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Exercise plasma improves traumatic brain injury outcomes in mice

Peyman Sahbaie[1,2,3]✉, Xiaoyou Shi[1,3], Tian-Zhi Guo[1,2], Wen-Wu Li[1,3], Karen-Amanda Irvine[1,2], QiLiang Chen[1,3] & J. David Clark[1,3]

Traumatic brain injury (TBI) is associated with pain and cognitive impairment although exercise may improve some adverse outcomes. We hypothesized that transfer of plasma derived from exercised mice would improve outcomes related to pain and memory after TBI. These studies used a concussive murine model of mild TBI. Plasma from sedentary or exercised mice was administered to TBI mice beginning 3 days after injuries. Mechanical nociceptive sensitization and descending control of nociception (DCN) were assessed. Object recognition memory and Y-maze were used to assess working memory. In addition, the spinal expression of Pdyn, Bdnf, Cxcl1 and Cxcl2 was measured 7 and 28 days after injuries. Levels of several candidate mediators of the exercise effects were measured in the transferred plasma. Mechanical allodynia following TBI recovered more quickly and DCN responses were partially preserved in animals receiving exercise plasma. Working memory was preserved in the same mice. The upregulation of spinal pain genes Pdyn and Bdnf was reduced by exercise plasma. Finally, exercise was associated with substantially higher plasma levels of TIMP2 and irisin. The present study suggests that developing therapies based on the administration of exercise plasma or its components may help reduce pain and cognitive loss after TBI.

Keywords Traumatic brain injury, Concussion, Pain, Memory, Exercise

Traumatic brain injury (TBI) is a common form of neurotrauma affecting individuals of all ages. The majority of TBIs are mild in severity with an annual incidence of 295 per 100,000 persons[1]. The cost to treat TBI patients can be high and long term, with one recent economic study estimating costs in the US to be $40.6 billion annually[2]. While most individuals survive their injuries, TBI can cause long term issues with cognition, emotion, motor control, sensory processing and chronic pain[3,4]. Amongst the most common chronic outcomes of TBI is pain experienced in the head, spine, and the extremities[5]. Recent clinical and preclinical studies suggest that chronic pain after TBI may be attributable to dysfunction of endogenous pain control circuits including descending pain control circuits[6–11].

A therapy proposed to reduce long-term disabilities that occur following a TBI is exercise[12]. Unfortunately, data examining the direct effects of exercise on pain in TBI patients is still lacking. Preclinically, several studies have demonstrated the benefits of exercise on cognitive outcomes after TBI[13]. Preservation of hippocampal function is one mechanism by which exercise may exert its positive effects[14]. A recent study using the closed head model of TBI revealed that mice using voluntary running wheels had enhanced recovery from early hindlimb and periorbital pain sensitization after injury, and partially preserved descending pain control for months after injuries[15]. Cognitive measures were improved as well. Exercise also reduced the enhanced spinal expression of genes associated with pain after TBI including prodynorphin (Pdyn), brain-derived neurotrophic factor (Bdnf), C-X-C Motif Chemokine Ligand 1 and 2 (Cxcl1 and Cxcl2). This study provided key insights into the mechanisms through which exercise may improve recovery following TBI[16–18].

Experimental models of neurodegenerative disorders (i.e., Alzheimer's disease) have shown that exercise can slow the progression of these diseases and preserve behavioral measures of cognition. These studies support the investigation into the identification of circulating factors that might confer these benefits. The transfer of plasma harvested from mice with access to running wheels into aged and neurodegenerative disease model mice revealed that exercise enhanced circulating levels of clusterin and glycoprotein phospholipase D (GPLD1) that may be involved in the procognitive effects of exercise[19,20]. Likewise irisin, an exercise-induced myokine, was effective

[1]Anesthesiology Service, Veterans Affairs Palo Alto Health Care System, 3801 Miranda Ave (112-A), Palo Alto, CA 94304, USA. [2]Palo Alto Veterans Institute for Research, 3801 Miranda Ave, Palo Alto, CA 94304, USA. [3]Department of Anesthesia, Perioperative and Pain Medicine, School of Medicine, Stanford University, Stanford, CA 94305, USA. ✉email: psahbaie@stanford.edu

in reducing cognitive changes in mouse models of Alzheimer's disease and in improving cognition and mood-related behaviors in sedentary mice[21–23]. Lastly, a naturally occurring tissue inhibitor of metalloproteinases (TIMP2), an inhibitor of metalloproteinases, is found in elevated levels in umbilical cord blood, and has been shown to enhance cognitive performance when transferred to aged mice[24]. This protein is of particular interest as enhanced activity of matrix metalloproteinase-9 (MMP9), a target of TIMP2, is associated with enhanced dendritic spine remodeling and degradation of the extracellular matrix in the brain after TBI[25].

The goal of the studies, therefore, was to determine whether bloodborne factors might mediate the beneficial effects of exercise on TBI outcomes. Such an observation might open the door to the development of novel beneficial therapies and a better understanding of the pathophysiology of TBI itself .

Results

The intravenous transfer of plasma from exercised mice accelerates recovery from nociceptive sensitization after TBI

A general timeline for the procedures carried out in the report is shown in Fig. 1. Mice were exposed to a single, closed-head impact to induce mild TBI (mTBI) above the right S1 cortex. This resulted in bilateral hindpaw allodynia (Fig. 2), similar to that shown in previous reports[11,15,18]. Three days after receiving impacts, mice began receiving intravenous plasma from sedentary or exercised mice twice per week, for 4 weeks. Mice receiving plasma from exercised mice recovered more rapidly from sensitization of both the contralateral (Fig. 2A: $F_{2,21} = 7.64$, $p < 0.01$; significant main effect of treatment) and ipsilateral (Fig. 2B: $F_{2,21} = 9.39$, $p < 0.01$; significant main effect of treatment) hindpaws when compared to TBI mice receiving plasma from sedentary mice. TBI mice treated with exercise plasma had a significant increase in paw withdrawal threshold at both 5- and 7-days post-injury (DPI) compared to TBI mice treated with sedentary plasma.

The intravenous transfer of plasma from exercised but not sedentary mice preserves descending control of nociception (DCN) after TBI

Deficits in DCN is linked to many forms of chronic pain including pain after TBI[26,27]. We used DCN as an index of the efficiency of endogenous pain control mechanisms after mTBI. Following full recovery of hindpaw nociceptive thresholds (at 4 weeks post-TBI), we measured DCN in the mice by first sensitizing the left hindpaw with an intraplantar injection of PGE_2 followed by injection of capsaicin into the right forepaw to activate DCN as we have previously shown[11,15]. Mice with no mTBI exhibit a robust analgesic response to the injection of forepaw capsaicin indicating strong DCN (Fig. 3). TBI mice treated with plasma from sedentary mice showed little analgesia after capsaicin indicating impaired DCN (Fig. 3), as we observed previously after mTBI alone[11,15]. In contrast, mTBI mice that received plasma from exercised mice demonstrated significant analgesic responses indicating a partially intact DCN response (Fig. 3: $F_{2,21} = 17.86$, $p < 0.001$; significant effect of group).

