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Effect of Polyarginine Peptide R18D Following a Traumatic Brain Injury in Sprague-Dawley Rats

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ABSTRACT

Background: Despite extensive studies, there are still no clinically available neuroprotective treatments for traumatic brain injury.
Objectives: In previous studies we demonstrated beneficial treatment effects of polyarginine peptides R18 (18-mer of arginine; 300 nmol/kg) and R18D (18-mer of D-arginine; 1000 nmol/kg) in a rat model of impact-acceleration closed-head injury.
Methods: We examined the efficacy of R18D when intravenously administered at a low (100 nmol/kg) and high (1000 nmol/kg) dose, 30 minutes after a closed-head injury in male Sprague-Dawley rats.
Results: At postinjury day 3, treatment with R18D at the high dose significantly reduced axonal injury ($P = 0.044$), whereas the low-dose treatment of R18D showed a trend for reduced axonal injury. Following assessment in the Barnes maze, both doses of R18D treatment appeared to improve learning and memory recovery compared with vehicle treatment at postinjury days 1 and 3, albeit not to a statistically significant level. Rotarod assessment of vestibulomotor recovery did not differ between R18D and the vehicle treatment groups.
Conclusions: R18D modestly decreased axonal injury only at the highest dose used but had no significant effect on functional recovery. These findings warrant further studies with additional doses to better understand peptide pharmacodynamics and provide information to guide optimal dosing.

Introduction

Due to its massive burden on affected patients, society and the economy, there is a need to develop a neuroprotective therapeutic for traumatic brain injury (TBI). This need is further compounded by the complex and multifactorial pathophysiology associated with TBI. Many previous approaches to developing a neuroprotective therapeutic agent have focused on targeting only a specific aspect of the TBI injury cascade. An alternative approach involves the use of potential therapeutic agents with multiple neuroprotective modes of action.

Recent studies have demonstrated that cationic arginine-rich peptides (CARPs) are beneficial in both in vitro and in vivo stroke-related injury models,[1–3] and there is increasing evidence that these peptides exert their neuroprotective actions through multiple mechanisms.[4,5] For example, we have previously demonstrated that CARPs can protect neurons from excitotoxicity by inhibiting intracellular calcium influx and reducing neuronal surface expression of the N-methyl-D-aspartate receptor subunit protein NR2B9c.[6,7] Moreover, CARPs have the capacity to reduce the activity and/or surface expression of other ion channels and receptors (eg, AMPAR, NCX, TRPV1, CaV2.2, CaV3.3, and TNFR) that may exacerbate brain injury associated with excitotoxicity or neurotrauma.[8–11] CARPs can also target and stabilize mitochondria, and reduce mitochondrial reactive oxygen species production, inhibit proprotein convertases that activate matrix metalloproteinases, modulate inflammatory responses, and activate pro-cell survival signalling.[12]

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The neuroprotective actions of CARPs are further supported by several studies that reveal this class of peptide can reduce cortical damage[13] by reducing inflammation and acute vasogenic edema,[14,15] increasing antioxidant activity,[13] and therefore functional recovery.[16] Studies in our laboratory have demonstrated that our current lead neuroprotective CARP, polyarginine-18 (R18) (18-mer of arginine), was able to significantly reduce the extent of injury in the corpus callosum in a closed head injury model in rats.[7] R18 was also shown to promote neurocytic cell growth as well as reduce apoptosis; brain water content; and activity of caspases 3, 8, and 9.[17] Furthermore, an 18-mer of D-arginine (R18D) significantly improved sensorimotor and vestibulomotor recovery, and reduced brain glial fibrillary acidic protein and interleukin 6 protein levels after closed head injury in male Long-Evans rats.[18]

Following our positive results with R18D, the aim of the current study was to further investigate the effectiveness of the peptide to improve functional (Barnes maze and rotarod) and histological (axonal injury) outcomes when administered at a low (100 nmol/kg) and high (1000 nmol/kg) dose following an impact-acceleration closed-head injury. Because male Sprague-Dawley rats are more widely used in TBI studies than the Long-Evans strain, this study was carried out using Sprague-Dawley rats to further inform our initial exploratory study.[7]

