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Li Shan Chiu

Ryan S. Anderton
The University of Notre Dame Australia, ryan.anderton@nd.edu.au

Jane L. Cross

Vince W. Clark

Neville W. Knuckey

See next page for additional authors

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Poly-arginine peptide R18D reduces neuroinflammation and functional deficits following traumatic brain injury in the Long-Evans rat

Li Shan Chiu [a, b*], Ryan S. Anderton [a, b, d, e], Jane L. Cross [a-c], Vince W. Clark [a-c], Neville W. Knuckey [a-c], Bruno P. Meloni [a-c]

[a] Perron Institute for Neurological and Translational Science, Nedlands, Australia

[b] Centre for Neuromuscular and Neurological Disorders, The University of Western Australia, Nedlands, Australia

[c] Department of Neurosurgery, Sir Charles Gairdner Hospital, QEII Medical Centre, Nedlands, Western Australia, Australia

[d] School of Heath Sciences, The University Notre Dame Australia, Fremantle, Western Australia, 6160, Australia

[e] Institute for Health Research, The University Notre Dame Australia, Fremantle, Western Australia, 6160, Australia

*Correspondence author.

Perron Institute for Neurological and Translational Science,

QEII Medical Centre,

8 Verdun St, RR Block, Nedlands, Western Australia, 6009, Australia.

E-mail: lishan.chiu@research.uwa.edu.au

Ph: +61 8 6457 0317

Fax: +61 8 6457 0333

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Abstract

We have previously demonstrated that the poly-arginine peptide R18 can improve histological and functional outcomes following traumatic brain injury (TBI) in the Sprague-Dawley rat. Since D-enantiomer peptides are often exploited in pharmacology for their increased stability and potency, the present study compared the effects of R18 and its D-enantiomer, R18D, following TBI in the Long-Evans rat. Following a closed-head impact delivered via a weight-drop apparatus, peptide was administered at a dose of 1000 nmol/kg at 30 minutes after TBI. Treatment with R18D, but not R18 resulted in significant reductions in sensorimotor ($p = 0.026$) and vestibulomotor ($p = 0.049$) deficits as measured by the adhesive tape removal and rotarod tests. Furthermore, treatment with R18 and R18D resulted in a significant reduction in brain protein levels of the astrocytic marker, glial fibrillary acidic protein ($p = 0.019$ and $p = 0.048$, respectively). These results further highlight the beneficial effects of poly-arginine peptides in TBI, however additional studies are required to confirm these positive effects.

Key words: neuroprotection; arginine-rich; peptide; TBI; inflammation

Introduction

In the absence of an efficacious neuroprotective therapeutic, traumatic brain injury (TBI) persists as a global burden. Survivors are often plagued with somatic complaints, cognitive dysfunction, and physical impairment for the remainder of their life. TBI also has long-term effects on society and the economy such as low return-to-work rates (Gabbe et al. 2016), considerable financial burden on the health care system, and proclivity for criminal behaviour in both males (Timonen et al. 2002; Schofield et al. 2006; Williams et al. 2010) and females (O'Sullivan et al. 2015). Therefore, a neuroprotective treatment that can minimise injury and improve outcomes after a TBI is urgently needed. The multifaceted pathophysiology of TBI presents significant challenges in developing an effective neuroprotective therapeutic. Thus, developing a multifunctional agent that targets several neuroprotective and/or neurodamaging pathways will likely provide the best translational opportunity for clinical success.

We have recently demonstrated that cationic arginine-rich peptides (CARP), including poly-arginine peptides, are highly neuroprotective in excitotoxic and oxygen glucose deprivation neuronal cell culture models (Meloni et al. 2015a, b, 2017; MacDougall et al. 2016; Chiu et al. 2017a), and in middle cerebral artery occlusion (Milani et al. 2016b, a, 2017, 2018; Meloni et al. 2017), hypoxic-ischemic encephalopathy (HIE) (Edwards et al. 2018), and TBI (Chiu et al. 2017a) animal models. In addition, our laboratory has also demonstrated that CARPs reduce excitotoxic neuronal calcium influx and reduce neuronal surface expression of the NMDA receptor subunit protein NR2B9c (MacDougall et al. 2016; Chiu et al. 2017a). Other studies have demonstrated that CARPs reduce the activity and/or surface expression of other ion channels and receptors (e.g. NCX, CaV2.2, TNFR) (Fotin-Mleczek et al. 2005; Brittain et al. 2011; Brustovetsky et al. 2014; Meloni et al. 2015a) that exacerbate ionic disturbances or cell death pathways that may be associated with a TBI. Additionally, CARPs

have other properties that may be beneficial in terms of neuroprotection such as the ability to target and stabilise mitochondria (Chen et al. 2015), mitigate ROS production (Szeto 2006; Dai et al. 2011; Szeto et al. 2011), activate pro-cell survival signalling (Gu et al. 2013; Yang et al. 2016), inhibit proprotein convertases that activate matrix metalloproteinases (Cameron et al. 2000; Kacprzak et al. 2004; Fugere et al. 2007; Ramos-Molina et al. 2015), and reduce inflammation (Laskowitz et al. 2006; Tu et al. 2017). Furthermore, CARPs have cell penetrating properties which allow them to cross the blood brain barrier and enter the brain.

