at post second-mTBI time-points of 3-, 7-, 14-, and 28-days for NfL\textsuperscript{high}, NfL\textsuperscript{low}, and sham rats. NfL\textsuperscript{high} rats had higher NfL levels at post second mTBI time-points of 3-, 7-, and 14-days (all p<0.0001) compared with NfL\textsuperscript{low} rats. (C) Violin plots show that the magnitude of change in NfL from the time of injury to 3-days post the second mTBI (ΔNfL) was greater in the NfL\textsuperscript{high} group than NfL\textsuperscript{low} group (p=0.0448). (D) ΔNfL was also greater in the NfL\textsuperscript{high} group than NfL\textsuperscript{low} group when analysis was restricted to 7- and 14-day interval rats only (p=0.01). (E) A greater number of observable neurological signs for the second mTBI was found in the NfL\textsuperscript{high} group when compared with the NfL\textsuperscript{low} group (p=0.0044). * p<0.05, ** p<0.01, **** p<0.0001.
Figure 3: The relationship between serum NfL levels and mTBI-related observable neurological signs.

The number of observable neurological signs immediately after the second mTBI correlated with serum NfL levels at the time of second mTBI (A), and post second-mTBI time-points of 3- (B), 7- (C) and 14-days (D). The number of observable neurological signs immediately after the second mTBI did not correlate to NfL levels at 28-days (E). A regression line is added to each scatterplot to assist visualisation of the spread of data. (F) Serum NfL at the time of injury had a good prognostic ability for the severity of visual signs (i.e., ≤2 versus >2 signs) immediately after the second mTBI with an AUROC value of 0.73 (sensitivity = 0.64; specificity = 0.78) at a 33.3 pg/mL cut-off.
Figure 4: Chronic white matter integrity in rats with low versus high serum NfL levels at the time of a second mTBI

In the left hemisphere, although not surviving multiple comparison corrections (α=0.017), a trend towards a decrease in FA for the NfL$^{\text{high}}$ group was found in the genu (A; p=0.046), and splenium (C; p=0.045), but not the body of the corpus callosum (B; p=0.269). In the right hemisphere, a trend for a decrease in FA in the NfL$^{\text{high}}$ group was found in the genu (D; p=0.071), but not the body (E; p=0.846), or splenium of the corpus callosum (F; p=0.293).
Figure 5: The relationship between inter-injury interval and serum NfL outcomes

(A) Box plots show the distribution of serum NfL levels at the time of second mTBI, and post second-mTBI time-points of 3-, 7-, 14-, and 28-days for sham, single mTBI, and the four inter-injury interval rmTBI groups. For full details of the post-hoc results for each time-point see supplementary table 2. (B) No inter-injury interval effect was found for ΔNfL.