Methods

Peptides used in this study

The R18D (H-rrrrrrrrrrrrrrrrrr-OH; r = D-arginine; net charge +18) peptide was synthesized by Mimotopes Pty Ltd (Mulgrave, Victoria, Australia) and purified by HPLC to 98%. Peptide used for dose response and serum uptake studies were prepared in 0.9% sodium chloride for injection (Pfizer, Sydney, New South Wales, Australia) before approximately 650 µL was aliquoted into 3 mL syringes. Reconstituted peptides were stored at −20°C until use.

TBI model

This study was approved by the Animal Ethics Committee of the University of Western Australia and follows the guidelines outlined in the Australian Code for the Care and Use of Animals for Scientific Purposes. Male Sprague-Dawley rats weighing 370 to 410 g were housed in pairs under controlled conditions with a 12-hour light–dark cycle and free access to food and water ad libitum before and after surgery.

A weight-drop impact-acceleration model of TBI was used to induce the injury as previously described.[7,18] Briefly, rats underwent anaesthesia induction with 5% halothane (mix 30% oxygen/70% nitrous oxide gas), were intubated, and maintained under 1% to 2% halothane during attachment of the metal disc to the skull, jugular vein cannulation, and the intravenous infusion of treatments. Animals were temporarily disconnected from the anaesthetic (<1 minutes) to induce TBI. Sham animals underwent the same surgery, but were placed adjacent to the weight-drop apparatus when the weight was released. Treatments were randomised and consisted of the vehicle control (0.9% sodium chloride for injection) and R18D at a low (100 nmol/kg) or high (1000 nmol/kg) dose administered in a blinded fashion at 30 minutes postimpact (600 µL over 6 minutes) through the right internal jugular vein using an infusion pump. Sham animals were administered the vehicle control via the same route.

A total of 26 animals underwent the procedure and 24 survived to the 3-day post-TBI end point (7.69% mortality). The 2 animal deaths consisted of 1 vehicle and 1 R18D-treated (100 nmol/kg) animal. Both animals experienced shallow, labored breathing during the surgical recovery phase and died within 1-hour post-TBI. Sham and vehicle treatment groups consisted of 6 animals each, and the low- and high-dose R18D treatment groups consisted of 5 and 7 animals, respectively.

Postsurgical animal care and monitoring

At the conclusion of surgery, pethidine (1 mg in 0.2 mL saline, IM) and bupivacaine were administered (0.1 mg in 0.2 mL saline per site, SC) to the head surgical wound. A 2 mL volume of injectable saline was also subcutaneously administered to aid hydration. Rat cages were placed on a heating mat during postsurgical monitoring, subsequently housed in a holding room maintained at 26°C to 28°C. Rats were monitored at least twice a day, and if animal behaviour suggested they were in pain, pethidine (1 mg in 0.2 mL saline) was administered. If weight did not steadily increase, saline (2 mL) was administered once daily. Rats were also provided with sweetened nourishments (eg, cereal or gel packs) to encourage food intake.

Functional assessments

The Barnes maze and rotarod (model No. MK-630B; Muromachi, Tokyo, Japan) tests are commonly utilized in TBI studies to identify functional deficits as previously described.[7,19,20] Briefly, the Barnes maze is a well-characterized test that encourages spatial learning and memory by using a rat's innate need to escape brightly lit areas,[21] and the rotarod is a reliable measure for vestibulomotor deficits.[20] However, because the adhesive tape test was relatively insensitive in detecting functional differences between peptide-treated and untreated Sprague-Dawley rats,[7] it was omitted for this study. Although experimenters were blinded to each animal's treatment status, latency for each assessment was measured in the early morning (commencing at 0730 hours) the day before surgery (baseline), then at days 1 and 3 postinjury. On each day, animals were given three consecutive attempts (trials) at each test. Each animal was given 180 seconds to locate the 1 darkened escape hole out of 20, and no time limit for the rotarod as animals attempted to stay on the rotating rod (4–40 rpm) for as long as possible. Mean latencies for each treatment group on postinjury days 1 and 3 were compared with baseline mean latency and presented as a percent change from baseline, which was then used for statistical analysis. A treatment group recording a positive or a negative value indicated an improvement or decline in functional recovery, respectively.