The use of chiral compounds in pharmacology has long been known to enhance stability and potency of the compound (Roques et al. 1993; Abadji et al. 1994). In the case of peptides, this can result in increased uptake into the brain (Wei et al. 2014). For example, peptides synthesised with D-enantiomer amino acids are more resistant to degradation by peptidases after intravenous administration (Wei et al. 2014). The poly-arginine peptide R18 (18-mer of arginine; R = arginine) and its D-enantiomer R18D have been assessed in both neuronal cell culture excitotoxic and *in vivo* stroke and HIE models in our laboratory (Milani et al., 2018; Edwards et al., 2018). The studies revealed a similar neuroprotective efficacy in the *in vitro* excitotoxic and animal HIE models, while a single-dose study in a permanent middle cerebral artery occlusion stroke model revealed that R18D reduced infarct volume to a greater extent than R18. However, both peptides reduced hemisphere swelling to a similar degree.

Considering our previous studies assessing R18 and/or R18D in TBI and in stroke-related models this study had several objectives. The first was to examine R18 and R18D efficacy in reducing functional deficits associated with TBI as functional improvement is the ultimate goal of any neuroprotective therapy. Secondly, we also examined global changes in the inflammatory response following treatment with R18 and R18D by measuring GFAP, IL-6,

TNFα, and IL-1β expression in total protein brain lysates following TBI. As only modest effects were observed with 300 nmol/kg R18 in our previous study, we increased the dose to 1000 nmol/kg. Furthermore, neuroprotective effects and injury outcomes can differ in different strains of rats. Given that our previous TBI study employed Sprague-Dawley rats, the present study used Long-Evans rats.

Methods

Peptides used in this study

R18 (H-RRRRRRRRRRRRRRRRRR-OH; net charge +18; R = L-arginine) and R18D peptide (H-rrrrrrrrrrrrrrrrrr-OH; net charge +18; r = D-arginine) peptides were purified by high performance liquid chromatography to at least 98% and subjected to peptide hydrolysis and liquid chromatography-mass spectrometry to obtain a precise measure of peptide content (Mimotopes). Lyophilised peptide stocks were resuspended in 0.9% sodium chloride for injection (Pfizer) and 650 μL was aliquoted into 3 mL syringes. Reconstituted peptides were stored at -20 °C until use.

Traumatic brain injury model and peptide administration

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 Long-Evans rats weighing 375 – 435 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 total of 40 animals underwent the TBI procedure. Two animals, one R18- and one R18D- treated animal, experienced breathing difficulties during surgical recovery within an hour of removal from ventilation and thus were euthanased. Two separate studies examining either R18 or R18D efficacy were performed.

The R18 study consisted of 6 peptide, 8 vehicle, and 5 sham treated animals. The R18D study consisted of 7 peptide, 9 vehicle, and 5 sham treated animals.

A moderate-severe, diffuse TBI was induced as previously described (Chiu et al. 2017a) using a well-characterised weight-drop impact-acceleration model (Foda and Marmarou 1994; Marmarou et al. 1994). Briefly, animals underwent anaesthetic induction with 5% halothane (mix 30% O_2/70% N_2O gas), intubation, and were maintained using 1 - 2% halothane. TBI was induced by releasing a 435 g brass weight from a 180 cm height, onto a steel disc (1 cm diameter and 2 mm thick) adhered to the skull of the animal with cyanoacrylate. At 30 minutes post-impact, treatments were administered intravenously (600 µL over 6 min) through the right internal jugular vein using an infusion pump. Treatments consisted of the vehicle control (0.9% NaCl for injection), and R18 or R18D at 1000 nmol/kg. Sham animals underwent the same surgical procedure and received vehicle treatment but were not subjected to TBI. All personnel carrying-out treatment administration and animal procedures were blinded to treatment status.