The intravenous transfer of plasma from exercised but not sedentary mice improves working memory deficits after TBI

Assessment of non-spatial working memory using the Object Recognition Memory (ORM) test revealed that mTBI mice treated with plasma from exercised mice had significantly improved novel object recognition ($p < 0.05$) compared to sedentary plasma treated mTBI mice ($p = 0.19$) (Fig. 4A). Supplemental figure S1 shows representative tracings of the ORM testing performed. Furthermore, performance on the Y-maze, a test of spatial working memory, revealed that sedentary plasma treated mTBI mice had significant reductions in short

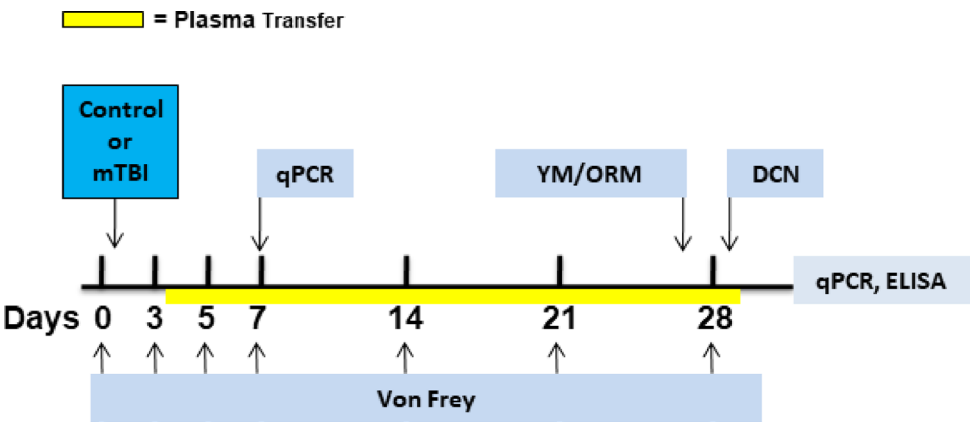

Fig. 1. Overview of the experimental timeline. After measurement of baseline von Frey sensitivity, animals received a mild TBI (mTBI) or sham procedure. After day 3 von Frey measurements, plasma transfer (200ul i.v. 2/wk, yellow line) was started and von Frey measurements continued on days 5, 7, 14, 21, and 28. DCN as well as memory and learning behavior tests were conducted near the completion of plasma treatment. In separate cohorts, animals received TBI with plasma transfer and tissues harvested on day 7 for analysis. Abbreviations: von Frey, mechanical sensitivity assay; DCN, descending control of nociception; YM, Y maze and ORM, object recognition memory.

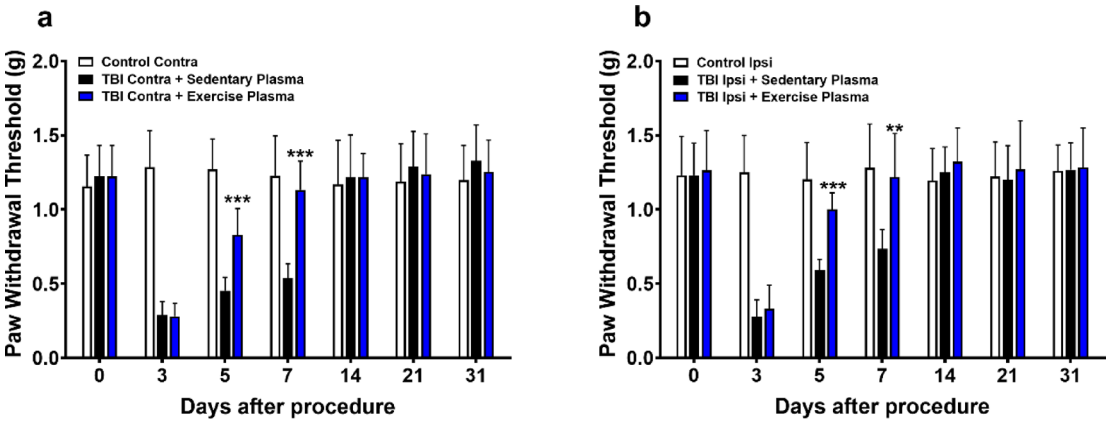

Fig. 2. The intravenous transfer of plasma from exercised mice accelerates recovery from nociceptive sensitization after TBI. Mice with mTBI received intravenous plasma from sedentary or exercised non-TBI mice 2/wk beginning post-TBI day 3 following mechanical sensitivity assessments. Mice receiving exercise plasma showed more rapid recovery from sensitization in hindpaws both contralateral (**A**) and ipsilateral (**B**) to the impacted side of the skull compared to mice receiving plasma from sedentary mice. Data were analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicates significant difference between TBI/Control plasma and TBI/Exercise plasma groups. Error bars: SD, $n = 8$/group.

term spatial memory compared to control mice (TBI/sedentary plasma: 48.94 ± 3.53 vs. control: 59.15 ± 4.30; Mean ± SD, $p < 0.05$) (Fig. 4B). In contrast, exercise plasma treated TBI mice did not demonstrate differences compared to control mice (TBI/Exercise plasma: 55.35 ± 1.95 vs. control: 59.15 ± 4.30; Mean ± SD, $p = 0.20$) (Fig. 4B).

The intravenous transfer of plasma from exercised mice reduces spinal cord pronociceptive gene expression early after TBI

We have previously shown that exercise reduced the spinal expression of several pain-related genes (Bdnf, Pdyn, Cxcl1, and Cxcl2) after mTBI compared to sedentary TBI mice[15]. We therefore measured the expression of these genes in the lumbar spinal cord of sedentary plasma treated mTBI mice compared to exercise plasma treated mice at 7 days after TBI when the differences in nociceptive thresholds between these groups and control mice were maximal (Figs. 2 and 5). Our results show that while mTBI mice treated with sedentary or exercised plasma have greatly elevated expression of Bdnf, Pdyn, Cxcl1, and Cxcl2 genes compared to control mice, the increase in the levels of Bdnf (**5 A**: $F_{2,21} = 24.68$, $p < 0.01$; significant main effect of treatment) and Pdyn (**5B**: $F_{2,21} = 81.18$, $p < 0.001$; significant main effect of treatment) were significantly lower in the exercise plasma treated mTBI mice compared to sedentary plasma treated mice. However, plasma from exercise mice did not significantly reduce the spinal expression of the Cxcl1/2 chemokine genes (Fig. 5C and D) compared to sedentary treated mTBI mice. Lumbar spinal cord tissue was further compared between sedentary and exercise plasma treated mTBI mice at 4 weeks post-injury soon after the last plasma treatment. Expression of our panel of nociception related genes revealed no changes in either sedentary or exercise plasma-treated mTBI mice compared to control mice at this later timepoint (Fig. 6).