Histological assessment for axonal injury

Three days after TBI, animals were killed with pentobarbital (100 mg/kg, IP) and transcardially perfused with normal saline, followed by 10% neutral buffered formalin. Brains were removed and postfixed in 4% formalin for 1 week before embedding in paraffin. Sectioned 10 µm coronal slices corresponding to bregma −4.5 were stained using Bielschowsky silver stain as previously described.[7] Following rehydration, slides were treated with a 10% silver nitrate solution for 8 to 10 minutes at 40°C, followed by ammoniacal silver (1 drop ammonium hydroxide in 10% silver nitrate) for 18 minutes, before reducing (50 mL developer stock solution: 0.25 g sodium citrate, 2 drops concentrated nitric acid, 10 mL 37% to 40% formaldehyde; 50 mL developer working solution: 8 drops each of concentrated ammonium hydroxide and developer stock solution) to a visible metallic silver. Slides were dehydrated in 1 change each of 95% and 100% ethanol, 2 changes of xylene, and mounted with Depex mounting medium. Stained sections were then imaged using light microscopy to qualitatively grade axonal injury (0–4) within the corpus callosum from 3 consecutive sections, while still blinded to the treatment groups. Axonal injury grading ranged

from 0 (indicating absence of injury) to 4. Increasing grade was indicative of increasing degree of axons displaying an increasing degree of disorganised architecture and orientation of oligodendrocyte nuclei, undulation, and varicosities. Images were captured using an Olympus DP-70 digital camera (Olympus, Tokyo, Japan) fitted to an Olympus IX70 inverted microscope.

Statistical analysis

All statistical analyses were conducted in R version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria) and presented as mean (SEM). Two-way and 1-way ANOVA were performed, followed by a Fisher LSD post hoc test when the omnibus ANOVA was significant. Data from the axonal injury assessment measurements were analyzed using the Kruskal-Wallis test with post hoc Bonferroni analyses. For statistical analysis of functional and histological outcomes, both R18D groups were compared with the vehicle treatment group. A value of $P < 0.05$ was considered statistically significant for all data sets.

Results

Functional outcomes

A 2-way ANOVA with repeated measures was initially performed. However, the interaction between treatment and day was not significant for either Barnes maze ($P = 0.395$) or rotarod performance ($P = 0.1333$). Therefore, we analyzed day 1 and day 3 separately, using a 1-way ANOVA.

On postinjury day 1, low and high R18D treatment groups resulted in an 8% and 30% decrease in performance from baseline in the Barnes maze assessment for learning and memory ($F_{3,20} = 1.31$; $P = 0.297$), whereas the vehicle treatment group exhibited a 148% decrease (Figure 1A). On postinjury day 3 ($F_{3,20} = 1.25$; $P = 0.317$), treatment with R18D at the low and high dose resulted in a 66% and 68% improvement, respectively, from baseline, whereas the vehicle group displayed a 36% improvement from baseline (Figure 1A). Although treatment with R18D appeared to provide positive effects on learning and memory recovery compared with vehicle, the results were not statistically significant. Sham animals did not appear to display any obvious functional deficits.

On postinjury day 1, rotarod assessment for vestibulomotor function ($F_{3,20} = 28.9$; $P < 0.001$) for the low- and high-dose R18D treatment groups resulted in a respective 90% ($P = 0.85$) and 72% ($P = 0.36$) decrease in performance from baseline, whereas the vehicle treatment group exhibited an 87% decrease (Figure 1B). On postinjury day 3 ($F_{3,20} = 4.21$; $P = 0.018$), the low- and high-dose R18D groups resulted in a respective 65% ($P = 0.80$) and 29% ($P = 0.24$) decrease in performance, whereas the vehicle group exhibited a 58% decrease (Figure 1B). Sham animals did not appear to display any obvious functional deficits.