Post-surgical animal care and monitoring

At the end of surgery, pethidine (IM: 1 mg in 0.2 mL saline) and bupivacaine were administered (SC: 0.1 mg in 0.2 mL saline per site) to the head surgical wound. A 2 mL volume of injectable saline was also administered subcutaneously to aid hydration. Cages were placed on a heating mat during a 1 to 2 hours post-surgical monitoring period, and subsequently housed in a holding room maintained at 26 - 28°C. Animals were monitored at least twice a day and provided with sweetened cereal and gel packs to encourage food intake.

Functional assessments

The functional assessments were used to identify deficits in spatial learning and memory (Barnes maze), sensorimotor (adhesive tape removal), and vestibulomotor (rotarod) function as previously described (Chiu et al. 2017a). All three assessments were carried out in the order described above on the day prior to surgery (day -1; baseline), and days 2 and 4 post-TBI. Each animal had three attempts and a maximum of 180 seconds to complete the Barnes maze and adhesive tape removal tests, while no time limit was set for the rotarod test. Functional recovery of each animal was determined by comparing the post-surgery latency for days 2 and 4 post-TBI for each test to its own pre-surgery (day -1; baseline) latency and expressed as percentage change from baseline. An animal recording a positive or a negative value on days 2 or 4 post-TBI indicated an improvement or decline in functional recovery, respectively.

Protein extraction and Western blotting

After the completion of functional measurements on day 4 post-TBI, animals were administered a lethal injection of pentobarbitone (325 mg/mL; Virbac), and transcardially perfused with ice-cold 0.9% saline. The brain was extracted, and total protein isolated from fresh frozen brain tissue using RIPA lysis buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA; 1% Triton X-100; 0.1% sodium deoxycholate; 0.1% SDS; 140 mM NaCl; 1 mM PMSF) containing protease inhibitor (one tablet in 10 mL; Roche). Protein lysates were centrifuged to collect supernatant which was subsequently stored at -80°C. Representative protein samples from each treatment group were then probed via Western blot.

Western blotting was performed as previously described (MacDougall et al. 2016). Briefly, proteins were separated by SDS-PAGE, transferred to PVDF membranes, and blocked in PBS-Tween 20 (0.1%) containing 1 mg/mL ovalbumin for 1 h. Membranes were then

incubated overnight at 4 °C in GFAP (1:1000; Sigma-Aldrich), IL-6 (1:1000; Invitrogen), TNFα (1:1000; Invitrogen), IL-1β (1:1000; Bio-Rad), or β-tubulin (1:3000; Invitrogen) primary antibodies prepared in PBS-Tween 20 (0.1%) with 1 mg/mL ovalbumin. Protein was detected by incubating for at least an hour at room temperature in goat anti-mouse DyLight 800 (1:10000; Bio-Rad) or goat anti-rabbit StarBright 700 (1:3000; Bio-Rad) with added hFab-Rhodamine (β-tubulin 1:10000; Bio-Rad) as the loading control. For β-tubulin, a HRP-conjugated goat anti-mouse secondary antibody (1:20000; Bio-Rad) was used. Blots were visualised using the ChemiDoc system (Bio-Rad). Quantification and band densitometry of Western blots was performed using ImageJ software (v1.51j8; National Institutes of Health, USA).

Statistical analysis

All statistical analyses of functional assessments and densitometry were conducted in R 3.5.1 and presented as mean ± standard error of the mean (SEM). An analysis of variance (ANOVA) was performed, followed by a Fisher's LSD post-hoc test. For statistical analysis of functional outcome and western blots, peptide treatment groups were compared to the vehicle treatment group. A value of $p < 0.05$ was considered statistically significant for all data sets.

Results

Functional assessments

The adhesive tape and rotarod tests demonstrated significant sensorimotor and vestibulomotor deficits in the TBI injured animal groups (Figures 1 – 2). In contrast, sham animals not subjected to TBI did not show deficits across any of the functional assessments (Figures 1 – 3).

For the adhesive tape test, at day 2 post-TBI, R18D-treated animals performed significantly better ($p = 0.026$) than vehicle-treated animals (Figure 1a), while there was no difference in performance between R18 and vehicle treated animals (Figure 1b). At day 4 post-TBI, R18D-treated animals demonstrated an improvement in performance from baseline, however this was not significantly different in performance from vehicle-treated animals, which remained below baseline (Figure 1c). Additionally, R18-treated animals demonstrated an improvement in performance approaching baseline, however this was not significantly different from vehicle treated animals, which remained below baseline (Figure 1d).

