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EVALUATION OF A NOVEL THERAPY TO IMPROVE RECOVERY FROM MODERATE TRAUMATIC BRAIN INJURY AFTER Critical Care Air Transport Team (CCATT) TRANSPORT – OPTIMIZING ADVANCED POINT OF INJURY TO EN ROUTE CARE

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14. ABSTRACT

Traumatic brain injury (TBI) is a major health issue that is particularly relevant in active duty populations. TBI is often associated with long-term disabilities; predominantly reduced learning and memory, as well as anxiety and executive function. Therapies to improve survival and long-term outcomes following TBI suffered on the battlefield are scarce. Strategies to both reduce brain injury and improve functional activity of the brain following TBI are critically important for the advancement of therapies from the bench to bedside. Consideration of war time TBI requires the understanding of the fact that TBI is often suffered in locations that require transport to optimal trauma centers (CCATT). In this proposal we use our recently developed mouse model of traumatic brain injury combined with CCATT to determine the effect of treatment with our novel Transient Receptor Potential M2 (TRPM2) inhibitor (tat-M2NX) on functional outcome after TBI+CCATT. We have identified the oxidative-stress sensitive ion channel, TRPM2, as a novel mediator of TBI-induced cognitive dysfunction and a key contributor to TBI-induced pathology. To confirm the functional benefit of acute inhibition of TRPM2 channels with tat-M2NX after TBI, we performed hippocampal-dependent neurobehavioral testing to measure memory function in mice 7 days after recovery from TBI. We found that that early pharmacological inhibition of TRPM2 channels with tat-M2NX provided sustained functional benefit. Our data indicates TRPM2 channel inhibition with tat-M2NX is a potential therapeutic during aeromedical transport for soldiers who have sustained TBI. The findings from our current study suggest that acute intervention at the time of CCATT is recommended and further development of our tat-M2NX is warranted.

15. SUBJECT TERMS
TBI, TRPM2, CCATT, oxidative stress.

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1.0 SUMMARY

Traumatic injuries are leading causes of preventable deaths in ongoing military efforts such as Operation Enduring Freedom (OEF) and Operation Iraqi Freedom (OIF). Traumatic brain injury (TBI) is a major health issue that is particularly relevant in active duty populations. TBI is often associated with long-term disabilities; predominantly reduced learning and memory, anxiety and executive function. Therapies to improve survival and long-term outcomes following TBI suffered on the battlefield are scarce. Clinical trials in civilian populations have shown that pre-clinically developed neuroprotective strategies (compounds known to reduce histological injury) are extremely difficult to translate to improved outcomes in humans. Clinically translatable strategies to reduce brain injury and to improve brain function following TBI are critically important. During wartime, TBI is often suffered in locations that require transport to optimal trauma centers, usually via the Critical Care Air Transport Team (CCATT). The influence of TBI + CCATT remains a critical en route care issue and most TBI therapeutics have not been validated under these conditions. We have developed a novel therapeutic, a Transient Receptor Potential M2 (TRPM2) inhibitor (tat-M2NX) that improves clinical outcomes, such as cognitive function in a preclinical mouse model of moderate TBI. However, the influence of CCATT on TBI outcomes and the efficacy of tat-M2NX1 under these conditions have yet to be tested.

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2.0 INTRODUCTION

2.1 Background

TBI is often associated with long-term disabilities; predominantly reduced learning and memory, anxiety and executive function. Unfortunately, therapies to improve survival and long-term outcomes following TBI suffered on the battlefield are scarce. Clinical trials in the civilian population have shown that neuroprotective strategies (compounds known to reduce histological injury) are extremely difficult to translate to improved outcomes in the human population. Therefore, strategies to both reduce brain injury and improve functional activity of the brain following TBI are critically important for the advancement of therapies from the bench to bedside. Consideration of war time TBI requires the understanding of the fact that TBI is often suffered in locations that require transport to optimal trauma centers (CCATT). *Relevant to the current proposal, we have developed a novel therapeutic that improves outcome following moderate TBI.* However, the influence of additional CCATT on TBI outcomes and the efficacy of our novel therapeutic are unknown. Therefore, we will take advantage of our recently developed mouse model of traumatic brain injury combined with CCATT to determine the effect of treatment with our novel compound on functional outcome after TB+CCATT. We have identified the oxidative-stress sensitive ion channel, TRPM2, as a novel mediator of TBI-induced cognitive dysfunction and a key contributor to TBI-induced pathology.

Specifically, our newly developed TRPM2 inhibitor (tat-M2NX[1]) improved cognitive function when administered acutely (within 2 hours (hr(s)) after TBI) or at more delayed timepoints (at least 1 month). To confirm the functional benefit of acute inhibition of TRPM2 channels with tat-M2NX after TBI, we performed hippocampal-dependent neurobehavioral testing to measure memory function in mice 7 days after recovery from TBI. Our preliminary data indicated that 2 millgrams/kilogram (mg/kg) tatM2NX administered 2 hr after TBI Intravenous (iv) appears to be effective in preventing TBI-induced memory dysfunction, although to a lesser degree than our initial dose of 20 mg/kg. A complete dose-response relation will be completed during the first year of the project.

Therefore, we propose to evaluate administration of tat-M2NX to improve outcome following TBI and air transport by evaluating histological and functional outcomes in mice treated 30min-3 hr after TBI+CCATT.