Histological outcomes

Based on Bielschowsky silver stain, axonal injury in the corpus callosum ranged from grade 0 to 3.5 ($P = 0.013$) across all treatment groups (Figure 2A and 2B). Vehicle-treated animals recorded the highest average axonal injury score (2.25), despite one animal displaying no apparent axonal injury (Figure 2C). Animals treated with the low and high doses of R18D reduced average axonal injury grades to 1.40 ($P = 0.121$) and 1.21 ($P = 0.044$), respectively, with the high dose significantly reducing injury compared with vehicle-treated animals. As expected, sham animals displayed no axonal injury (grade 0). Further analysis found no correlation between axonal injury scoring and either functional outcome.

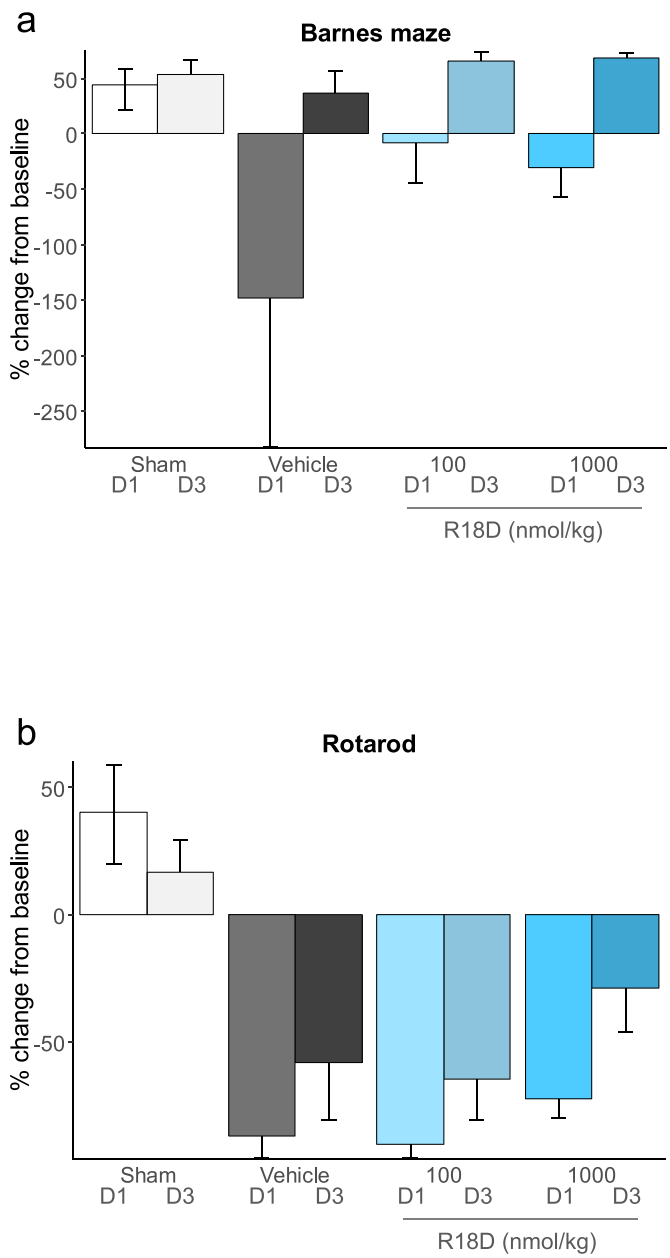

Figure 1. (A) Learning and memory and (B) vestibulomotor functional recovery on the Barnes maze and rotarod. Postinjury day 1 (D1) and day 3 (D3) for each group (N = 5–7). Data are presented as mean (SEM). The mean latency (SEM) of each treatment group on the Barnes maze is shown in Supplemental Table 1 in the online version at doi:XXXXXXXX. The mean latency (SEM) of each treatment group on the rotarod is shown in Supplemental Table 2 in the online version at doi:XXXXXXXX.