For the rotarod test, at day 2 post-TBI, R18D-treated animals performed significantly better ($p = 0.049$) than vehicle-treated animals (Figure 2a), while there was no difference in performance between R18 and vehicle treated animals (Figure 2b). At day 4 post-TBI, R18D-treated animals demonstrated an improvement in performance compared to vehicle treated animals, albeit not to a statistically significantly level (Figure 2c), while there was no difference in performance between R18 and vehicle treated animals (Figure 2d).

At days 2 and 4 post-TBI, Barnes maze performance remained above baseline and did not differ significantly between peptide and vehicle treated and sham animals (Figure 3a – d).

Western blot analysis for neuroinflammatory response

Western blot analysis of brain lysates collected on day 4 post-TBI, demonstrated that treatment with R18D ($p = 0.048$) and R18 ($p = 0.019$) resulted in significantly reduced GFAP expression by 52% and 67% respectively, compared to treatment with vehicle (Figures 4a). However, neither peptide was significantly better than the other ($p = 0.50$) at reducing GFAP

expression. Treatment with R18D ($p = 0.11$) and R18 ($p = 0.37$) also reduced cytokine IL-6 protein levels by 44% and 23% respectively in the brain, albeit not to a statistically significant level (Figures 4b). Contrastingly, R18 ($p = 0.062$) treatment increased TNFα (Figure 4c), and IL-1β increased in both R18 ($p = 0.59$) and R18D ($p = 0.19$) treatment groups (Figure 4d), however none were significant when compared to vehicle.

Discussion

The present study has demonstrated that treatment with R18D reduced TBI-associated sensory and vestibular motor deficits in the Long-Evans rat. Moreover, both R18 and R18D reduced TBI-associated reactive astrocytic cellular (GFAP) responses. These findings are consistent with our proposed application of CARPs as a therapeutic in TBI (Chiu et al. 2017b), and previous data from our laboratory which has demonstrated that treatment of Sprague-Dawley rats with R18 following a TBI reduced the extent of axonal injury and provided positive trends for functional recovery (Chiu et al. 2017a). While the present study has confirmed that both the L- and D-isoform R18 peptides can provide beneficial outcomes following TBI, the more favourable effects observed following R18D treatment could be related to the superior stability of the D-enantiomer peptides. Interestingly, the Barnes maze test did not demonstrate any obvious deficits in performance following TBI, which suggests that it may not be sufficiently sensitive for use following this TBI model in the Long-Evans rat.

Increased levels of GFAP within the brain is associated with the proliferation of astrocytes and gliosis, which play a key role regulating neuroinflammation, and can have both detrimental and regenerative functions following brain tissue injury. Elevated GFAP expression following TBI is a commonly used biomarker of astrocytic activation and

neuronal degeneration (Huang et al. 2015), and correlates with TBI severity (Hsieh et al. 2017). Therefore, measurement of GFAP is a good pre-clinical biomarker for the assessment of potential neuroprotective treatments in TBI. In this regard, the present study has for the first time demonstrated that R18 and R18D can reduce GFAP levels in the brain following TBI, which is in line with previous studies demonstrating that other CARPs such as COG1410 (AS[Aib]LRKL[Aib]KRLL; net charge +4) (Hoane et al. 2007) and APP96-110 (NWCKRGRQCKTHPH; net charge +5) (Plummer et al. 2018) also reduced GFAP expression following brain injury. The exact mechanism whereby CARPs such as R18 and R18D reduce GFAP expression is not known but may be due to the neuroprotective actions of the peptide limiting neural injury directly, or by inhibiting pathways responsible for astrocyte activation and inflammation.

The suggestion that R18 and R18D could reduce levels of the pro-inflammatory cytokine IL-6 in the brain of rats following TBI is of potential interest. IL-6 expression increases in rodent brain (Taupin et al. 1993; Shohami et al. 1994) and in human serum soon after TBI (Yousefzadeh-Chabok et al. 2015; Gill et al. 2017), with increased levels associated with worse outcomes. Clinically, serum IL-6 has been implicated in elevated intracranial pressure following TBI (Hergenroeder et al. 2010), however the exact role of elevated brain levels of the cytokine with respect to brain injury is poorly understood (Chakraborty et al. 2016). Other CARPs administered following neurotrauma such as COG133 (Ac-LRVRLASHLRKLRKRLL-NH_2, net charge +7.1), CN-105 (Ac-VSRRR-NH_2, net charge +3), and PACAP (Ac-HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK-NH_2, net charge +10.1) have demonstrated downregulation of IL-6 mRNA (Laskowitz et al. 2017) and/or expression of other inflammatory cytokines (Lynch et al. 2003; Mao et al. 2012). In addition, CARPs can reduce the release of IL-6 from C2C12 myotubes exposed to TNF-α

(Lightfoot et al. 2015), synovial fluid mononuclear cells of rheumatoid arthritis patients exposed to VEGF (Yoo et al. 2005), dendritic cells stimulated with toll-like receptor ligands (Kandler et al. 2006), and reduce serum levels in collagen-induced arthritic mice (Yoo et al. 2005).