Levels of some circulating mediators associated with the beneficial effects of exercise are increased

Some exercise protocols have been shown to elevate circulating levels of several mediators that can enhance cognition in aging animals or in animal models of neurodegenerative diseases[28]. We therefore compared the plasma levels of 4 such mediators, TIMP2, irisin, GPLD1 and clusterin, between sedentary and exercised mice collected 28 days after starting exercise. Biochemical analysis revealed that the levels of TIMP2 (Fig. 7A) and irisin (Fig. 7B) were elevated 4 to 6-fold after exercise compared to plasma from sedentary mice ($p < 0.0001$) whereas the expression of GPLD1 (Fig. 7C) was significantly decreased in plasma from exercised donor mice. Exercise had no effect on the expression levels of clusterin (Fig. 7D) compared to plasma derived from sedentary mice.

Discussion

Mild traumatic brain injury (mTBI) is a common form of neurotrauma with limited treatments available to enhance the recovery of cognitive and/or sensory-related outcomes. Exercise in various forms is commonly recommended as a treatment for persistent symptoms of mTBI and to improve recovery[12,29]. While exercise has been associated with improvements in neurocognitive outcomes after mild TBI[30], differences in the specific outcomes measured in these exercise-based studies and poor data quality have limited the ability to quantify the overall impact of exercise on TBI[29,31]. Adherence to exercise-based therapies in TBI patients is often poor as a consequence of coexisting injuries, lack of available equipment or supervision, and limited self-efficacy

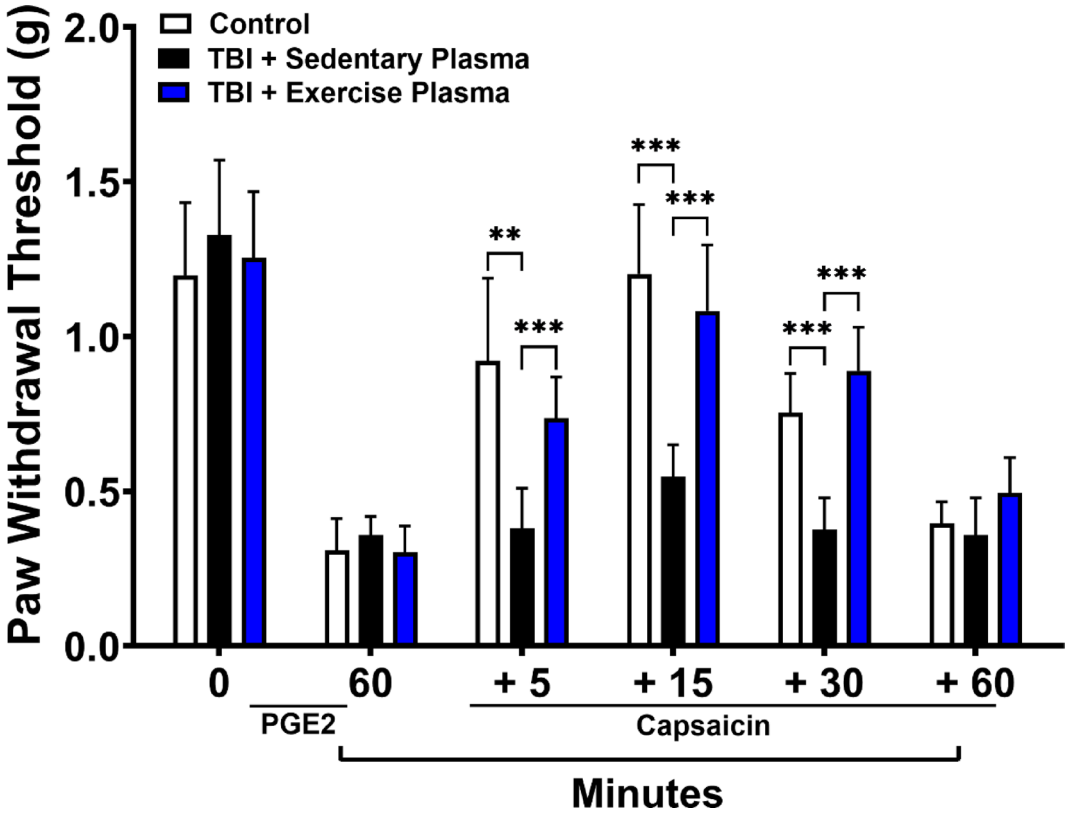

Fig. 3. The intravenous transfer of plasma from exercised but not sedentary preserves descending control of nociception (DCN) after TBI. Once hindpaw nociceptive thresholds had recovered to preinjury levels by 4 weeks post-injury, DCN was measured using a noxious stimulation–induced analgesia protocol. Mice were first sensitized with subcutaneous hindpaw PGE_2 administration and von Frey measurements were conducted. This was followed by forepaw capsaicin administration to activate DCN. Control mice with no TBI showed a strong analgesic responses to forepaw capsaicin indicative of intact DCN pathways. mTBI mice having received plasma from sedentary mice showed little analgesia after capsaicin indicating impaired DCN, while mice that received plasma from exercised mice demonstrated preservation of mTBI induced DCN impairment. Data were analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. ** $p < 0.01$, *** $p < 0.001$ indicates significant difference between groups. Error bars: SD, $n = 8$/group.

among other factors[32]. Our understanding of the mechanisms by which exercise can improve recovery from mTBI and which injury-based sequelae are most amenable to exercise therapy is limited. However, exercised induced circulating factors have been proposed to mediate the beneficial effects of exercise on cognition[20,23,24,33]. Recent experiments from our group suggest that voluntary aerobic exercise using home cage running wheels after mTBI enhances nociceptive and cognitive outcomes in a murine model[15]. The present series of experiments demonstrate that the intravenous transfer of plasma from exercised mice to recipients with a mTBI results in, (1) more rapid resolution of acute nociceptive sensitization and the preservation of descending pain modulatory circuits, (2) reduced cognitive deficits, and (3) reductions in the TBI-induced up-regulation of Pdyn and Bdnf in spinal cord tissue. Results showing rapid improvements in sensitization after TBI in exercise plasma recipients could be relevant to acute forms of post-injury pain, and the preservation of DCN responses suggests that endogenous pain control may also benefit from exercise plasma. Chronic pain of many types including TBI may be more severe in individuals with inefficient or absent endogenous pain control mechanisms that might be preserved or restored with exercise-based treatments[26,27]. The endogenous metalloproteinase inhibitor TIMP2 and the exercise-associated myokine, irisin, were strongly elevated in the plasma purified from mice after 1 month of access to home cage running wheels. Both of these proteins are thought to mediate the beneficial effects of exercise in other systems, and are candidates for mediating the positive effects of exercise after mTBI.