Discussion

In line with our previous studies examining the effectiveness of R18D in a rat closed-head injury model, the current findings indicate that intravenous administration of R18D at 1000 nmol/kg has therapeutic potential in TBI. Histological examination demonstrated that R18D treatment could reduce the extent of axonal injury in the corpus callosum. Furthermore, R18D treatment displayed a tendency to improve functional recovery in the Barnes maze and on the rotarod, although these changes were not robust.

Diffuse axonal injury is an important feature of TBI caused by both biomechanical and biochemical disturbances. Although the biomechanical primary phase of brain trauma is not amenable to

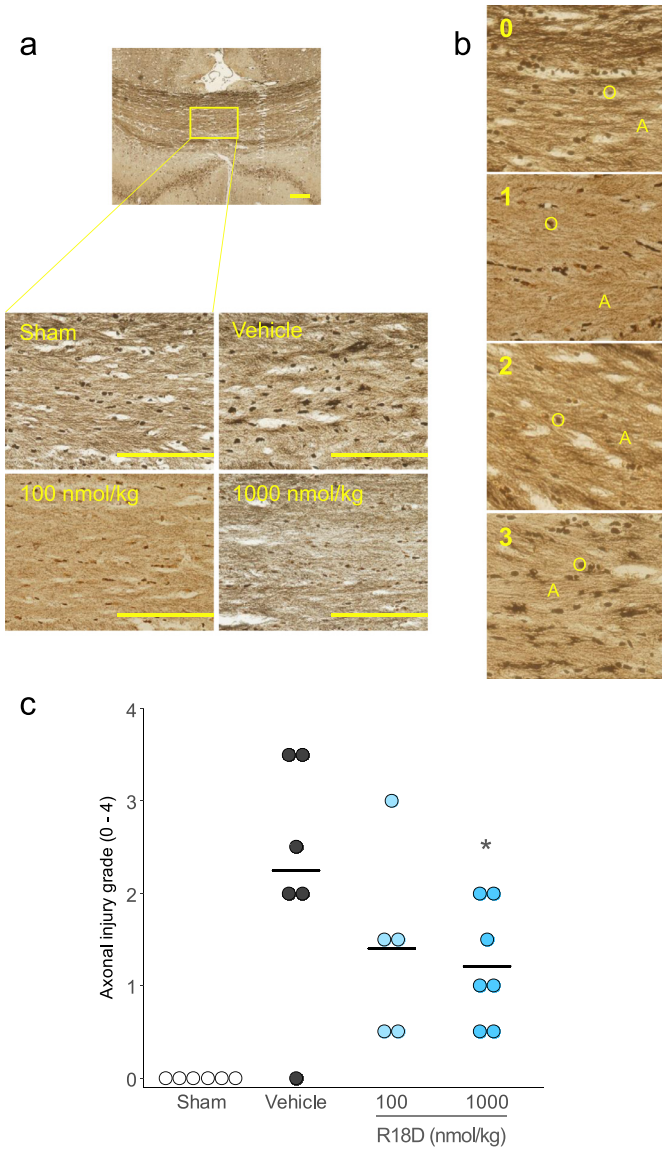

Figure 2. Representative images of each treatment status and the corpus callosum where images were obtained (A) following Bielschowsky silver stain, with total magnification at 200 × (horizontal yellow bar = 200 μm), and (B) axonal injury grading criteria from 0 to 3. No samples were graded at 4. (C) Grading of the extent of axonal injury in the corpus callosum. Horizontal bar represents mean grade (N = 5–7). $^{*}P < 0.05$ when compared with vehicle treatment group. [O]Oligodendrocyte nucleus. [A]Axonal fibers.

pharmacological treatment, the secondary pathophysiological consequences associated with the biochemical aspect can be targeted. Secondary injury processes such as excitotoxicity, mitochondrial dysfunction, oxidative stress, and inflammation contribute to disrupted axonal transport and degeneration.[22] Histologically, axonal injury is characterized by axons exhibiting a beads-on-a-string appearance and, in more severe cases, axonal retraction bulbs. The ability of R18D at the high dose, and low dose to a lesser degree, at reducing the extent of axonal damage is in line with an earlier study in our laboratory demonstrating that R18 at a 300 nmol/kg dose reduced axonal injury.[7]