3.0 METHOD, ASSUMPTION AND PROCEDURES

3.1 Experimental Set up

We will use our model of experimental traumatic brain injury using the controlled cortical impact (CCI) model (Figure 1). Specifically, the CCI model as previously published by our laboratory: Anesthesia is induced using 4 percent (%) inhaled isoflurane, followed by maintenance with 1.5-2.5% inhaled isoflurane. A 4 mm circular right craniotomy is completed using a micromotor drill (Stoelting, Wood Dale, IL) centered 2.5 mm anterior to the lambdoid suture and 2.7 millimeter (mm) lateral to the sagittal suture using a stereotactic setup. Following exposure of the dura mater, a 3 mm flat-tipped pneumatic impact device (Impact One Stereotaxic Impactor for CCI, Leica Biosystems, Buffalo Grove, IL) is used to deliver a single, rapid impact using the following standardized parameters: angle 10° to the right of vertical, speed 3 ± 0.02 meters/second (m/s), duration 500 milliseconds (msec). Depth of impact can be varied from 0 mm (sham) to 2 mm (severe), although this proposal will use 1 mm depth throughout (Figure 2). Following impact, hemostasis is achieved, the bone flap replaced, and the skin closed.

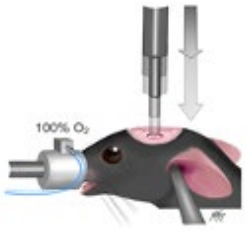

Figure 1. TBI Rodent Model

Hypobaria to mimic CCATT is achieved using custom pressure chambers that have the ability to modulate pressure and oxygen levels. We will perform studies to test the effect of altitude variances by altering pressure to 655 millimeters of mercury (mmHg) to model 4000 feet (ft) and 565 mmHg pressure to model approximately 8000ft. altitude, which approximates cabin pressure during CCATT (Figure 2). The timing of onset of CCATT is standardized to 4 hr. post TBI, to model rapid evacuation to trauma center. In order to control for the confounding effect of being located in Denver, CO which is at 5200ft. altitude, mice will be purchase from vendors at sea level and immediately upon arrival in Denver will be housed in our chambers set with pressures equivalent to sea level (760 mmHg). All animals will be housed before and after experiments in the sea level chambers to directly compare the effects of CCATT from sea level.

Figure 2. Experimental design

TBI injury followed by IV – 10 mg M2NX/Saline, mice are then exposed to hypobaric flight conditions (fly) or ground level Controls (no fly) for 4 hours, placed at ground level conditions for 7 days, followed by Behavior and euthanasia on day 7.

With sight restrictions in place, mice are placed in a random treatment group of TBI+no transport, TBI+CCATT4k, TBI+CCATT8k and analysis performed on functional outcomes and histological injury as described below. In a second set of experiments, mice of each group will be either untreated or treated with 2 mg/kg tat-M2NX) administered IV at the time of TBI. Mice are allowed to survive for 7 days and behavioral testing performed to assess cognitive function

(memory) and post traumatic stress disorder (PTSD)-like behavior (anxiety). Memory is tested using the Contextual Fear Conditioning paradigm. The contextual fear conditioning (CFC) paradigm was utilized as a hippocampal-dependent memory task. Figure 3, CFC apparatus consisted of two fear conditioning chambers with shock grid floors, consisting of 16 stainless steel rods connected to a shock generator (Colbourn Instruments, Model H13-15, Whitehall, PA, USA). Mice were transported in white buckets during the training and testing sessions. During training on day 7 after TBI+CCATT, mice are allowed to habituate the conditioning chamber for two separate 2 minute pre-exposure sessions followed by a foot shock (2-seconds/1.0 milliAmp (mA) electric shock) immediately after the second exposure. Following shock, mice were returned to their home cages. Testing occurred 24 hrs later, mice were transported in white buckets and placed back into the fear conditioning chambers. Freezing behavior was measured in 10 sec intervals across a 5 minute test by a blinded observer, and was defined as the absence of movement except for heart beat/respiration. Similarly, using the same apparatus, immediate shock paradigm will be utilized to assess anxiety. Following behavioral testing (7 days post-injury), animals were transcardially perfused under isoflurane anesthesia with phosphate-buffered saline for five minutes, followed by five minutes of fixation with 4% paraformaldehyde (PFA). Their brains were removed, allowed to post-fix in 4% PFA for 24 hours, and embedded in paraffin, as previously described[2, 3]. Coronal sections were cut in 6 micrometer (μm) thickness, and every sixth section was mounted in series onto slides for further processing. The interval between serial slide groups was 100 μm. For quantification of injury volume, one set of serial tissue sections stained with hematoxylin and eosin (H&E) was analyzed using quantitative stereologic analysis to quantify injury volume according to the Cavalieri principle. A Leica photomicroscope (Leica Microsystems, Buffalo Grove, IL) and a StereoInvestigator program (Stereologer 2000, SRC, Tampa, FL) were used for this purpose. For each animal, a total of 8 standardized sections were analyzed to calculate the global injury volume in cubic millimeters. Injured area was traced at 1.25x magnification, and tissue depth was calculated at 40x magnification.