In addition to assessing the effect of plasma from exercised mice on TBI related behavioral changes, we also measured the alterations in gene expression in the spinal cord. We have previously shown an increase in the spinal expression of several genes (Bdnf, Pdyn, Cxcl1, and Cxcl2) that have been shown to be involved in mediating the nociceptive outcomes seen acutely after TBI[15]. Treatment with exercise plasma reduced the elevated expression of Pdyn and Bdnf in tandem with reductions in hindpaw mechanical allodynia after mTBI. Spinal expression of these genes has also been shown to increase in other models of pain involving hindlimb injury[16–18]. After hindlimb injury, intrathecal injection of the kappa opioid receptor antagonist, nor-binaltorphimine, or the TrkB receptor antagonist, ANA-12 can provide analgesia suggesting that spinal prodynorphin and BDNF are capable of supporting allodynia[34]. However, a broader assessment of transcriptional changes after injury and the effects

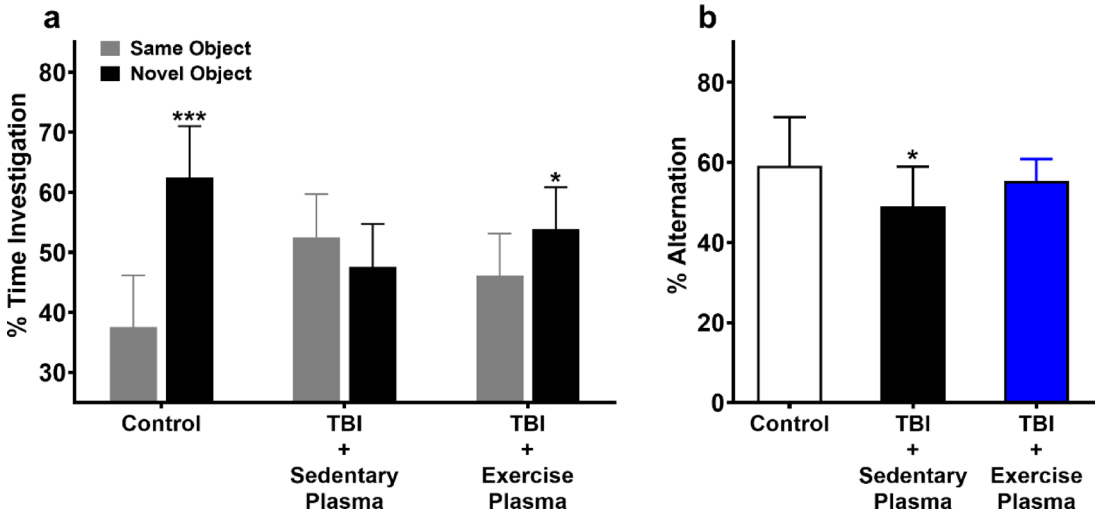

Fig. 4. The intravenous transfer of plasma from exercised but not sedentary mice improves working memory deficits after TBI. Working memory evaluations were in control and TBI mice that received plasma from either sedentary or exercised non-TBI mice. Non-spatial working memory was measured using Object Recognition Memory (ORM) tests. (**A**): Control mice explored the novel object for significantly greater time than the familiar object that was indicative of an intact non-spatial memory. In contrast, mTBI mice that had received sedentary plasma demonstrated a lack of novel object exploration with no significant difference between the novel and familiar object. mTBI mice that had received exercise plasma did explore the novel object for significantly longer time than the familiar object, suggesting that the exercise plasma had preserved the non-spatial memory in these mice. (**B**): Evaluation of spatial working memory was done using Y maze testing. Control mice showed a preference for investigating a new arm of the maze rather than returning to one that was previously visited (Spontaneous alternation). mTBI mice having received sedentary plasma showed no preference between a new or previously visited arm suggesting a reduction in short term spatial memory. mTBI mice that had received the exercise plasma did not significantly differ compared to control mice. * $p < 0.05$, *** $p < 0.001$ indicates significant time difference between novel and familiar objects (**A**); * $p < 0.05$ indicates significant difference between groups (**B**). Error bars: SD, $n = 8$/Group.

of exercise and exercise plasma on those changes may help us to more fully understand the role of exercise in reducing TBI induced pain. We do acknowledge that when looking at the acute effects of plasma transfer, we have not determined whether the transferred plasma has a direct effect on nociceptive signal transmission or acts through an indirect or multi-step process to reduce sensitization. However, we used dialyzed plasma eliminating components smaller than about 3.5 kDa including many metabolites with signaling properties.

Our earlier work has revealed an up-regulation in the expression of the chemokines, Cxcl1 or Cxcl2 in the lumbar spinal cord during the maximal increase in mechanical hypersensitivity of both hindpaws at 7 days post-TBI[18]. CXCR2 is a receptor for CXCL1 or CXCL2, and when injured mice were injected intrathecally with the CXCR2 antagonist, SCH527123 at 7 days post-TBI it resulted in analgesia suggesting important roles for these chemokines in pain after TBI[18,35]. The upregulation of Cxcl1 and Cxcl2 after injury is dependent on descending serotonergic signaling as the intrathecal administration of the serotonergic neurotoxin, 5,7-DHT, prevents their up-regulation as well as hindpaw sensitization after mTBI[18]. Plasticity in descending serotonergic nociceptive signal regulation after injury is well documented in both mouse and rat mTBI models[6–8,11,18]. The transfer of plasma from exercised mice failed to reduce Cxcl1 or Cxcl2 spinal cord expression at 7 days post-TBI when a behavioral effect of the transfer of exercised plasma on nociception was noted. Therefore, the transfer of plasma from exercised mice may be more limited in its impact on the consequences of TBI when compared to TBI mice that are actively exercising. At 4 weeks post-injury, measurements of the Pdyn, Bdnf, Cxcl1 and Cxcl2 gene expression revealed no significant differences between the TBI and control groups, with no effects of plasma transfer. Other rodent TBI studies have shown the elevated expression of some genes for a month or longer [36]. Techniques that can measure expression changes of the whole genome across a broader time course e.g., microarray analysis or RNAseq, and in other key CNS tissue such as the hippocampus plausibly involved in the memory effects may provide a more comprehensive assessment of effects of exercise plasma on the CNS after transfer. Some proteins such as BDNF may be differentially regulated in specific CNS tissues.

