Prophylactic Treatment With CN-105 Improves Functional Outcomes in a Murine Model of Closed Head Injury

David Van Wyck (david.vanwyck@socom.mil)
US Army Special Operations Command https://orcid.org/0000-0002-6949-2803
Bradley J. Kolls
Duke University Hospital
Haichen Wang
Duke University
Viviana Cantillana
Duke University Medical Center: Duke University Hospital
Maureen Maughan
AegisCN LLC
Daniel T. Laskowitz
Duke University Medical Center: Duke University Hospital

Research

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Abstract

Introduction: Traumatic brain injury (TBI) has become the signature wound of recent military operations and training over the past two decades. A treatment that would protect against secondary brain injury in TBI and improve the long-term functional outcomes of traumatic brain injury on service personnel is therefore of great interest. One agent that has shown promise in modifying the post-traumatic neuroinflammatory response in murine closed-head injury models of TBI is a novel, small 5 amino acid apolipoprotein E (ApoE) mimetic peptide, CN-105. The goal of this study was to determine whether CN-105 would maintain its neuroprotective effects if administered prior to closed-head injury in a clinically relevant murine model.

Materials and Methods: CN-105 was synthesized by Polypeptide Inc. (San Diego, CA) to > 99% purity. We examined the efficacy of prophylactic administration of CN-105 in a well-established closed head injury model associated with reproducible vestibulomotor deficits. CN-105 was dissolved in sterile 0.9% saline and administered to male 12 week old C57-BL/6 mice intravenously (IV) through a tail vein and/or by intraperitoneal (IP) injection in a volume of 100 microliters at various time points prior to injury. Vehicle treated animals received IV and/or IP injection of 100 microliters of normal saline at the same time points. Animals were randomly assigned to treatment groups immediately following injury and all behavioral observations were conducted by investigators blinded to treatment. Vestibulomotor function was assessed using an automated Rotarod (Ugo Basile, Comerio, Italy). An in vivo assessment of the pharmacokinetics of CN-105 following IV or IP administration in healthy fed adult male CD-1 mice was conducted by Charles River (Worcester, MA). Mouse brains were harvested and sectioned 7 days post TBI with 25 ml phosphate buffered saline (PBS) and fixed overnight in 4% paraformaldehyde in PBS. Brains were left in sucrose 30% prior to being sectioned in 40 µm sections by microtome. Microglial activation in rat hippocampi was assessed in 5 vehicle and 5 CN-105 treated rats who had been received CN-105 three hours prior to injury using F4/80 immunohistochemical staining.

Results: Intravenous (IV) administration of CN-105 up to thirty minutes prior to closed head injury significantly improved durable vestibulomotor function compared to vehicle control-treated animals. In pharmacokinetic studies in uninjured CD-1 mice, IP administration of CN-105 resulted in an ~ 3-fold increase in the time to reach maximal plasma concentration (Tmax) and an ~1.5-fold increase in mean plasma residence time (MRT) compared to IV administration although the terminal elimination half-lives ($T_{1/2}$) were similar. When CN-105 was co-administered by IP and IV dosing 6 hours prior to injury, a durable improvement in vestibulomotor function was observed up to 28 days following injury. Microglial counted in CN-105 treated specimens were significantly less ($p = 0.0327$) than those counted in vehicle specimens.

Conclusion: CN-105 improves functional outcomes and reduces hippocampal microglial activation when administered prior to injury. This may be adaptable in the future as a means of preventing or reducing TBI-related secondary brain injury and improving clinical outcomes in service members who are identified as high-risk for TBI, are pre-treated, and subsequently suffer a TBI in the line of duty.

Introduction

Traumatic brain injury (TBI) has come to be known as the “signature injury” of the Global War on Terror.[1,2] Furthermore, TBI has become increasingly recognized as a complication of military training exercises such as combatives (hand-to-hand fighting), obstacle courses, airborne operations, demolitions ranges, and heavy-weapons training.[2-6] According to the most recent data there have been 430,720 total TBIs of all severities reported across the Department of Defense (DoD) between 2000 and the third quarter of 2020[7] with projected health care costs related to the care of TBI of approximately $14 billion over the next 20 years.[8] Unfortunately, there are no neuroprotective pharmacological therapies that have been demonstrated to improve long term functional outcome following TBI, and the treatment options for patients with persistent TBI symptoms remains primarily supportive.[9] Considering the detrimental impact of TBI on the health and safety of individual service members and the resulting strain on individual and unit health and readiness, filling this therapeutic vacancy has become a top priority for the DoD.