Both TNFα and IL-1β were elevated in the brain following treatment with R18 and/or R18D, but not to statiscally significant levels. Although there is still uncertainty surrounding the exact role of the neuroinflammatry response after injury, there are suggestions that cytokines may have both deleterious and beneficial effects at different phases of secondary injury (Shohami et al. 1999; Ziebell and Morganti-Kossmann 2010). Given that sensorimotor and vestibulomotor deficits were significantly reduced, but profound changes were not observed in IL-6, TNFα, or IL-1β may be attributable to their dual role in the inflammatory reponse. Similarly, CN-105 demonstrated neuroprotective properties in TBI, despite not reducing mRNA levels of IL-6 and TNFα (Laskowitz et al. 2017).

There are several mechanisms whereby CARPs may suppress the acute inflammatory response following a TBI. For example, CARPs can inhibit the TLR4/MyD88/NF-κβ signalling pathway (Mao et al. 2012) through specific inhibition of NF-κβ. In support of this, the CARP AIP6 (RLRWR, net charge +3) can block binding of the NF-κβ p65 sub-unit to DNA and inhibit the transcription of pro-inflammatory genes (Wang et al. 2011). Furthermore, proteasomal activity increases following TBI, and CARPs such as R8 (RRRRRRRR, net charge +8) (Kloß et al. 2009), PR39 (RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP, net charge +10) (Gao et al. 2000), and PR11 (RRRPRPPYLPR, net charge +5) (Gaczynska et al. 2003; Anbanandam et al. 2008) are potent inhibitors of the proteasome, an effect that would inhibit proteasomal

degradation of the NF-κβ inhibitory sub-unit I-κβ and maintain NF-κβ in an inactive state. In addition, CARPs may affect the cell-surface levels of receptors and/or interfere with binding of ligands to cell-surface receptors such as tumour necrosis factor receptor (Fotin-Mleczek et al. 2005), vascular endothelial growth factor receptor (Yoo et al. 2005), and toll-like receptor (TLR) (Mao et al. 2012) that mediate inflammatory responses. In this regard, the TLR4/MyD88/NF-κβ signalling pathway is also known to activate the JAK-STAT pathway, which is known to regulate GFAP and astrogliosis (Woodcock and Morganti-Kossmann 2013).

Limitations

Two separate studies examining R18 and R18D efficacy at only one dose were performed, thereby precluding a direct side-by-side dose and statistical comparison between the two peptides. In addition, relatively low animal numbers were used, and the study had a short 4-day end point. Future studies examining peptide mechanisms of action, dosing regimen, extended end-points, and additional functional tests (e.g. ladder test, Morris water maze, wire hang) are needed to confirm peptide efficacy after TBI. It would be important in future studies to undertake histological analysis of brain tissue following TBI to confirm that improvements in functional parameters correlate with reductions in brain injury, and to confirm efficacy in other models of TBI. Furthermore, a more segmented analysis of the inflammatory markers may have uncovered more differential effects in different brain regions.

Additionally, the current study did not include sham control animals treated with R18 and R18D as we were not anticipating any confounding effects from the peptides given we have never observed side effects from peptide treatment. Regardless, we acknowledge that the

inclusion of these groups would have been beneficial in understanding the safety and tolerability of intravenously administered R18 and R18D in healthy, male Long-Evans rats.

Conclusions

In line with CARPs having multiple potential mechanisms of neuroprotection following TBI (Chiu et al. 2016, 2017b), we have demonstrated in a weight-drop impact-acceleration model of TBI that both R18 and R18D poly-arginine peptides can reduce GFAP and, to a lesser extent, IL-6 expression in brain tissue, and that R18D reduced the severity of functional deficits after TBI. These findings provide further evidence that CARPs represent a novel class of neuroprotective agent with promising application as an acute treatment for TBI.