Several laboratories have conducted plasma transfer from exercised animals into models of neurodegeneration including age-related changes and Alzheimer's disease in order to identify circulating factors that may explain the beneficial effects of exercise in these models. Other studies have used plasma or plasma derived-components from umbilical cord blood or young animals based on the rationale that young individuals often demonstrate greater neuro-regenerative abilities or better recovery from neurotrauma[24,36]. In the current study we have extended this approach to the setting of TBI. The duration of exercise of the plasma donor mice, plasma preparation and the administration schedule were taken from previous successful studies[19,20], although the required period of donor exercise remains undefined. Consistent with the reports in aged and Alzheimer's model mice, positive

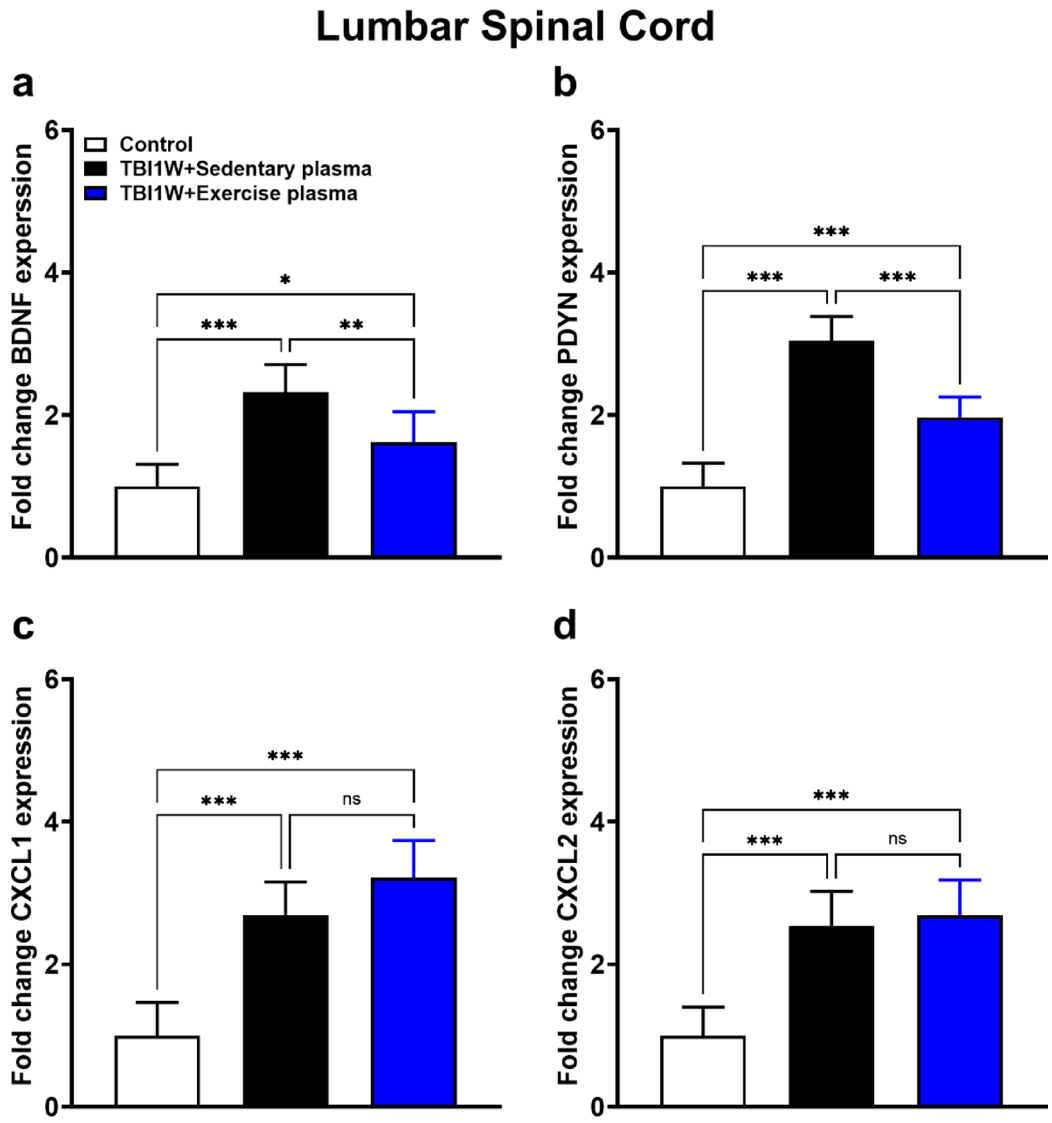

Fig. 5. The intravenous transfer of plasma from exercised donor mice reduces spinal cord early pronociceptive gene expression after TBI. The expression of several known pronociceptive genes in lumbar spinal cord tissue were measured 7 days after mTBI when the differences in nociceptive thresholds between sedentary plasma and exercise plasma treated mice were maximal. TBI mice treated with sedentary plasma had elevated expression of BDNF (**A**), PDYN (**B**), CxcL1 (**C**) and CXCL2 (**D**) genes, while levels of BDNF and PDYN were lower in the exercise plasma treated mice. The exercise plasma treatments did not reduce expression of the chemokine genes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicates significant difference between groups. Error bars: SD, $n = 8$/group.

effects of exercise plasma transfer on cognition were found in our studies. Moreover, we demonstrate that the antinociceptive effects of the exercise plasma is evident even after a single dose. To better characterize the plasma being transferred, we measured the levels of clusterin, GPLD1, irisin and TIMP2 in plasma from exercise versus sedentary mice, as these were reported to be elevated after exercise by other investigators[19,20,22,24]. Exercise has also been reported to reduce plasma GPLD1, and that is what we observed[37]. Only irisin and TIMP 2 were elevated in our preparations.

Individually or together both irisin and TIMP2 may contribute to the observed positive effects of exercise. Irisin is the cleaved product of fibronectin type III domain-containing protein 5 (FNDC5). Recent evidence suggests that this protein is expressed both peripherally in muscle and within the CNS. Knockdown of irisin expression not only reduces cognitive performance measures in mice, but also abrogates the effects of exercise on cognitive enhancement[21]. Irisin is more broadly felt to link exercise to the amelioration of neurological disease[28]. Irisin-induced expression of Bdnf and other genes within the hippocampus may underlie irisin's pro-cognitive effects[38]. It is notable that while exercise and irisin may upregulate Bdnf in hippocampal tissue, exercise and plasma transfer downregulate Bdnf in spinal cord tissue suggesting that irisin's signaling systems may be complex. Likewise, TIMP2, a naturally occurring inhibitor of multiple metalloproteinases[39], is also a