Primary brain injury, or the injury to brain tissue that occurs as a direct result of head trauma, is frequently exacerbated by indirect, secondary brain injury that consists of cellular energy depletion, excitotoxicity, and ischemia in the acute phase, followed by neuroinflammation in the intermediate phase. The corresponding secondary injury typically leads to cerebral edema, oxidative damage, and intracranial hypertension.[3,10,11,12] Thus, for optimal benefit, a pharmacological intervention that targets these processes should be administered as quickly as possible post-injury. Alternatively, prophylactic administration to soldiers in high-risk environments might also be of benefit. This would require adequate tissue concentrations of the drug to be achieved by the time of the primary injury, and for the intervention to effectively slow the progression of secondary injury. Although no proposed neuroprotectants improve long-term functional outcomes, several investigational agents have shown promise in preclinical studies.[9] For military applications, a TBI intervention should not only be effective at limiting secondary injury and improving functional outcomes, it should also be safe and tolerable (i.e. have no impact on duty or combat performance), have a long shelf life for use on deployments, be stable throughout a range of climate extremes, have a minimally or non-invasive route of administration, and be cost effective.

Increasing evidence suggests that functional outcomes after acute brain injury are modified by genetic factors, which may influence neuroinflammation and secondary neuronal injury as well as plasticity and recovery.[13,14] One of the most robust genetic associations with outcome after TBI is the apolipoprotein E (ApoE) polymorphism. [15,16] ApoE is a 34 kD lipoprotein composed of 299 amino acids, and is the primary apolipoprotein produced in the brain where its synthesis is upregulated after injury. ApoE has been demonstrated to reduce glial activation and inflammatory cytokine release in vitro and in vivo closed head injury models.[16] Although ApoE does not readily cross the blood brain barrier, we developed CN-105, a 5-amino acid peptide (Ac-VSRRR-COOH) derived from the polar face of the ApoE receptor binding region. This peptide readily crosses into the CNS compartment, is well tolerated, and improves long-term histological and functional outcome in multiple preclinical models of acute brain injury, including subarachnoid hemorrhage, intracranial hemorrhage, stroke, blast injury, and closed head injury.[17,18,19]

Moreover, CN-105 is an excellent candidate for clinical translation, and has demonstrated an excellent safety profile, as well as a linear and predictable pharmokinetic profile in phase 1 escalating dose studies involving healthy volunteers.[20] We now test the hypothesis that CN-105 retains its neuroprotective and anti-inflammatory effects when administered prior to injury in a clinically relevant murine model.

Materials And Methods

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Duke University Institutional Animal Care and Use Committee, Durham North Carolina as well as Department of Defense Animal Care and Use Review Office (ACURO).

Closed Head Injury Model: The murine closed head injury model used in this study has been previously described. [17] The closed head impact results in injury to selectively vulnerable neurons in the cortex and hippocampus and is associated with acute vestibulomotor deficits. Briefly, 10–12-week-old C57Bl/6J male mice (Jackson Laboratories, Bar Harbor, ME) were used. The trachea was intubated after anesthesia induction with 4.6% isoflurane and the lungs were mechanically ventilated with 1.6% isoflurane in 30% O2/70% N2. Core body temperature was servo regulated at 37° C via rectal probe. To avoid basilar skull fracture, ear bars were not used. The animal was secured in a stereotactic device, mechanically ventilated, and the head shaved to identify anatomical landmarks. After a midline scalp incision, a concave 3-mm metallic disc was adhered to the skull immediately caudal to the bregma. A 2.0-mm diameter pneumatic impactor (Air-Power Inc., High Point, NC) was used to deliver a single midline impact to the disc surface. The impactor was discharged at 6.8 +/- 0.2 m/second with a head displacement of 3 mm. After impact, the animals were allowed to recover. Once spontaneous ventilation resumed the mice were extubated. Mice were allowed free access to food and water. Sham mice were treated identically but had no induced head injury. All mice were housed in the same facility.

Drug Administration: CN-105 (Ac-VSRRR-amide) was synthesized by Polypeptide Inc. (San Diego, CA) to a purity of > 99%. It was dissolved in sterile, 0.9% saline and administered by IV through a tail vein and/or by IP injection at a dose of 0.05 ~ 0.5mg/kg. Vehicle treated animals received IV and/or IP injection of 100 µL of normal saline at the same time points. Animals were randomly assigned to treatment groups by a coded study identification number after injury using Graphpad online randomizer.

Testing of Functional Deficits. Mice were randomly assigned to treatment groups immediately following injury and all behavioral evaluations were performed by investigators blinded to treatment. An automated Rotarod (med associates inc., Georgia, Vermont) was used to assess vestibulomotor function. 2 days prior to injury, mice (n = 10–15 mice per group) underwent one training trial at an accelerating rotational speed (2-20 rpm in 5 min) for 300 seconds and a daily three l test trials with an accelerating rotational speed of 4 to 40 rpm in 5 min.. On the second day of training the average time to fall from the rotating cylinder in the test trials was recorded as baseline latency. Mice were tested on consecutive days post-injury and received three consecutive daily trials with accelerating rotational speed (inter-trial interval = 15

minutes; n = 10–15 mice). The average latency to fall from the rod was recorded. Mice unable to grasp the rotating rod were given a latency value of 0 seconds.