Compliance with Ethical Standards

Conflicts of interest: Bruno P. Meloni and Neville W. Knuckey are the holders of several patents regarding the use of arginine-rich peptides as neuroprotective treatments. The other authors declare no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Figure legends

Fig. 1 Adhesive tape test. Performance at days 2 and 4 post-TBI as determined by comparing percentage change of each animal's own latency prior to surgery. (a) R18D-treated animals at day 2 post-TBI. (b) R18-treated animals at day 2 post-TBI. (c) R18D-treated animals at day 4 post-TBI. (d) R18-treated animals at day 4 post-TBI. R18 cohort: sham N = 5, vehicle N = 8, R18 N = 6; R18D cohort: sham N = 5, vehicle N = 9, R18D N = 7. Data are presented as mean ± SEM. *$p < 0.05$ when compared to vehicle treatment group

Fig. 2 Rotarod test. Performance at days 2 and 4 post-TBI as determined by comparing percentage change of each animal's own latency prior to surgery. (a) R18D-treated animals at day 2 post-TBI. (b) R18-treated animals at day 2 post-TBI. (c) R18D-treated animals at day 4 post-TBI. (d) R18-treated animals at day 4 post-TBI. R18 cohort: sham N = 5, vehicle N = 8, R18 N = 6; R18D cohort: sham N = 5, vehicle N = 9, R18D N = 7. Data are presented as mean ± SEM. *$p < 0.05$ when compared to vehicle treatment group

Fig. 3 Barnes maze test. Performance at days 2 and 4 post-TBI post-TBI as determined by comparing percentage change of each animal's own latency prior to surgery. (a) R18D-treated animals at day 2 post-TBI. (b) R18-treated animals at day 2 post-TBI. (c) R18D-treated animals at day 4 post-TBI. (d) R18-treated animals at day 4 post-TBI. R18 cohort: sham N = 5, vehicle N = 8, R18 N = 6; R18D cohort: sham N = 5, vehicle N = 9, R18D N = 7. Data are presented as mean ± SEM

Fig. 4 Western blot analysis of total brain lysates from representative animals treated with vehicle (N = 3), R18D (N = 3), R18 (N = 3) and subjected to TBI or sham (N = 1) surgical procedure. Brain samples collected at day 4 post-TBI or sham surgery (a) GFAP, (b) IL-6,

(c) TNFα, and (d) IL-1β protein expression in peptide treatment groups are presented relative to vehicle treatment group, which was set at 1-fold. Data are presented as mean ± SEM. *$p < 0.05$ when compared to vehicle treatment group.

Figure1

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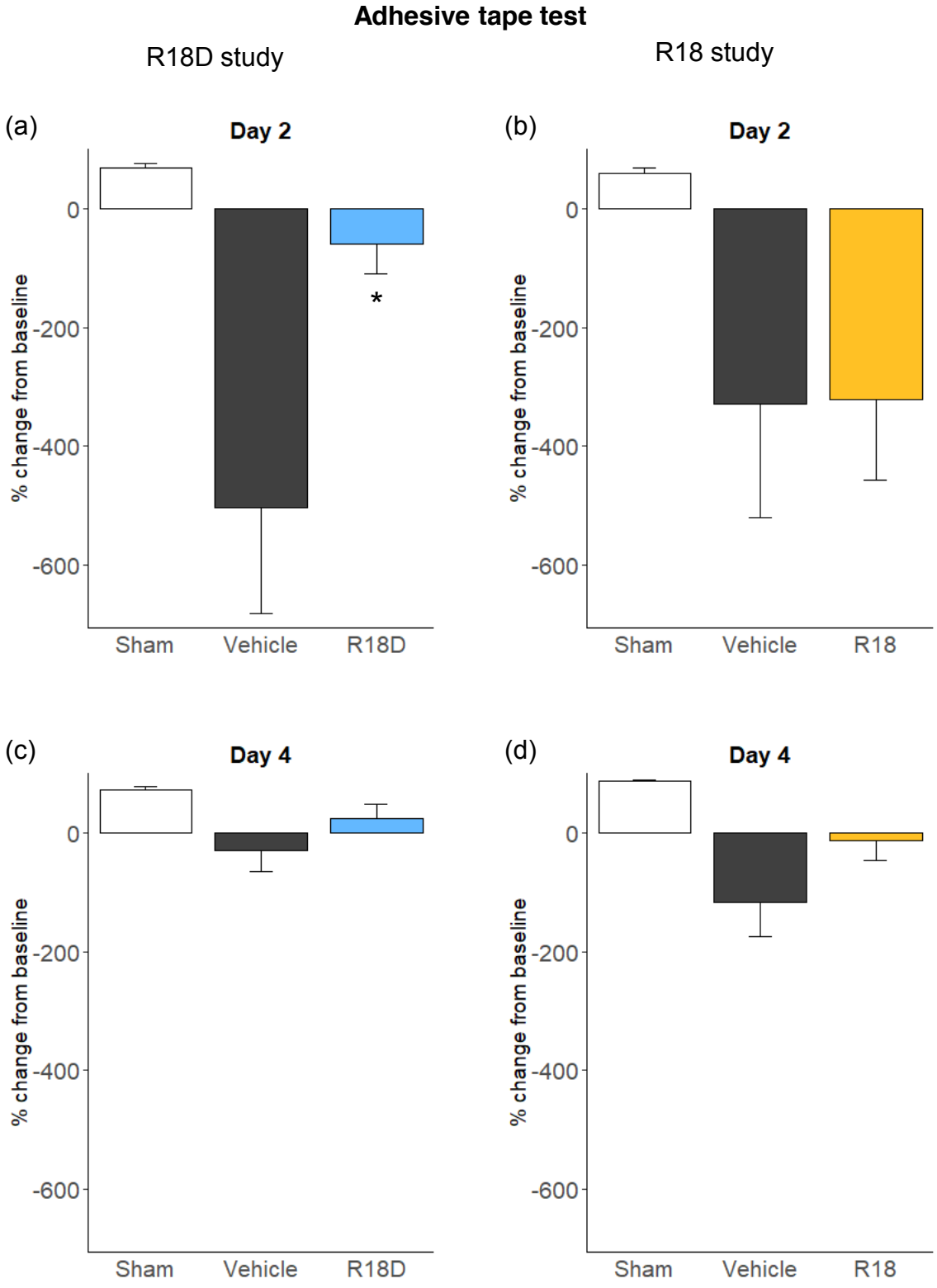