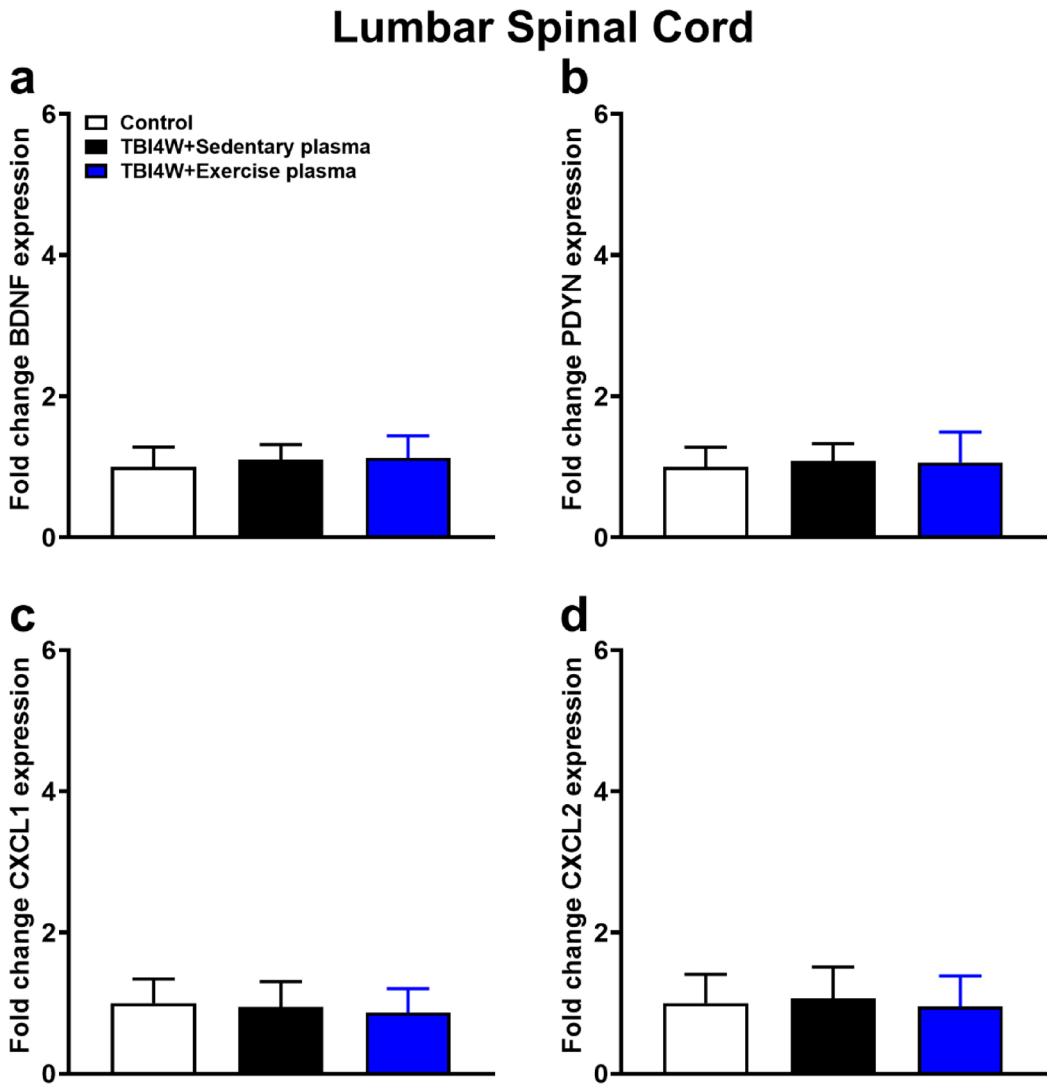

Fig. 6. The intravenous transfer of plasma from exercised donor mice does not reduce spinal cord late pronociceptive gene expression after TBI. The expression of pronociceptive genes in lumbar spinal cord tissue were measured soon after the final plasma injection at 28 days post-TBI. At this time all mTBI mice regardless of treatment showed no significant difference in the expression of the genes analyzed: BDNF (**A**), PDYN (**B**), CxcL1 (**C**) and CXCL2 (**D**). Error bars: SD, $n = 8$/group.

plausible mediator of positive exercise effects observed after TBI. Metalloproteinases activated after TBI act through the degradation of the extracellular matrix and other mechanisms to disrupt the blood brain barrier (BBB), support inflammation and impact neurogenesis[25]. On the other hand, metalloproteinase inhibitors, including specific and non-specific MMP9 inhibitors, show promise for limiting CNS damage and dysfunction after TBI[40]. Furthermore, recent studies demonstrate a non-MMP mediated mechanism for TIMP2 preservation of the BBB; TIMP2 was shown to interact with $\alpha_3\beta_1$ integrin on endothelial cells thus reducing permeability after TBI[41]. It should be emphasized that additional proteins such as Cathepsin B are elevated by exercise. Cathepsin B is capable of stimulating hippocampal progenitor cells and is linked to both exercise and memory function in humans[42].

We do not believe the beneficial potential of exercise plasma or its components is necessarily limited to mild closed head TBI. Aside from models of neurodegenerative disorders already shown to respond positively to exercise plasma, exercise has positive effects on neurotrauma of other types including spinal cord injury and stroke[43,44]. The techniques outlined in our studies for the generation and testing of exercise plasma have broad applicability. Therefore, examining the effects of exercise plasma on brain pathology and cognitive disorders seen after lateral fluid percussion or blast TBI may provide new insights for developing novel treatments for other forms of CNS injury[45,46].

While these observations regarding the positive effects of plasma transfer on several adverse outcomes of TBI are important, there still remains significant limitations to these studies. It is of critical importance to define the administration parameters for efficiently transferring plasma from exercised donors to the recipients. These include optimal administration frequency of doses, volume of plasma and duration to achieve the beneficial

effects. The effects of sex and age of both the donor and recipient animals are still largely unexplored and would be critical if translational studies are considered. The composition of plasma is complex and many other proteins, miRNAs or additional plasma components may mediate the positive effect of exercise plasma. The studies did not include a careful comparison of sedentary plasma versus simple saline which might reveal effects of plasma not related to exercise. Sedentary plasma, however, did not yield behavioral or gene expression results qualitatively different from control groups in our previous reports. We also did not measure recipient mouse plasma protein levels. Ultimately, defining the components of exercise plasma that are important for its positive effects may then provide a guide to assembling an effective treatment mixture not requiring use of a blood derived product.

Methods

Animals

All experimental procedures and protocols were approved by the Veterans Affairs Palo Alto Health Care System Institutional Animal Care and Use Committee (Palo Alto, CA, USA) in accordance with the guidelines of National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications 8th edition, 2011) and with ARRIVE guidelines. All experiments used 12 week old male C57Bl/6J mice (Jackson Laboratories; Bar Harbor, MA, U.S.A). Upon arrival at our animal facility, mice were acclimated for at least 7 days prior to the start of any experiments. The mice were housed 2–4 per cage under standard vivarium conditions of 12 h light/dark cycle and were given food and water *ad libitum*. Mice were habituated to handling by the experimenters prior to starting any behavioral experimentation, behavioral testing was done during the light cycle.

A general timeline for the procedures carried out in the report is shown in Fig. 1. Experimenters were blind to the identity of treatments or experimental conditions. Based on preliminary data and our prior publications group sizes were determined for pain-related outcomes by power analysis for 25% differences detectable with 80% power at alpha 0.05.