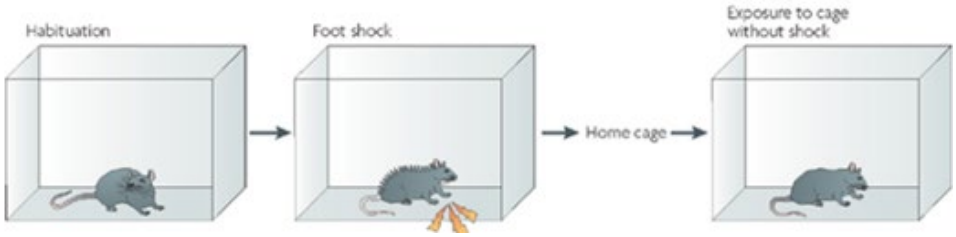

Figure 3. Rodent Contextual Fear Conditioning Model

3.1.1 Statistical Analysis Aim 1

The primary goal of Aim 1 is to determine the impact of altitude profiles and treatment on functional outcome (CFC) following moderate TBI. Secondary measures are anxiety behavior and injury volume. Efficacy was previously demonstrated using TBI+tatM2NX compared to TBI+tatSCR. Using these CFC data, sample size and power analyses were performed.

A power analysis indicated that a sample of 8 animals per group (a total of 48 animals over two experiments) would be sufficient to detect a moderate effect of $f = 0.6$ with 80% power and an adjusted alpha of 0.006 (to account for multiple comparisons) in a two-way analysis of variance (ANOVA). Previous data were observed to be normally distributed, as determined by the

Shapiro-Wilk normality test. Therefore, parametric tests will be used. The Shapiro-Wilk test will be used on all data sets and in the event that data are not normally distributed, non-parametric tests will be used.

For Aim 1, we will conduct a 2 (no treatment, 2 mg/kg tat-M2NX) × 3 (TBI only, TBI+CCATT4k, TBI+CCATT8k) two-way ANOVA with CFC as the primary outcome. This analysis will allow us to test both hypotheses of Aim 1 in the interaction effect and subsequent post-hoc pairwise comparisons with the Bonferroni adjustment for multiple comparisons applied.

Specifically, for Aim 1 Hypothesis 1 we will compare the mean CFC scores of the altitude profiles (TBI only, TBI+CCATT4000, TBI+CCATT8000) for animals that did not receive tat-M2NX. For Aim 1 Hypothesis 2 we will compare the mean CFC scores of the altitude profiles (TBI only, TBI+CCATT4k, TBI+CCATT8k) for animals that received tat-M2NX. Additionally, we will compare CFC scores between animals who did and did not receive tat-M2NX within each altitude profile to determine whether tat-M2NX resulted in better outcomes than no treatment for TBI only, TBI+CCATT4k, and TBI+CCATT8k. This model will be repeated for the two secondary outcomes of anxiety behavior and injury volume. The Benjamini-Hochburg procedure will be applied to control the false discovery date.

3.1.2 Statistical Analysis Aim 2

For Aim 2, we will determine whether the effect of tat-M2NX on outcome following TBI+CCATT8000 is dose-dependent. Specifically, we will compare the CFC outcome between varying doses of tat-M2NX after TBI+CCATT-8k using a one-way ANOVA. To determine differences in efficacy across all treatments and doses, we will use a Tukey post-hoc test for multiple comparisons. Secondary outcomes will also be compared using one-way ANOVAs with Tukey post-hoc tests. The Benjamini-Hochburg procedure will be applied to control the false discovery date.

3.2 Assumptions

We do not anticipate technical complications implementing our model of TBI+CCATT as we have extensive experience with all the methods proposed in the current study. There is technical risk around the time and cost needed to perform specific aim 1. It is possible that the efficacious dose of tat-M2NX in the TBI+CCATT conditions will be difficult to find. We have confidence we will identify the efficacious dose, but if multiple dose escalations are needed, this could increase time and cost.

There is some concern regarding potential adverse events based upon the peripheral expression of TRPM2 channels in the heart and vasculature, raising the possibility of acute physiological effects of TRPM2 inhibition. Fortunately, we recently published that intravenous administration of tat-M2NX had no significant effects on blood pressure or heart rate, reducing the risk of concern regarding side-effects. An important factor that may have contributed to failure of pre-clinical studies to translate to the human population is the over-reliance on histological analyses; histological brain injury may not be a reliable predictor of functional recovery. Therefore, alternative analyses are needed to demonstrate pre-clinical efficacy. We are uniquely poised to perform these experiments, as our laboratory routinely uses a combination of electrophysiology and neurobehavioral tests to assess the health of the neuronal networks following brain injury.

Further, we are poised to advance this protocol into a swine model that replicates swine injuries. Regarding the collaboration of this team, we have worked together at UC Denver (UCD) for over three years. Regarding collaborating with military scientists and with this team, we have > 10 Department of Defense (DoD) funded projects at UCD with military CO-investigators and CO-primary investigators with many high impact joint publications, presentations, and clinical reports impacting CPGs and drug/device development. We have at least two CRADA and several research contracts and thus can anticipate or resolved anticipated technical risks in contracting, work execution, joint authorship, and high impact dissemination of the work to combatant commands and DoD/59th HPW research leaders.

4.0 RESULTS AND DISCUSSIONS

Acute inhibition of TRPM2 channels fails to reduce injury following TBI.