Pharmacokinetic methods: An *in vivo* assessment of the pharmacokinetics of CN-105 following 1mg/kg IV or intraperitoneal (IP) administration in healthy fed adult male CD-1 mice was conducted by Charles River (Worcester, MA). This was derived from dosing used in previously conducted phase I trials.[20] Briefly, CN-105 was aseptically resuspended in 0.9% sodium chloride and filtered through a 0.22-micron PVDF syringe filter prior to IV or IP dosing. Terminal blood was collected at 0.083, 0.25, 0.5, 1, 2, 4, and 8 hours following IV or IP administration (n=3 per dose group) in K_2EDTA tubes and stored on wet ice until processed to plasma by centrifugation at 3500 rpm for 10 min at 5^0C within 30 min of collection. Plasma was transferred to fresh tubes containing HALT protease inhibitor cocktail (ThermoFisher Scientific) at a final concentration of 1%, vortexed, frozen and stored at -80^0C. Plasma CN-105 levels were determined by liquid chromatography (Ajilent 1290)-tandem mass spectrometry (API6500+). The analytical range was 5 ng/ml to 5000 ng/ml (R2 = 0.995). Pharmacokinetic parameters were determined by non-linear compartmental analysis using WinNonlin (Certara, Princeton, NJ).

Brain harvesting and sectioning: Mice were perfused 7 days post TBI with 25 ml phosphate buffered saline (PBS) and fixed overnight in 4% paraformaldehyde in PBS. Brains were left in sucrose 30% in PBS prior to being sectioned in 40 µm sections by microtome.

F4/80 immunohistochemistry : 1 of every 8 sections was permeabilized in saponin 1%, for 1 hour at room temperature (RT). The sections were blocked for 30 min in tris buffered saline (TBS) plus 10% goat serum. F4/80 antibody (Invitrogen cat # PA5 21399, 1:30000 in TBS plus 1% Goat serum) was incubated overnight at 4°C. Goat anti-mouse biotinylated antibody (vector cat # BA-9400, 1:3000 in TBS plus 1% goat serum) was incubated for 2 hours at RT. Secondary antibody was labeled using Vectastin Elite ABC-HRP Kit (Vector cat # PK 6100) for 1hr at RT. The peroxidase activity was detected using DAB substrate kit (vector cat SK 4100) for 5 min at RT.

Unbiased stereology: All sections 0.36 mm to 2.40 mm lateral position were counted using optical fractionator probe. Counting frame 200x200 µm, counting grid 400x400 µm. Evaluation interval 320 µm. The first coefficient of error Schmitz- Hof was 0.054 ± 0.003 for CN105 treated mice and 0.045 ± .004 for the vehicle mice.

Statistical analysis. Serial tests of functional performance, including Rotarod and MWM performance, were compared with a two factor repeated measures analysis of variance (ANOVA) with time as the repeated variable. When F-value was significant for group effect, pairwise comparison was performed using *post-hoc* Scheffe test for correcting multiple comparisons. To test the hypothesis that the behavioral improvement associated with treatment was associated with a reduction in the number of hippocampal number of F4/80 positive cells, a one-tailed T test was used. Parametric values are expressed as mean ± standard error (SE). Significance was assumed if $p < 0.05$.

Results

We first designed a series of experiments to assess whether CN-105 could reduce functional deficits as defined by rotorod latency when administered intravenously prior to injury. Previously we demonstrated neuroprotection when CN-105 was administered post-treatment, and this was used as a positive control.[17] At 10 and 20 minutes prior to injury, IV administration of 0.05 mg/kg CN-105 was associated with durable motor improvements in TBI-associated functional deficits as compared to administration of vehicle control (Fig. 1A). Significant functional benefit was also observed when CN-105 was administered 30 minutes prior to injury, although there was only a trend towards benefit when administration occurred 60 minutes prior to injury (Fig. 1B).

While our data supports prophylactic use, the known half-life of CN-105 in mice is 30 minutes.[19] To address this limitation, we conducted a series of pharmacokinetic experiments to explore the possibility that intraperitoneal injection of drug would result in higher sustained tissue concentrations. **Figure 2** shows the mean CN-105 plasma concentrations as a function of time in uninjured CD-1 mice following IV or IP administration of 1 mg/kg CN-105 in 0.9% sterile sodium chloride. Following IV infusion, CN-105 plasma concentration declined in a polyphasic manner. Following IP administration, a short distribution phase was seen followed by a polyphasic reduction in CN-105 plasma concentration that closely mirrored that seen following IV infusion. Individual plasma concentration versus time profiles of CN-105 following IV versus IP administration were analyzed by non-compartmental analysis to determine the PK parameters (**Table 1**). The bioavailability of CN-105 after IP dosing was 93%. Although the CN-105 terminal elimination half-life ($T_{1/2}$) was similar following IP versus IV administration (T1,2's ~ 1.1 – 1.3 hours), the Tmax was ~ 3-fold longer, and the mean residence time was ~ 1.5-fold longer following IP administration.

Based on these results, we modified the dosing paradigm to include co-administration of CN-105 by IP (0.5 mg/kg) as well as IV (0.1 mg/kg) dosing at 3 or 6 hours prior to injury. These dose ranges were determined based on similar dosing regimens used in prior clinical models.[17-20] As shown in **Figure 3**, this dosing regimen was associated with an improved trajectory of vestibulomotor recovery that was durable when longitudinal testing was performed out to 28 days following injury.