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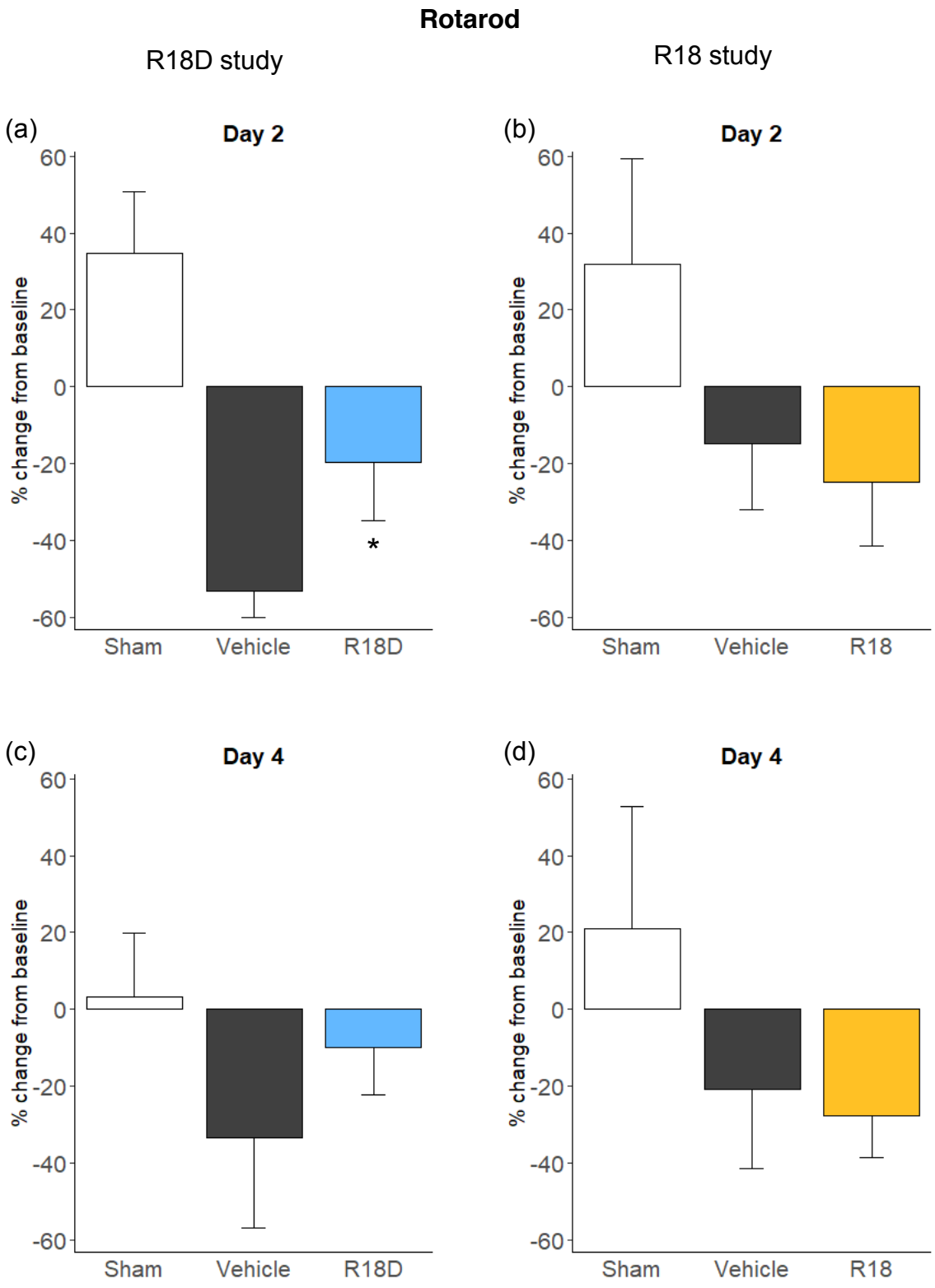