To determine if inhibiting TRPM2 channels with our novel peptide, tat-M2NX would decrease cortical injury volume in male mice, mice were administered intravenously tat-M2NX at various doses 2 hrs post injury with stereological analysis at 7 days after TBI. Figure 4A WT sham operated mice displayed a small cortical injury associated with the craniectomy (2.978 ± 0.3487; n=6). Male TBI mice (figure 4B) showed a significant increase in injury volume (12.06±1.663,

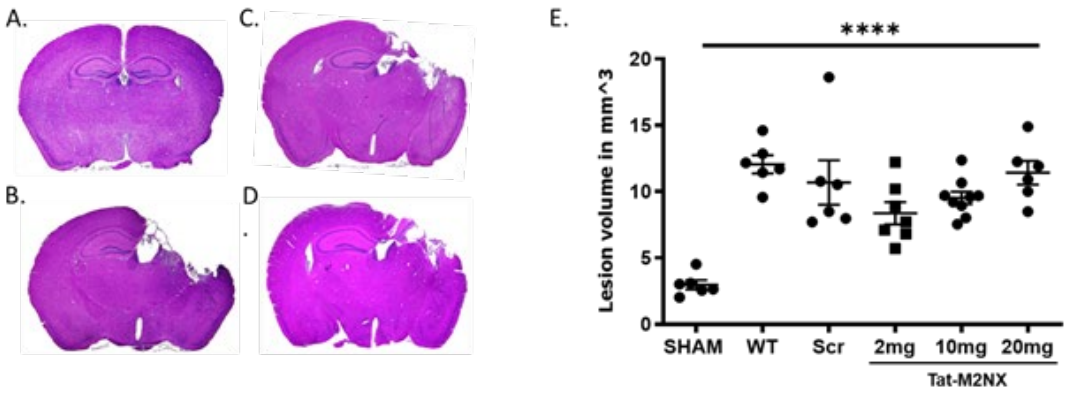

Figure 4. Acute tatM2NX administration fails to reduce injury
Stereologic, quantitative analysis of injury volume. Gross examination of the brain 7 days after TBI demonstrates that a dose response did not decrease brain injury volume between TBI (B), Tat-Scr (C) and Tat-M2NX (10mg) treated mice (D) when compared to sham (A). Summary bar graph across all groups demonstrates statistically significant injury volume in comparison to sham, analyzed by 1-way ANOVA n=5–7 animals per group. Error bars indicate standard error of the mean.

n=6) when compared to sham injured mice ($p<.05$). Figure C shows mice treated with Tat-Scr, and figure D shows mice treated with Tat-M2NX (10mg). Figure 4E shows mice treated with tat-M2NX at 2, 10 and 20 mg/kg (8.371± 2.234, n=7; 9.525± 1.418, n=9; 11.42±2.182, n=6; respectively) did not significantly decrease injury volume when compared to tat-M2NX scrambled TBI treated mice (10.69± 4.104, n=6) ($p<.05$). These data suggest that pharmacologically blocking TRPM2 receptors fail to provide neuroprotection.

Acute inhibition of TRPM2 channels preserves synaptic plasticity and memory function following TBI.

In these set of experiments we tested the effect of our TRPM2 channel antagonist, tat-M2NX, on plasticity and memory outcomes. Figure 5 shows a significant impairment of long-term potentiation (LTP) (117.3± 8.18% (n=8) 7 days after TBI when compared to sham controls ($p<0.05$). To investigate whether TRPM2 channels are involved in the impairment of LTP, tat-M2NX or tat-SCR (0.2, 2.0, and 10 mg/kg) was administered 2 hours post-TBI intravenously. Figure 5 shows that tat-M2NX administered 2 hours after TBI at the lowest dose (0.2 mg/kg) did not enhance LTP compared to scrambled tat-M2NX TBI group (129.9 ± 5.558, n=5; 110.4± 10.87% n=6, respectively). However, treatment with tat-M2NX at 2.0 or 10 mg/kg dose prevented

TBI-induced LTP impairment (165.5 ± 15.7, n=8 and 186.9 ± 17.28, n=6 respectively) when compared to mice treated with tat-SCR ($p<0.05$). These results demonstrate that mice that acute administration (2 hours) of tat-M2NX can provide sustained improvement of hippocampal plasticity, observed 7 days after TBI.

To assess the ability to pharmacologically target TRPM2 channels to improve cognitive recovery following TBI, we used our novel tat-M2NX peptide. To investigate whether tat-M2NX had effects on learning and memory 7 days following TBI, a dose response (0.2, 2.0, 10 and 20 mg/kg) was administered 2 hrs post-TBI intravenously. TBI mice treated with tat-M2NX at the lowest dose (0.2 mg/kg) did not have any significant effect on freezing behavior (figure 5D), a measurement of learning and memory, when compared to TBI treated with tat-SCR (22.44 ± 2.256; 30.14 ± 3.316 N=22, $p<0.05$, respectively). However, a one-way ANOVA found that treatment with 2.0, 10 and 20 mg/kg (47.9 ± 8.204 n=10; 52.11 ± 4.721 n=9; 62.29 ± 5.06 n=7; respectively), significantly prevented TBI-induced memory impairment when compared to tat-SCR ($p<0.05$).

Together, these results demonstrate that TRPM2 channels play a role in learning impairment and there is a dose response for tat-M2NX in the prevention of TBI-induced learning impairments.