Next, to assess neuroinflammatory responses, we quantified F4/80 immunoreactivity, which stains activated microglia and cells of monocyte lineage in mouse brains harvested 7 days post-TBI. Five vehicle treated mouse brains and five mouse brains treated with CN-105 three hours prior to TBI were evaluated. Microglia were counted in hippocampal sections of both vehicle and CN-105 treated animals. The estimated polulation of hippocampal microglia was significantly higher in the vehicle treated mice as compared to the CN-105 treated mice (14288 ± 1290 vs 22568 ± 4520, p= 0.04). The histological differences in microglial activation in vehicle and CN-105 treated mouse hippocampi are demonstrated in **Figure 4**.

Discussion

Traumatic brain injury is a heterogenous disorder that is the result of both primary and secondary injury mechanisms. It can vary in severity and is frequently associated with high morbidity in all forms that negatively impacts the health and readiness of individual soldiers. As a result, considerable attention has been given to understanding the complex array of secondary injury mechanisms in order to develop neuroprotective therapies. Although many interventions have been evaluated for this purpose, over 30 phase III prospective clinical trials evaluating various therapies have failed to reach their primary endpoints.[21-26] Furthermore, many of these therapies have been evaluated as post-injury interventions. While this may serve civilian TBI patients, where approximately 60-75% of patients present for medical evaluation shortly after injury[27,28] evaluation and documentation rates following acute TBI, particularly mild TBI, has historically been low.[29] A number of factors unique to military service have been identified as barriers to acute evaluation and care, including various in-theater assessment barriers, exposure to other, concurrent injuries that are often more severe, symptoms that are common to various other diagnoses or attributed to the stresses of military service, and co-existing mental health comorbidities. This is further complicated by a history of underreporting amongst military personal for a disease process that continues to rely on accurate reporting of history and clinical symptoms to make a diagnosis. Suggested hypotheses to explain this underreporting have included concerns amongst affected soldiers that reporting symptoms may result in removal from their units, colleagues and responsibilities, fear of delays return to home and family following deployment, beliefs that existing symptoms are minor and will resolve on their own, fear of stigmatization and a reduced ability to recognize TBI symptoms until return to less structured garrison and civilian lifestyles.[30,31,32] Prophylactic neuroprotection for high-risk training and operations would theoretically address some of these issues. In the current study, we demonstrate that CN-105 improves functional outcomes and reduces microglial activation in rat hippocampi when administered prior to TBI. These findings, along with its ease of administration and safety profile, make it an attractive prospect for providing a degree of neuroprotection to soldiers who are deemed by military leadership and military healthcare providers to be at high risk for TBI.

The development of ApoE mimetic neuroprotective therapies was based on initial observations demonstrating that endogenous ApoE played an adaptive role following brain injury by reducing neuroinflammation and secondary neuronal injury.[33,34,35] Although the intact ApoE lipoprotein is too large to cross the blood brain barrier and thus cannot serve as a viable therapeutic[36], ApoE peptides can be created from the apoE receptor binding region, which is believed to mediate its anti-inflammatory and neuroprotective effects via interactions with the glial LRP-1 receptor.[37,38] Importantly, there is convergent evidence that ApoE-based neuroprotectants improve outcomes in a variety of preclinical animal models that recreate the different features of TBI pathology, including closed head injury, parenchymal and subarachnoid hemorrhage, and ischemia (**Table 2**).

CN-105 was developed to optimize potency and CNS penetration by linearizing the polar surface of the helical receptor binding region of ApoE. CN-105 has been demonstrated to reduce glial activation in vitro, and in vivo and to improve histological and functional outcomes in a number of preclinical models of

brain injury. Moreover, CN-105 is stable and can be stored in lyophilized form or in solution. Importantly, phase 1 single and multiple dose escalation studies have demonstrated linear and predictable pharmacokinetic profile, and a favorable toxicity profile both in the Phase 1 and ongoing Phase 2 trials.[20] Our current observations demonstrating prophylactic efficacy of CN-105 reducing vestibulomotor deficit was likely a function of adequate blood levels at the time of injury. Of note, the measured half-life in humans ~3.5 hours, is considerably longer than in rodent models (<1.5 hours) (Table 1 and Ref. 20) and increases the feasibility of prophylactic administration in the military setting. This data indicates that prophylactic dosing of CN-105 may be effective in improving functional outcomes and microglial activation in the hippocampus when administered prior to traumatic brain injury. Considering that balance skills have been associated with increased hippocampal volumes[59], the reduction in microglial activation may have contributed to the improved vestibulomotor function in CN-105 treated mice. However, the short half-life associated with intravenous dosing in a murine model may limit the prophylactic window required to achieve therapeutic tissue concentrations at the time of injury.