With evidence that CARPs, including polyarginine-18 peptides (eg, R18 and R18D), have beneficial immunomodulatory effects[15,18,23] and can reduce the toxic accumulation of intracellular calcium,[6,7] it is becoming increasingly apparent that this class of peptide may provide a viable neuroprotective therapeutic agent for TBI. Furthermore, as previous neuroprotective agents developed for TBI have generally targeted a single pathophysiological event such as glutamate receptor antagonists (eg, dexanabinol, selfotel, and magnesium) and calcium channel blockers (eg, nimodipine and nicardipine)[24] for excitotoxicity and mitochondria-interacting agents (eg, cyclosproin A),[25] all have failed clinically. Therefore, R18D represents a therapeutic with a multitude of neuroprotective actions[5] that greatly enhances its neuroprotective potential and translational clinical effectiveness.

In a previous study, we demonstrated that treatment with R18D reduced TBI-associated sensorimotor and vestibulomotor deficits,[18] whereas in the present study only a positive trend for improved recovery was observed for learning, memory, and vestibulomotor function. There could be several reasons for different functional outcomes observed in the 2 studies. Most notably, a different strain of rat was used for the current study. Strain-specific differences in injury susceptibility and recovery outcome have been well documented in TBI.[26] However, a lack of significant findings suggests that the postinjury examination time points and functional assessment regimen may not have been optimal. Additionally, higher animal numbers and a longer study end point may have reduced variability and uncovered statistically significant differences with R18D treatment.

The current and previous studies suggest that R18D has potential to improve motor and cognitive outcomes after TBI. The molecular mechanisms contributing to these improvements may be attributed to its ability to reduce neuronal apoptosis and promote outgrowth.[7,17] Furthermore, other CARPs have also demonstrated the ability to improve functional outcome in TBI models. For example, the peptides COG1410 (Ac-AS(Aib)LRKL(Aib)KRLL-NH_2, net charge: +4) and CN-105 (Ac-VSRRR-NH_2, net charge: +3) provided significant improvements in spatial learning and memory in mouse TBI models.[15,27] Furthermore, pretreatment with PACAP38 (Ac-HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK-NH_2, net charge: +9.1) improved learning and memory function in rats subjected to a TBI.[28] Uninjured healthy rats treated with PACAP38 demonstrated an improvement in spatial memory and increased expression of antioxidative enzymes.[29]

Conclusions

This study has demonstrated that R18D only modestly reduced axonal injury at a dose of 1000 nmol/kg and had no effect on functional recovery. To elucidate whether R18D may be at least partly responsible for these effects, further dose-response and long-term studies are required.

CRediT authorship contribution statement

Li Shan Chiu: Data curation, Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Ryan S. Anderton:** Conceptualization, Supervision, Writing - review & editing. **Vince W. Clark:** Data curation, Methodology. **Jane L. Cross:** Supervision. **Neville W. Knuckey:** Conceptualization, Funding acquisition, Supervision. **Bruno P. Meloni:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Writing - review & editing.

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Conflicts of Interest

B. P. Meloni and N. W. Knuckey are the holders of several patents regarding the use of arginine-rich peptides as neuroprotective treatments. The authors have indicated that they have no other conflicts of interest regarding the content of this article.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.curtheres.2020.100584.