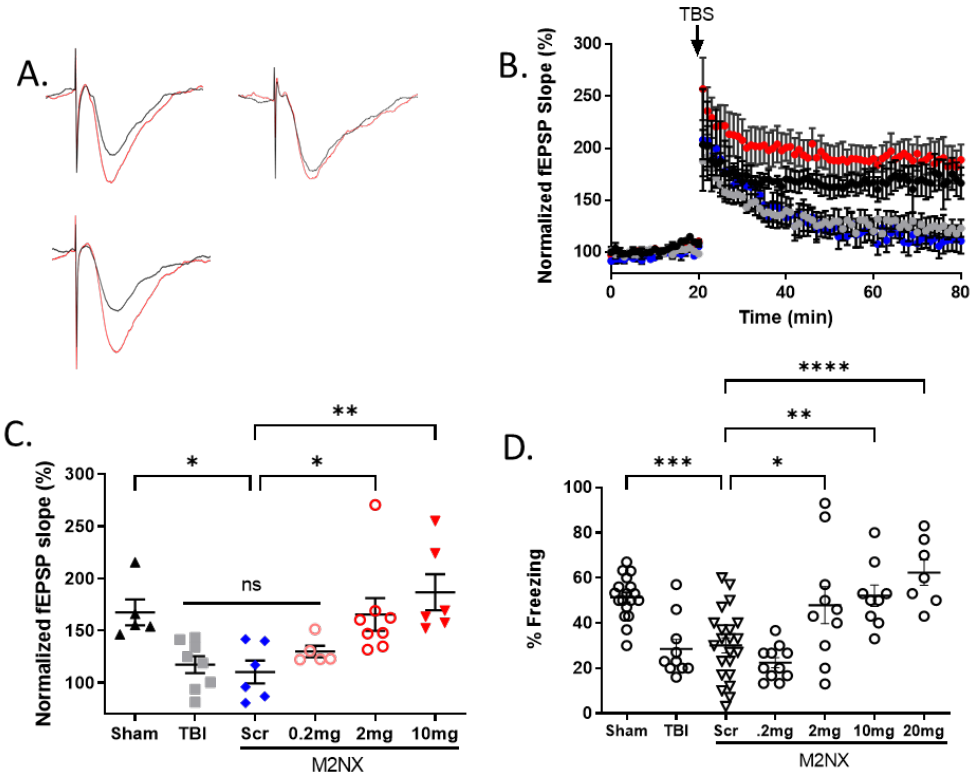

Figure 5. Acute tat-M2NX administration enhances hippocampal plasticity and memory function 7 days after recovery from TBI.

(a) Example fEPSPs from sham-operated control mice at 7 days after injury before (black) and after (red) TBS in sch-CA1 hippocampal pathway. (b) Time-course of fEPSP slope (mean ± SEM) from the hippocampus in sham mice (black filled circles), TBI (gray filled circles) and tat-scrambled treated mice in (blue filled diamonds) and tat-M2NX treated (red filed) 7 days after TBI. Arrow indicates timing of TBS (40 pulses). (c) Quantification of change in fEPSP slope after 60 min following TBS normalized to baseline, set at 100% (mean ± SEM).(d) Memory impairment 7 days after TBI. Adult mice displayed memory dysfunction after contextual fear conditioning. Quantification of freezing behavior 24 h after contextual fear conditioning in a novel environment (n = 8-18/group). *p < 0.05 compared to sham; behavior was scored. (n = 8-18/group. *p < 0.05 compared to sham controls).

Inhibiting TRPM2 receptor activity preserves synaptic LTP function in mice 30 days following TBI.

To determine long-term effects of tat-M2NX on hippocampal synaptic plasticity, mice were administered 10mg/kg two hours following TBI, with Sch-CA1 field potential recordings performed 30 days later. Following a brief Theta-burst stimulation (TBS) (40 pulse TBS), extracellular field recordings in CA1 area of acute hippocampal slices from sham (figure 6B,C) operated control mice resulted in LTP of 166.3 ± 9.152 (n=7). LTP in TBI mice 30d after injury showed impairment (125.0% ± 8.061 (n=5) when compared to sham controls. Mice treated with tat-Scr (10mg/kg) 2 hrs after injury and tested 30 days later showed no significant difference when compared to TBI mice (124.4% ± 13.63 (n=5). However, mice treated with tat-M2NX (10mg/kg)

2 hrs after injury and tested 30d later showed a significant protective effect of function with LTP expression at (201.8 ± 12.14, n=6).

Inhibiting TRPM2 receptor activity preserves Cognitive function in mice 30 days following TBI.