Although CN-105 represents an excellent candidate for clinical translation in the setting of traumatic brain injury, there are several potential limitations, which should be addressed. As a peptide, CN-105 has minimal oral bioavailability, and current clinical trials utilize intravenous administration.[16,17,18,20] Although this does not represent a challenge for administration following injury, it would not be optimal for repeated prophylactic administration. To this end, we are exploring minimally or noninvasive routes of delivery, such as intranasal, subcutaneous, or transdermal administration. The mechanism(s) by which CN-105 exerts its neuroprotective effects remains incompletely defined, although convergent data suggests that both apoE and the apoE mimetic peptides exert direct neuroprotective and anti-inflammatory effects via interaction with the LRP-1 receptor, which is present on neurons and glia.[37,38] A better understanding of the physicochemical nature of this interaction may allow the rational development of small molecule therapies. Finally, although we demonstrate as proof of principle that prophylactic administration of CN-105 improves recovery and functional outcome after TBI, as long as adequate blood/tissue concentrations are achieved, intraperitoneal administration is not feasible in the clinical setting. Moreover, rodent models are not always ideal for studies of human clinical pharmacokinetics or disease intervention; for example, the half-life of CN-105 in a clinical trial is approximately 3.5 hours, which compares favorably to the much shorter half-life in rodents (Guptill paper) Nevertheless, the positive effects on vestibulomotor function in the study group of this trial are encouraging. With safety and tolerability in humans previously established[20], future studies should aim to determine if the neuroprotective effects of CN-105 seen in murine TBI trials can translate into outcome benefits in clinical trials.

Conclusions

Our results demonstrate that administration of CN-105 prior to an induced murine closed head injury produced a durable improvement in vestibulomotor function. Further, there was a statistically significant reduction in F4/80 positive cells counted in CN-105 treated hippocampal sections compared to vehicle

treated hippocampi. These findings suggest that based on the longer half-life of the drug observed in humans, CN-105 may be an effective prophylactic strategy for improving functional outcomes in soldiers at high risk for head injury in both training and combat environments.

Declarations

Acknowledgments

None.

Author Contributions

DVW played primary role in data analysis, writing of manuscript.

HW performed animal experiments and played a role in editing.

BJK played a role in data interpretation and editing.

VC performed immunohistochemistry and editing of the manuscript.

MM reviewed and edited the manuscript final draft.

DTL played a role in experimental design, data analysis, writing and drafting.

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Availability of Data and Materials

All available data and materials will be made available on request.

Declarations

Ethical Approval and Consent to Participate

All experiments were approved by and conducted in accordance with the Duke University Institutional Animal Care and Use Committee.

Consent for Publication:

Not applicable

Competing Interests

Dr. Laskowitz is an officer and has equity in Aegis CN, LLC which supplied the study drug. Dr. Wang serves as a consultant for Aegis CN, LLC. Aegis CN, LLC had no editorial control over the study design, its execution, or the writing of this manuscript. Duke University has equity and an intellectual property stake in CN-105.

Author's Information

DVW: U.S. Army Special Operations Command, 3rd Special Forces Group; Duke University Hospital, Department of Neurology.

HW: Duke University School of Medicine, Department of Neurology.

BJK: Duke University School of Medicine, Department of Neurology.

VC: Duke University School of Medicine, Department of Neurology.

MM: Officer, Aegis CN, LLC.

DTL: Duke University School of Medicine, Department of Neurology and Department of Neurobiology; Officer, Aegis CN LLC.

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Tables

Due to technical limitations, table 1-2 is only available as a download in the Supplemental Files section.