References

1. Milani D, Bakeberg MC, Cross JL, et al. Comparison of neuroprotective efficacy of poly-arginine R18 and R18D (D-enantiomer) peptides following permanent middle cerebral artery occlusion in the Wistar rat and in vitro toxicity studies. Mongin AA, ed. *PLoS One*. 2018;13(3). doi:10.1371/journal.pone.0193884.
2. Milani D, Cross JL, Anderton RS, Blacker DJ, Knuckey NW, Meloni BP. Delayed 2-hour post-stroke administration of R18 and NA-1 (TAT-NR2B9c) peptides after permanent and/or transient middle cerebral artery occlusion in the rat. *Brain Res Bull*. 2017.
3. Milani D, Cross JL, Anderton RS, Blacker DJ, Knuckey NW, Meloni BP. Neuroprotective efficacy of poly-arginine R18 and NA-1 (TAT-NR2B9c) peptides following transient middle cerebral artery occlusion in the rat. *Neurosci Res*. 2017;114:9–15. doi:10.1016/j.neures.2016.09.002.
4. Meloni BP, Milani D, Edwards AB, et al. Neuroprotective peptides fused to arginine-rich cell penetrating peptides: Neuroprotective mechanism likely mediated by peptide endocytic properties. *Pharmacol Ther*. 2015;153:36–54. doi:10.1016/j.pharmthera.2015.06.002.
5. Chiu LS, Anderton RS, Knuckey NW, Meloni BP. Peptide Pharmacological Approaches to Treating Traumatic Brain Injury: a Case for Arginine-Rich Peptides. *Mol Neurobiol*. 2017;54(10):7838–7857. doi:10.1007/s12035-016-0287-3.
6. MacDougall G, Anderton RS, Edwards AB, Knuckey NW, Meloni BP. The Neuroprotective Peptide Poly-Arginine-12 (R12) Reduces Cell Surface Levels of NMDA NR2B Receptor Subunit in Cortical Neurons; Investigation into the Involvement of Endocytic Mechanisms. *J Mol Neurosci*. 2016;12:1–12. doi:10.1007/s12031-016-0861-1.
7. Chiu LS, Anderton RS, Cross JL, et al. Assessment of R18, COG1410, and APP96-110 in excitotoxicity and traumatic brain injury. *Transl Neurosci*. 2017;8(1):147–157. doi:10.1515/tnsci-2017-0021.
8. Ferrer-Montiel A V. Merino JM, Blondelle SE, Perez-Payà E, Houghten RA, Montal M. Selected peptides targeted to the NMDA receptor channel protect neurons from excitotoxic death. *Nat Biotechnol*. 1998;16(3):286–291. doi:10.1038/nbt0398-286.
9. Brustovetsky T, Pellman JJ, Yang X-F, Khanna R, Brustovetsky N. Collapsin Response Mediator Protein 2 (CRMP2) Interacts with N-Methyl-d-aspartate (NMDA) Receptor and Na+/Ca2+ Exchanger and Regulates Their Functional Activity. *J Biol Chem*. 2014;289(11):7470–7482. doi:10.1074/jbc.M113.518472.
10. Planells-Cases R, Aracil A, Merino JM, et al. Arginine-rich peptides are blockers of VR-1 channels with analgesic activity. *FEBS Lett*. 2000;481(2):131–136.
11. García-Caballero A, Gadotti VM, Stemkowski P, et al. The Deubiquitinating Enzyme USP5 Modulates Neuropathic and Inflammatory Pain by Enhancing Cav3.2 Channel Activity. *Neuron*. 2014;83(5):1144–1158. doi:10.1016/j.neuron.2014.07.036.
12. MacDougall G, Anderton RS, Mastaglia FL, Knuckey NW, Meloni BP. Mitochondria and neuroprotection in stroke: Cationic arginine-rich peptides (CARPs) as a novel class of mitochondria-targeted neuroprotective therapeutics. *Neurobiol Dis*. 2019;121(May 2018):17–33. doi:10.1016/j.nbd.2018.09.010.
13. Miyamoto K, Tsumuraya T, Ohtaki H, et al. PACAP38 Suppresses Cortical Damage in Mice with Traumatic Brain Injury by Enhancing Antioxidant Activity. *J Mol Neurosci*. 2014:370–379. doi:10.1007/s12031-014-0309-4.