To assess the chronic effects of TRPM2 channels on the hippocampal tasks of learning and memory following TBI, contextual fear conditioning was used to measure memory function in mice 30 days after recovery from TBI. To investigate whether tat-M2NX had effects on learning and memory 30 days following TBI, a dose of 10mg/kg was administered 2 hours post-TBI retro-

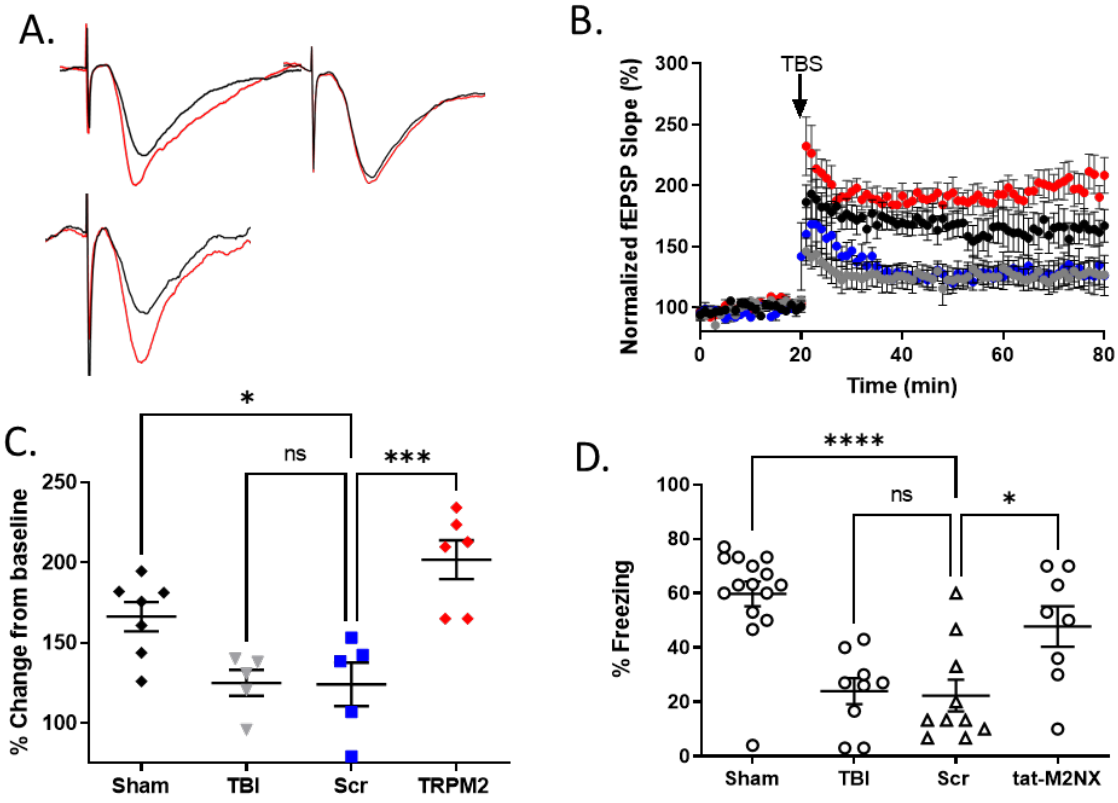

Figure 6. Acute taM2NX administration enhances hippocampal plasticity and memory function 30 days after recovery from TBI

(a) Example fEPSPs from sham-operated control mice at 30 days after injury before (black) and after (red) TBS in sch-CA1 hippocampal pathway. (b) Time-course of fEPSP slope (mean ± SEM) from the hippocampus in sham mice (black filled circles), TBI (gray filled circles) and tat-scrambled treated mice in (blue filled diamonds) and tat-M2NX treated (red filed) 30 days after TBI. Arrow indicates timing of TBS (40 pulses). (c) Quantification of change in fEPSP slope after 60 min following TBS normalized to baseline, set at 100% (mean ± SEM).(d) Memory impairment 30 days after TBI. Adult mice displayed memory dysfunction after contextual fear conditioning. Quantification of freezing behavior 24 hrs after contextual fear conditioning in a novel environment (n = 8-18/group). *p < 0.05 compared to sham; behavior was scored. (n = 8-18/group. *p < 0.05 compared to sham controls).

orbitally (figure 6D). A one-way ANOVA group analysis showed that TBI injured mice did not show significant difference on freezing behavior when compared to TBI treated with tat-SCR (23.96±4.731, n=9; 22.3± 5.790, n=10; $p<0.05$, respectively). However, sham and treatment with 10 mg/kg (59.77 ± 4.604 n=15; 47.75 ± 7.480 n=8; respectively), significantly prevented TBI-induced memory impairment when compared to tat-SCR ($p<0.05$). This suggest that TRPM2 channels are chronically impairing cognitive deficits and with a single dose of the tat-M2NX inhibitor 2 hrs post injury, chronically protected against memory impairment.

Hypobaric exposure did not affect time exploring in the open field (inner zone) or distance traveled.

To assess the behavioral effects of hypobaric exposure on local motor activity, we used the open field test. This test measured total time (seconds) spent in the inner zone of the chamber and distance traveled (meters) at 7 days after injury (Figure 7). A one-way ANOVA group analysis done on sham and TBI treated hypobaric +/- tat-M2NX mice did not show significant difference on either time spent in inner zone ($P>0.05$).