Figures

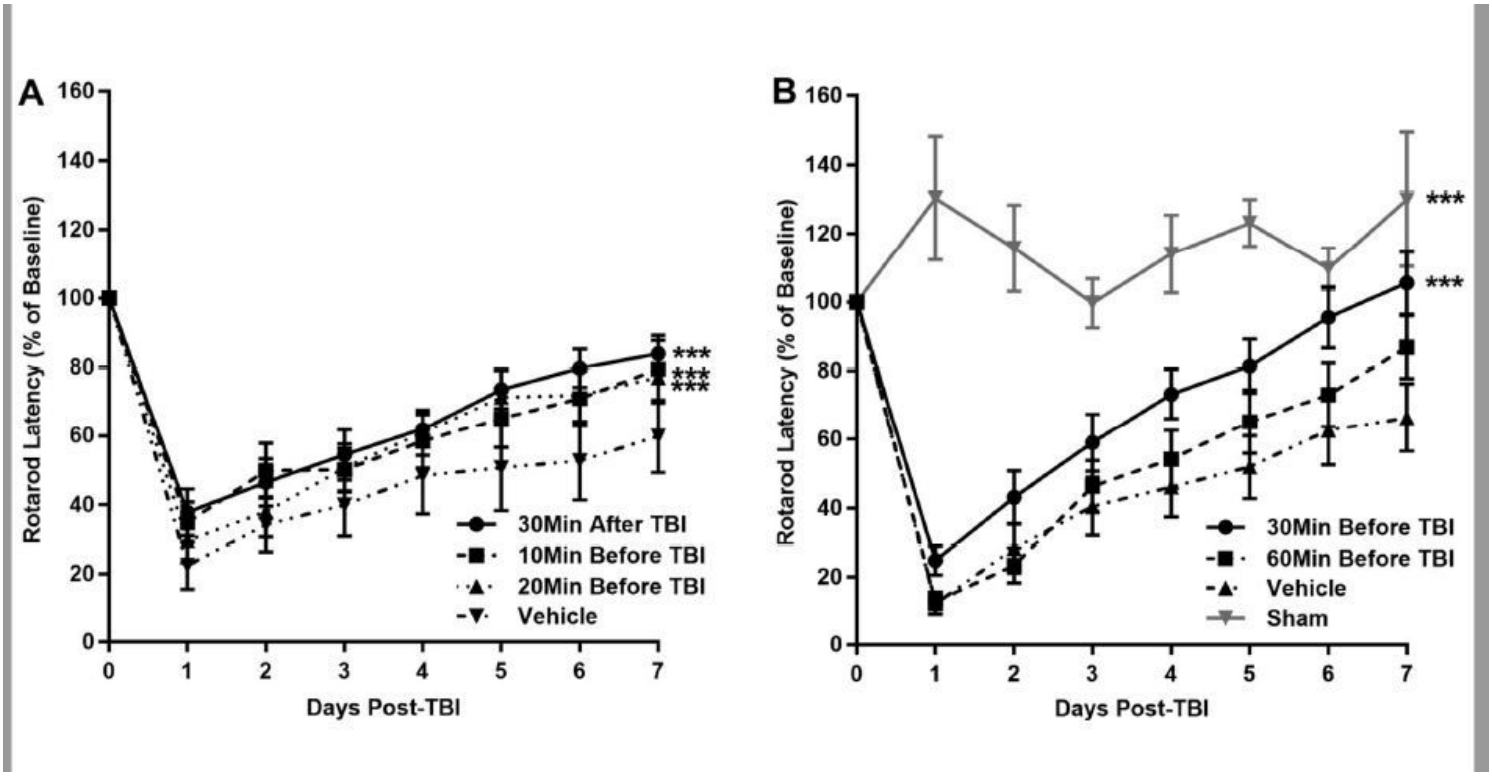

Figure 1

(A) A single intravenous administration of CN-105 (0.1 mg/kg) improved vestibulomotor function as assessed by Rotarod when administered 10 min (A), 20 min (A) or 30 min (B), but not 60 min (B) before the induction of TBI.