14. Cao F, Jiang Y, Wu Y, et al. Apolipoprotein E-Mimetic COG1410 Reduces Acute Vasogenic Edema following Traumatic Brain Injury. *J Neurotrauma*. 2016;33(2):175–182. doi:10.1089/neu.2015.3887.
15. Laskowitz DT, Wang H, Chen T, et al. Neuroprotective pentapeptide CN-105 is associated with reduced sterile inflammation and improved functional outcomes in a traumatic brain injury murine model. *Sci Rep*. 2017;7(October 2016):46461. doi:10.1038/srep46461.
16. Plummer SL, Corrigan F, Thornton E, et al. The amyloid precursor protein derivative, APP96-110, is efficacious following intravenous administration after traumatic brain injury. *PLoS One*. 2018;13(1). doi:10.1371/journal.pone.0190449.
17. Batulu H, Du G, Li D, Sailike D, Fan Y, Geng D. Effect of poly-arginine R18 on neurocyte cell growth via autophagy in traumatic brain injury. *Exp Ther Med*. March 2019:1–7. doi:10.3892/etm.2019.7423.
18. Chiu LS, Anderton RS, Cross JL, Clark VW, Knuckey NW, Meloni BP. Poly-arginine Peptide R18D Reduces Neuroinflammation and Functional Deficits Following Traumatic Brain Injury in the Long-Evans Rat. *Int J Pept Res Ther*. 2019;25(4):1563–1572. doi:10.1007/s10989-018-09799-8.
19. Fox GB, Fan L, Levasseur RA, Faden AI. Effect of traumatic brain injury on mouse spatial and nonspatial learning in the Barnes circular maze. *J Neurotrauma*. 1998. doi:10.1089/neu.1998.15.1037.
20. Hamm RJ, Pike BR, O'Dell DM, Lyeth BG, Jenkins LW. The Rotarod Test: An Evaluation of Its Effectiveness in Assessing Motor Deficits Following Traumatic Brain Injury. *J Neurotrauma*. 1994;11(2):187–196. doi:10.1089/neu.1994.11.187.
21. Barnes CA. Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat. *J Comp Physiol Psychol*. 1979;93(1):74–104. doi:10.1037/h0077579.
22. Maxwell WL, Domleo A, McColl G, Jafari SS, Graham DI. Post-acute alterations in the axonal cytoskeleton after traumatic axonal injury. *J Neurotrauma*. 2003;20(2):151–168.
23. Lynch JR, Wang H, Mace B, et al. A novel therapeutic derived from apolipoprotein E reduces brain inflammation and improves outcome after closed head injury. *Exp Neurol*. 2005;192(1):109–116. doi:10.1016/j.expneurol.2004.11.014.
24. McConeghy KW, Hatton J, Hughes L, Cook AM. A review of neuroprotection pharmacology and therapies in patients with acute traumatic brain injury. *CNS Drugs*. 2012;26(7):613–636. doi:10.2165/11634020-000000000-00000.
25. Hatton J, Rosbolt B, Empey P, Kryscio R, Young B. Dosing and safety of cyclosporine in patients with severe brain injury. *J Neurosurg*. 2008;109(4):699–707. doi:10.3171/JNS/2008/109/10/0699.
26. Reid WM, Rolfe A, Register D, Levasseur JE, Churn SB, Sun D. Strain-related differences after experimental traumatic brain injury in rats. *J Neurotrauma*. 2010;27(7):1243–1253.
27. Laskowitz DT, McKenna SE, Song P, et al. COG1410, a novel apolipoprotein E-based peptide, improves functional recovery in a murine model of traumatic brain injury. *J Neurotrauma*. 2007;24(7):1093–1107. doi:10.1089/neu.2006.0192.
28. Mao SS, Hua R, Zhao XP, et al. Exogenous administration of PACAP alleviates traumatic brain injury in rats through a mechanism involving the TLR4/MyD88/NF-kappaB pathway. *J Neurotrauma*. 2012;29(10):1941–1959. doi:10.1089/neu.2011.2244.
29. Ladjimi MH, Barbouche R, Ben Barka Z, et al. Comparison of the effects of PACAP-38 and its analog, acetyl-[Ala15, Ala20] PACAP-38-propylamide, on spatial memory, post-learning BDNF expression and oxidative stress in rat. *Behav Brain Res*. 2019;359:247–257.