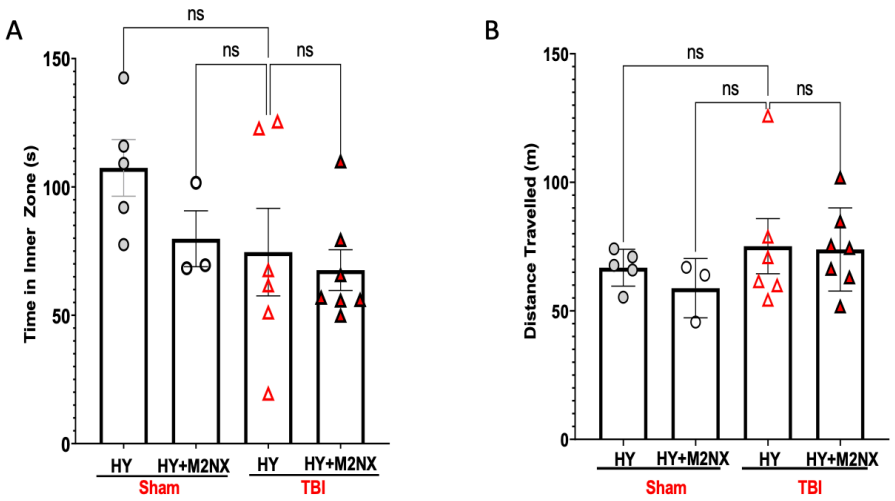

Figure. 7. TBI + Hypobaric exposure (CCATT) did not effect sensorimotor or anxiety behavior in the open field task

(A) There were no effects of injury or hypobaric exposure on time spent in the inner zone. (B) There were no effects of injury or hypobaric exposure on perimeter distance traveled. The graph shows mean±S.E.M. (n=3 - 7).

Hypobaric exposure did not effect limb preference.

To assess the behavioral effects of hypobaric exposure on preferred limb use, we used the cylinder test. The cylinder test is intended to evaluate locomotor asymmetry in rodent models of following brain injuries or disorders. The mice are placed in a transparent cylinder (diameter = 9 centimeter (cm)), wide enough for the mouse to move freely, with an overhead camera used to analyze rearing behavior. Limb use was analyzed by calculating the percentage use of the ipsilateral paw placements before and after injury (figure 8). A one-way ANOVA group analysis done on sham and TBI treated hypobaric +/- tat-M2NX mice did not show significant difference either contralateral limb use or that of both ($p>0.05$)

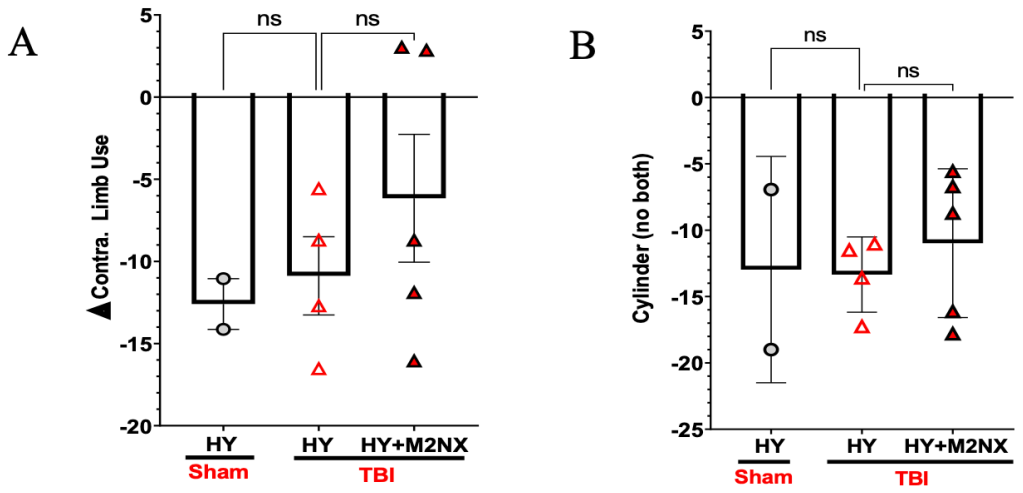

Figure 8. TBI +/- CCATT does not show significant impairment in Limb use

A) Analyzing both left and right limb cylinder touches did not show sensorimotor deficits 7 days after TBI/HY/M2NX. B) Analyzing left limb use only did not show sensorymotor deficits 7 days after TBI/HY/M2NX. mice unsuccessfully showed functional recovery of the impaired forelimbs asymmetry (b).The graph shows mean±S.E.M. (n=2 - 5).

TRPM2 receptor inhibition preserves memory 7 days after TBI+ Hypobaric.

To investigate whether tat-M2NX had effects on learning and memory 7 days following injury, mice were administered drug 2 hours post-TBI retro-orbitally. Figure 9 statistical analysis showed that TBI Hypobaric+tatM2NX 10mg/kg did showed a significant difference on freezing behavior when compared to sham hypobaric alone. (38.33±3.920, n=6; 64.00±.2.66; $p<0.05$, respectively). Both Hypobaric sham and TBI+tatM2NX 10 mg/kg (66.67 ± 3.33 n=3; 64.29 ± 4.031 n=7; respectively), significantly prevented TBI-induced memory impairment when compared to tat-SCR ($p<0.05$). This suggest that TRPM2 channels are chronically impairing cognitive deficits and with a single dose of the tat-M2NX inhibitor 2 hrs post injury, chronically protected against memory impairment.