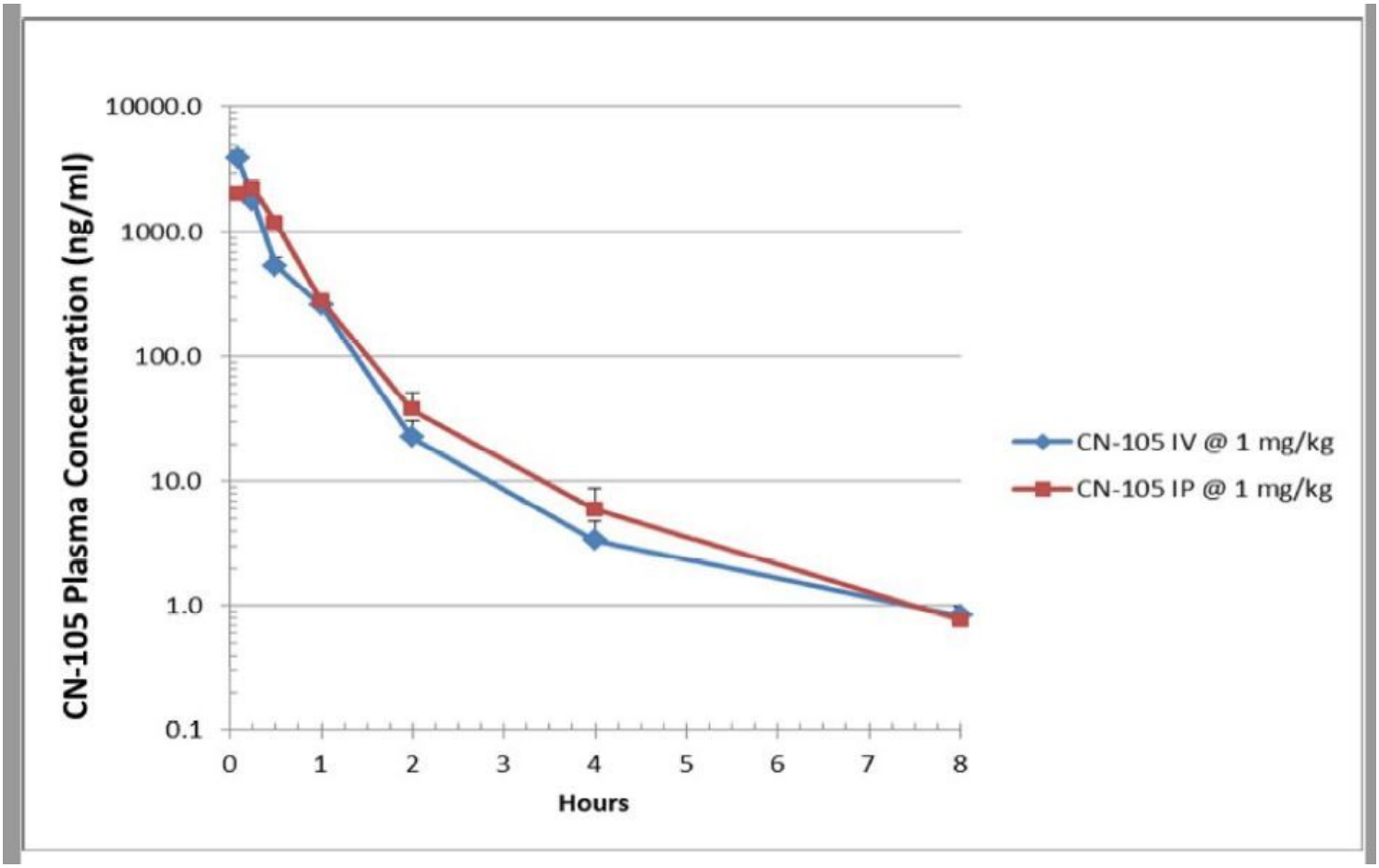

Figure 2

Mean CN-105 plasma concentration as a function of time following an intravenous (IV) or intraperitoneal (IP) injection of 1 mg/ml CN-105 in sterile saline. The data represent the mean + the standard error of 3 animals per time point per dose group.

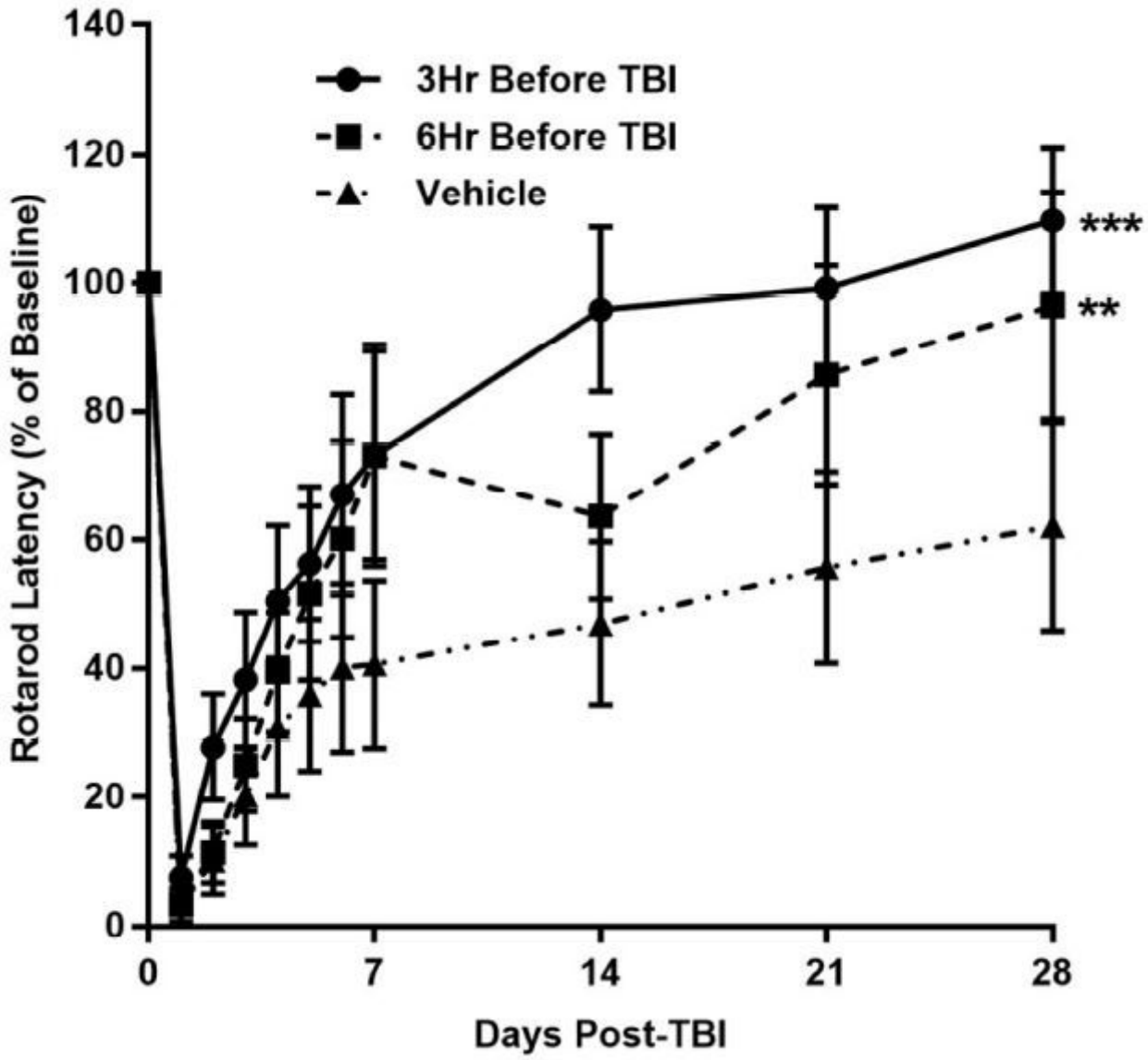

Figure 3

Co-administration of CN-105 by intravenous (0.1 mg/kg) and intraperitoneal injection (0.5 mg/kg) for 3 or 6 hours before the induction of TBI resulted in a durable improvement of vestibulomotor function up to 28 days after injury as assessed by Rotarod.