Drugs

Prostaglandin E2 [100ng/15µl] (PGE_2, 14010, Cayman Chemicals, MI, USA) is a principal mediator of inflammation and pain hypersensitivity. Stock PGE_2 solutions were made in 100% ethanol and further diluted in 0.9% sterile saline prior to use. Capsaicin (CAP, M2028, Millipore-Sigma, MO, USA) causes hyperalgesia to mechanical stimuli at and around the injection site. Capsaicin was dissolved in sterile saline containing 0.25% DMSO, 0.25% ethanol and 0.125% Tween 80, and was prepared daily prior to use.

Closed-head model of mild traumatic brain injury (mTBI)

The closed-head mild TBI (mTBI) and control procedures were based on our previously established protocols[11,15,18,47]. Briefly, a benchmark stereotaxic impactor (MyNeurolab, St. Louis, MO, USA) actuator was mounted on a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) at a 40° angle with a 5-mm impactor tip. The parameters of the procedure have been validated, and no skull fractures were observed previously[48]. After isoflurane anesthesia induction, mice were placed on a foam mold in prone position on the stereotaxic frame, and maintained under anesthesia for the duration of the procedure. The stereotaxic arm was adjusted so that the head impact was at a fixed point relative to the right eye and ear, corresponding to the primary somatosensory (S1) cortex. An impact of 5.8–6.0 m/s with a dwell time of 0.2 s and depth of 5 mm was applied to the head. After impact, the mice were allowed to recover from anesthesia on a warming pad prior to returning to their home cages. For control sham mTBI groups, the above procedure was performed except that the impact device was discharged in the air.

Mechanical nociceptive assay

Mechanical sensitivity was assessed using nylon von Frey filaments (Stoelting Co., IL, USA) according to the "up-down" algorithm developed by Chaplan et al.[49]. We have applied this technique previously to estimate withdrawal thresholds in mice after mTBI[11]. After acclimating mice on the wire mesh platform inside plastic enclosures (10 cm radius), sequential fibers with increasing stiffness were applied to the plantar surface of hind limb and left in place for 5 s or until a paw withdrawal response was noted. If no response was noted, the next stiffest fiber was applied. If a response was noted, the next lightest fiber was applied. When 4 fibers had been applied after the first response, the testing was terminated. Mechanical withdrawal threshold was determined by a data fitting algorithm. The mechanical withdrawal thresholds were measured in both hindpaws, contralateral and ipsilateral, to the mTBI impact side.

Descending control of nociception (DCN)

DCN was assessed using a noxious stimulation–induced analgesia protocol optimized for use in mice as a gauge of endogenous control of nociception[47]. Briefly, DCN assessments in the mTBI groups were done after mice had recovered to baseline mechanical threshold levels (4 weeks post-injury). Next, an intraplantar (i.pl.) injection of PGE_2 (the test stimulus) into the hindpaw contralateral to the mTBI was used to produce hypersensitivity. After an hour, withdrawal thresholds of the PGE_2 injected hindlimb were measured using the von Frey filaments as described above. Subsequent to mechanical threshold evaluations, mice were given an injection of capsaicin (the conditioning stimulus) into the dorsal surface of the forepaw ipsilateral to the TBI to induce DCN. At 5, 15, 30, and 60 min after capsaicin, withdrawal thresholds were measured using the von Frey filaments. All intraplantar injections were administered under light isoflurane anesthesia.

Working memory tests

Spatial working memory was assessed using Y-maze testing (YM) as previously described[50]. The arena consisted of 3 symmetrical arms (arms A, B and C) at 120° angles with a dimension of 20 × 8 × 16 cm (L × W × H) for