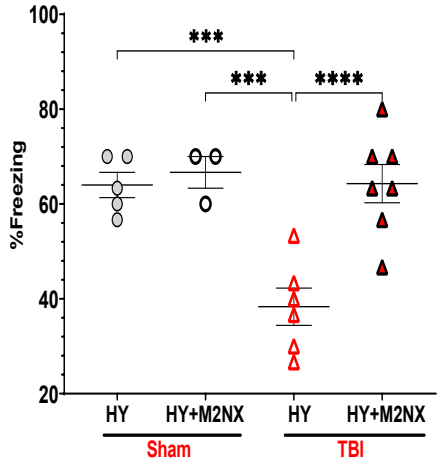

Figure 9. TRPM2 channel inhibition preserves memory 7 days after TBI+CCATT

TBI+Hypobaric treatment displayed memory dysfunction after contextual fear conditioning. However, mice treated with Tat-M2NX 2 hours after injury and tested 7 days later rescued memory impairment when compared to Hypobaric exposed TBI mice. Quantification of freezing behavior 24 h after contextual fear conditioning in a novel environment. Each point plotted represents each animal tested. Data presented as mean ±SEM, *$p < 0.05$ by ANOVA. (N=3-7/group).

4.1 Narrow Discussion of Results

The current study shows that moderate to severe traumatic brain injury causes acute histological injury that results on subacute and chronic impairment of

hippocampal synaptic plasticity and loss of short-term memory. We observed that the oxidative-stress sensitive ion channel TRPM2 contributes to acute injury in males only. In summary, we observed that early pharmacological inhibition of TRPM2 channels with the novel peptide inhibitor tat-M2NX failed to reduce histological injury, but did provide sustained functional benefit.

Behavioral analysis using open field and cylinder task in mice treated with tat-M2NX showed no significant difference between TBI, hypobaric exposure and tat-M2NX administration, suggesting that both anxiety and locomotor activity are not affected by the drug. Our data further shows that tat-M2NX given 2 hrs after TBI results in improved memory function compared to sham controls. This suggests that tat-M2NX is protective. Therefore, targeting TRPM2 channels activity may be a potential therapeutic during aeromedical transport for soldiers who have sustained a TBI.

4.2 Discussion

The current study shows that moderate to severe traumatic brain injury causes acute histological injury that results on subacute and chronic impairment of hippocampal synaptic plasticity and loss of short-term memory. We observed that the oxidative-stress sensitive ion channel TRPM2 contributes to acute injury in males only. The use of global TRPM2 channel knockout mice demonstrate a role for TRPM2 channels in both acute histological injury and long-term functional recovery in males. In contrast, we observed that early pharmacological inhibition of TRPM2 channels with the novel peptide inhibitor tat-M2NX failed to reduce histological injury, but did provide sustained functional benefit.

Traumatic brain injury can be classified into primary and secondary injury, albeit a somewhat oversimplification. Primary injury is the immediate consequence of the physical forces on the brain, while secondary injury occurs in the hours to days after the impact and contribute to ongoing cell death and neuroinflammation. The secondary injury proceeds for a few days and has been observed to exhibit ongoing injury up to 7 days after injury. Therefore, histological injury in the current study was performed 7 days after TBI, to analyze the effects of both primary and secondary injury. Secondary injury is a complex inter-related set of processes consisting of brain edema, (blood–brain barrier) BBB breakdown, ionic imbalances, neuroinflammation and oxidative stress. Unfortunately, there are currently no treatments available to enhance recovery following imoderate to severe TBI. The current study is the first to utilize the TRPM2 knockout mouse to assess the role of TRPM2 channels in TBI-induced injury. We observed reduced secondary injury in male knockout (KO) mice, with no effect observed in female mice. This data is remarkably similar to recent studies in ischemia-reperfusion models showing male-specific neuroprotection following knockout of TRPM2 channels. In contrast, we did not observe reduced secondary injury in mice treated with the TRPM2 antagonist tat-M2NX. In contrast, we observed tat-M2NX neuroprotection male mice following models of global and focal cerebral ischemia. A potential explanation for the current finding is that tat-M2NX is effectively taken into neurons, with less impact on microglia and other neuro-immune pathways. Indeed, the study by cook et al. implicated increased TRPM2 expression in microglia following rat TBI[4]. Further studies are needed to determine the reason that TRPM2 inhibition failed to reduce injury.

5.0 CONCLUSION

In conclusion we found that that early pharmacological inhibition of TRPM2 channels with the novel peptide inhibitor tat-M2NX provided sustained functional benefit. Our data indicates TRPM2 channel inhibition with tat-M2NX is a potential therapeutic during aeromedical transport for soldiers who have sustained TBI.

RECOMMENDATIONS

The current study indicates that acute intervention at the time CCATT is advisable. Further, it is recommended that the novel therapeutic tat-M2NX be further developed in a preclinical, translational large animal model.

6.0 REFERENCES

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

%	percent
mmHg	millimeters of mercury
ft	feet
µm	micrometers
ANOVA	Analysis of variance
CCATT	Critical Care Air Transport Team
CCI	Controlled Cortical Impact
CFC	Contextual Fear Conditioning
DoD	Department of Defense
Hg	Mercury
hr	Hour
iv	Intravenous
k	Thousand (8k, 4k)
Kg	Kilogram
LTP	Long-term Potentiation
OEF	Operation Enduring Freedom
OIF	Operation Iraqi Freedom
M	Meter
Ma	Milliamp
mg	Milligram
mm	Millimeter
msec	Milliseconds
PFA	Paraformaldehyde
SEM	Standard Error of the Mean
Tat-SCR	Control peptide drug
Tat-M2NX	TRPM2 channel antagonist
TBI	Traumatic Brain Injury
TBS	Theta-burst stimulation
TRPM2	Transient Receptor Potential M2