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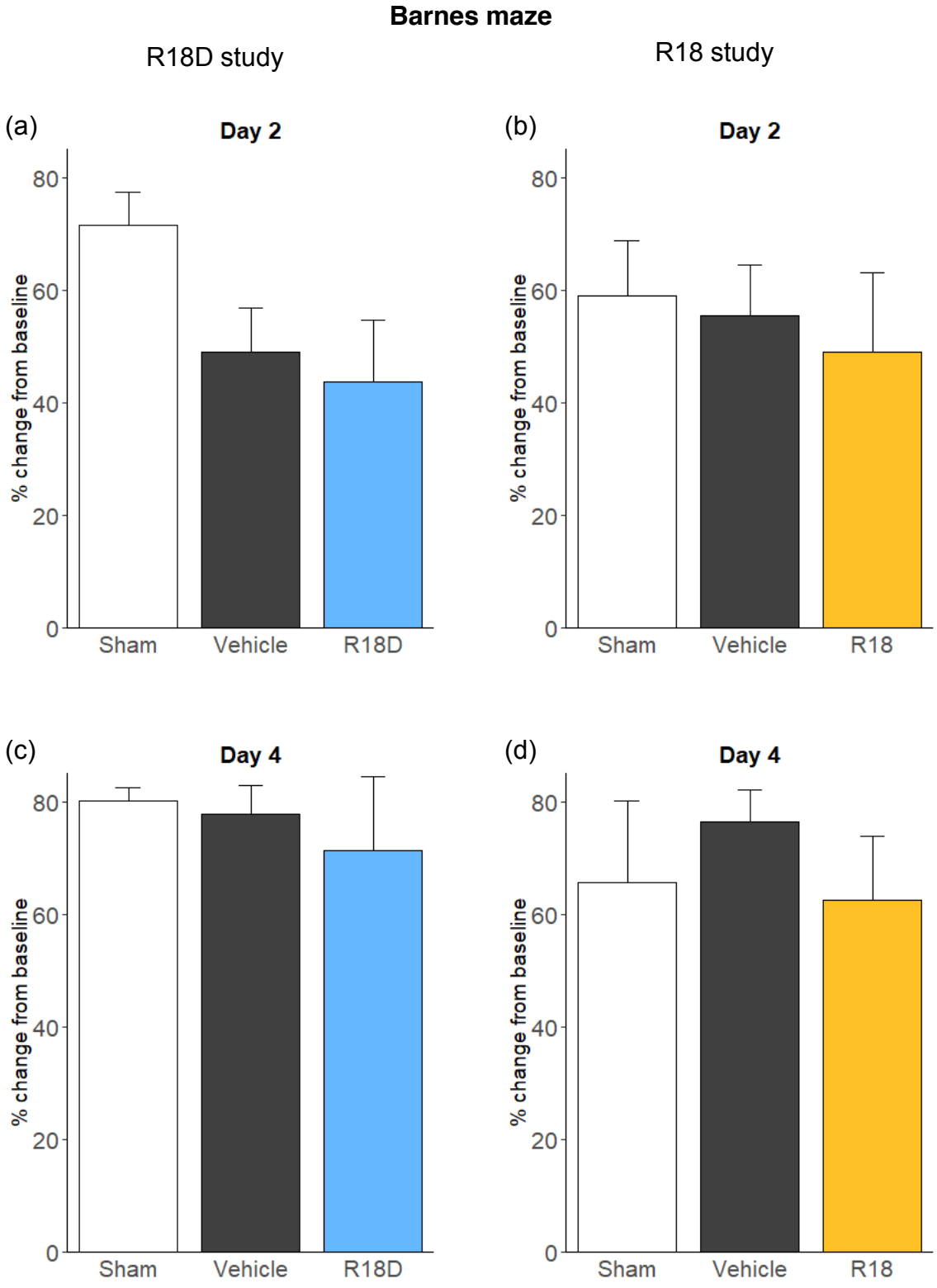

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