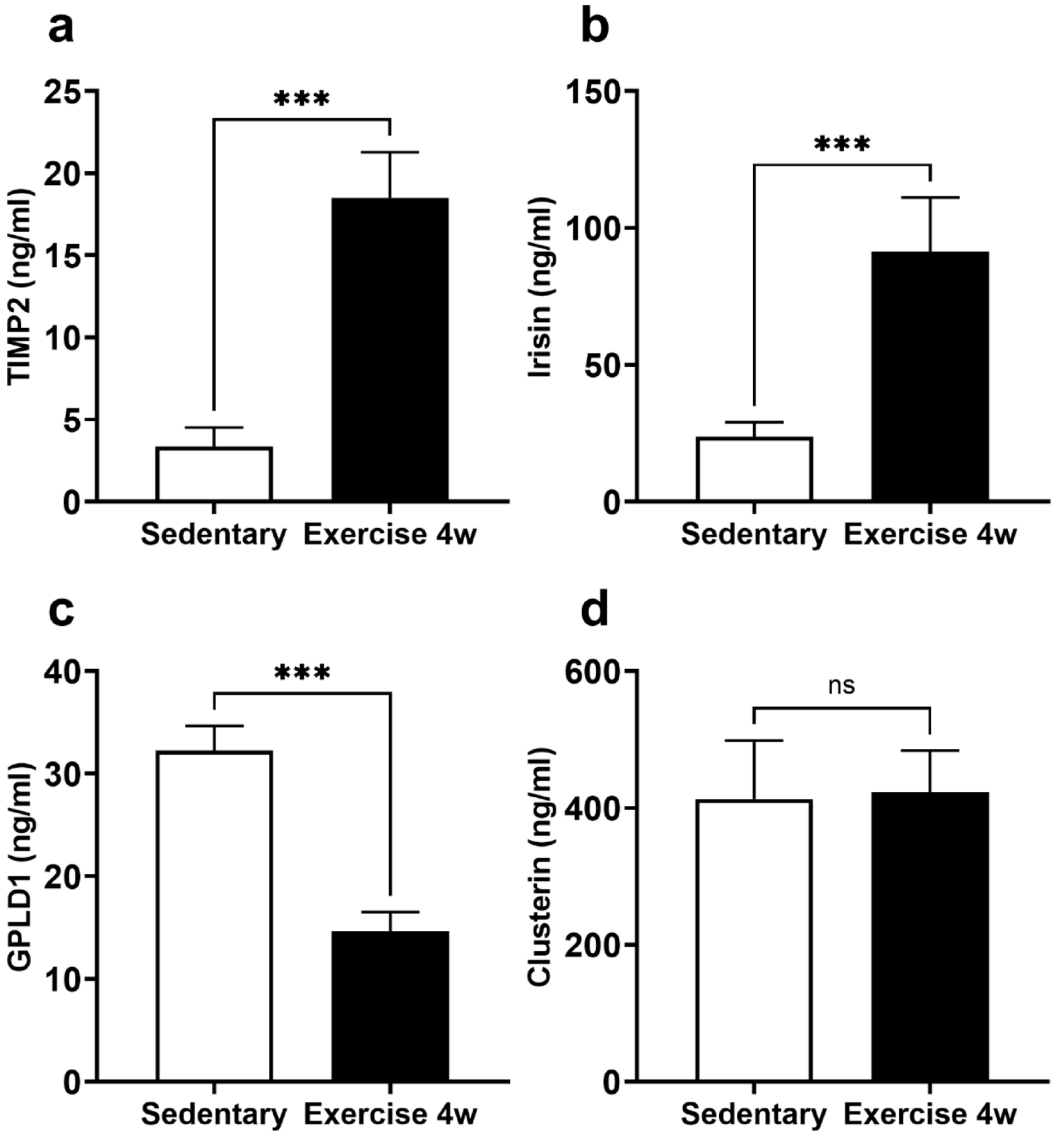

Fig. 7. The level of circulating mediators associated with the beneficial effects of exercise were increased in exercised compared to sedentary mice plasma. The expression of several exercise induced circulating mediators known to enhance cognition were compared in plasma derived from sedentary and exercise animals. Analysis of the plasma from exercised mice showed significantly elevated levels of TIMP2 (**A**) and irisin (**B**) compared to plasma from sedentary mice. The levels of GPLD1 was significantly lower in the plasma derived from exercise mice when compared to sedentary mice (**C**). There was no difference in the expression of clusterin between sedentary and exercised plasma (**D**). *** $p < 0.001$ indicates significant difference between sedentary and exercise groups. Error bars: SD, $n = 12$/group.

each arm. The mice were placed in the center of the arena and arm entry was recorded for 10 min. Unique triad combination of consecutive arm entries was used as a measurement of spontaneous alternation behavior. Typically, rodents prefer to investigate a new arm of the maze rather than returning to one that was previously visited. The triads of arm entries in which the mouse sequentially visited each possible arm without repeating, i.e., ABC, ACB, BCA, BAC, CBA and CAB were used as a measure of spontaneous alteration. Percentage of alternation was calculated as: (number of unique triad combination)/(total number arm entries – 2).

Object recognition memory (ORM) experiments were used to assess nonspatial working memory after TBI[15]. Mice were initially habituated to 2 identical objects after being placed in the middle of the open field arena, as previously described [35]. Subsequently mice were returned to their home cages for 5-minutes. Next, during a 10-minute trial, mice were returned to the arena with one of the previous identical objects was replaced with a novel one and exploratory behavior (investigation time) was recorded. As mice explore novel objects more than familiar ones, time spent exploring the novel compared with familiar was used to assess nonspatial working memory. All recordings from the above experiments were analyzed in real-time by TopScan software (Version 3.0, CleverSys, Reston, VA).

Voluntary exercise

Uninjured 12 weeks old C57BL/6J male mouse donors used for plasma preparation were kept in cages with a running wheel in place for 28 days prior to sacrifice. The computer-monitored exercise cages were purchased from Lafayette Instruments (Lafayette, IN). Exercise mice ($n = 36$) were housed in the activity cages in pairs and had free access to wheels: sedentary mice ($n = 36$) were housed in pairs in cages with locked wheels. Additional sedentary and exercise donor mice ($n = 14$ each group) were used for the qPCR 7 days post TBI and plasma transfer analyses. The exercise donor mice used in these experiments established a daily wheel running average of 5.50 ± 0.16 km/day (Mean ± SD) between days 7 and 28. Previous observations suggest that it takes C57Bl/6J mice about 7 days to acclimate to the wheel cages and reach stable running performance[15].

Processing and administration of plasma

The donor mouse blood was collected using cardiac puncture with 30 µl of 250 mM EDTA (Thermo Fisher Scientific, Cat# 15575020). Cells were removed from the blood by centrifugation for 15 min at 1,000 g using a refrigerated centrifuge. The plasma from the resulting supernatant was pooled together and dialyzed using the Slide-A-Lyzer dialysis cassettes with 3.5 kDa molecular weight cut-off (Thermo Fisher Scientific, Cat# 66330). Then the plasma was aliquoted and stored at − 80 °C until required. For plasma transfer experiments, non-exercised mTBI mice were injected retro-orbitally with 200 µl of donor plasma, from either exercised or sedentary mice, twice per week for 4 weeks, beginning on day 3 after mTBI[19,24].

Quantitative real-time polymerase chain reaction

Mouse lumbar spinal cord (L4 to L6) tissues were collected using rapid dissection after euthanasia. Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Cat# 74104), and the purity and concentration were determined spectrophotometrically. Then, complementary DNA was synthesized from 1 µg RNA using RT² First Strand Kit (Qiagen, Cat# 330404). Real-time polymerase chain reactions were performed on the QuantStudio 7 Flex Real-Time PCR system (Thermo Fisher Scientific) using RT² qualitative polymerase chain reaction (qPCR) Primer Assay and the RT² SYBR Green qPCR Mastermix (Qiagen, Cat# 330523). Brain-derived neurotrophic factor (BDNF) (Qiagen, Cat# PPM03006C), prodynorphin (PDYN) (Qiagen, Cat# PPM25340A), C-X-C Motif Chemokine Ligand 1 (CXCL1) (Qiagen, Cat# PPM03058C), C-X-C Motif Chemokine Ligand 2 (CXCL2) (Qiagen, Cat#PPM02969F) and 18 S (Qiagen, Cat# PPM72041A) primer sets were validated on dissociation curves to document single product formation. The data from real-time polymerase chain reaction experiments were analyzed by the comparative CT ($2^{-\Delta\Delta Ct}$) method (Normalized to 18 S expression levels) as described in the manufacturer's manual.

Enzyme immunoassay

The mouse plasma protein levels of several circulatory mediators associated with effects of exercise were assayed for TIMP2 (Tissue inhibitor of metalloproteinase 2; MyBioSource, MBS2701309), irisin (MyBioSource, MBS2600628), GPLD1 (Glycoprotein phospholipase D1; MyBioSource, MBS2886115) and clusterin (MyBioSource, MBS2884040) ELISA kits. Manufacturer's recommendation of using 100ul of sample per well were followed.

Data analysis

All data are presented as mean ± S.D. Two-way repeated measures (RM) analysis of variance (ANOVA) was performed for hindpaw allodynia and DCN experiments. Tukey's post hoc test for multiple comparisons was used to assess differences between the groups within each time point. Data obtained from PCR experiments were analyzed using one-way ANOVA followed by Sidak's multiple comparisons correction. The ORM and ELISA experiments were analyzed using unpaired t-tests. Data from YM were analyzed by one-way analysis of variance with for between group evaluations. Statistical significance was established a priori at a confidence level of 95%. All statistical analyses were conducted in Graphpad Prism Software (La Jolla; CA). For all experiments, group sizes were calculated to have approximately 80% power to detect 25% changes at the $p < 0.05$ level.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

P.S. performed animal procedures and behavioral testing. X.Y.S. performed qPCR, protein isolation and ELISA experiments. T.Z.G and W.W.L performed the plasma transfer exeriments. P.S. and X.Y.S. analyzed the data. J.D.C. and P.S. were involved in conceptualisation and supervision of the the study. J.D.C. and P.S. wrote the mauscript. QL.C., K.A.I. reviewed and edited the final version of the mauscript and contributed to the experimental design. All authors reviewed and accepted the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to P.S.

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