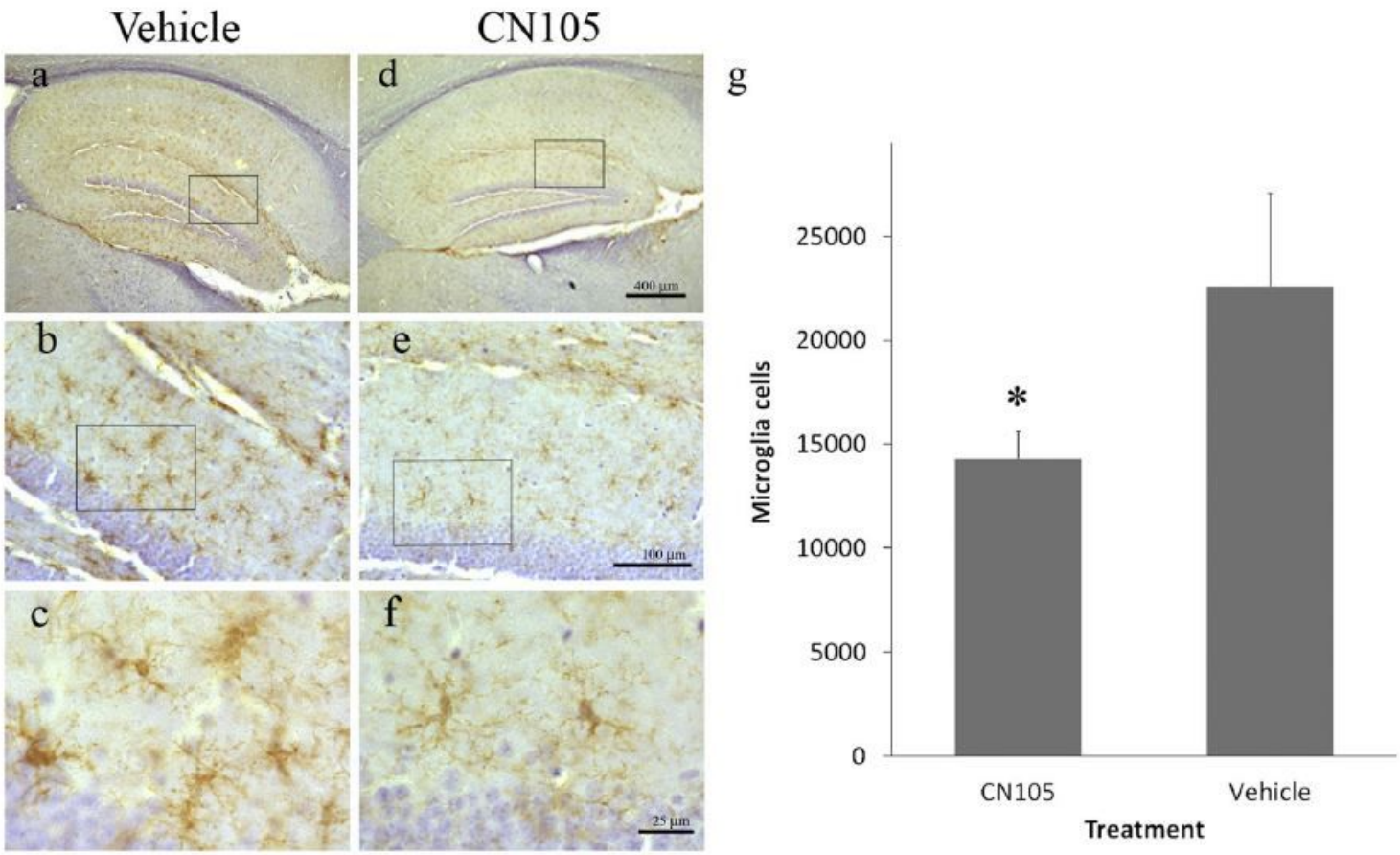

Figure 4

Reduction in F4/80+ cells following treatment with CN-105 (a) as compared to vehicle (d). At higher magnifications, ramified microglial morphology is more evident in untreated mice (b vs. e, and c vs. f). Formal unbiased stereology revealed a reduction in number of hippocampal F4/80 positive cells as a function of treatment (g).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.pdf
- CN105ProphylaxisTable2Apr2021.pdf