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Pharmacologically Induced Hypothermia Attenuates Traumatic Brain Injury in Neonatal Rats

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Abstract

Neonatal brain trauma is linked to higher risks of mortality and neurological disability. The use of mild to moderate hypothermia has shown promising potential against brain injuries induced by stroke and traumatic brain injury (TBI) in various experimental models and in clinical trials. Conventional methods of physical cooling, however, are difficult to use in acute treatments and in induction of regulated hypothermia. In addition, general anesthesia is usually required to mitigate the negative effects of shivering during physical cooling. Our recent investigations demonstrate the potential therapeutic benefits of pharmacologically induced hypothermia (PIH) using the neurotensin receptor (NTR) agonist HPI201 (formerly known as ABS201) in stroke and TBI models of adult rodents. The present investigation explored the brain protective effects of HPI201 in a P14 rat pediatric model of TBI induced by controlled cortical impact. When administered via intraperitoneal (i.p.) injection, HPI201 induced dose-dependent reduction of body and brain temperature. A six-hour hypothermic treatment, providing an overall 2–3°C reduction of brain and body temperature, showed significant effect of attenuating the contusion volume versus TBI controls. Attenuation occurs whether hypothermia is initiated 15 min or 2 hr after TBI. No shivering response was seen in HPI201-treated animals. HPI201 treatment also reduced TUNEL-positive and TUNEL/NeuN-labeled cells in the contusion area and peri-injury regions. TBI-induced blood brain barrier damage was attenuated by HPI201 treatment, evaluated using the

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Evans Blue assay. HPI201 significantly decreased MMP-9 levels and Caspase-3 activation, both of which are pro-apoptotic, while it increased anti-apoptotic Bcl-2 gene expression in the peri-contusion region. In addition, HPI201 prevented the up-regulation of pro-inflammatory tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and IL-6. In sensorimotor activity assessments, rats in the HPI201 treated group exhibited improved functional recovery after TBI versus controls. These data support that PIH therapy using our NTR agonist is effective in reducing neuronal and BBB damage, attenuating inflammatory response and detrimental cellular signaling, and promoting functional recovery after TBI in the developing brain, supporting its potential for further evaluation towards clinical development.

Keywords

Drug-induced hypothermia; Neonates; Traumatic brain injury; Cell death; Brain protection; Functional recovery

Introduction

Traumatic brain injury (TBI) is a common clinical disorder in neonates and young children. TBI can cause significant brain damage involving neuronal cell death, gliosis, blood brain barrier disruptions, brain edema, ischemia, inflammation and other pathological events (Sharp, et al., 2014). In the developing brain, TBI may also cause neonatal seizures and epilepsy due to the hyperexcitability of neurons and neural circuits, resulting in long-term functional impairments (Choe, et al., 2012, Finnie, 2012). Unfortunately, effective treatments to prevent the pathological and functional deficits after neonatal TBI have not been developed.

Mild to moderate hypothermia has shown strong protective effects in both pre-clinical and clinical studies. Therapeutic hypothermia using physical cooling methods has been studied as treatments for a number of brain and peripheral organ disorders, including ischemic and hemorrhage strokes (Sheng, et al., 2012, Wu and Grotta, 2013), epileptic seizures (Motamedi, et al., 2013, Srinivasakumar, et al., 2013), spinal cord injury (Ahmad, et al., 2014), perinatal asphyxia (Rey-Funes, et al., 2013) and others. In contrast, the potential benefits of hypothermic therapy for the treatment of TBI, especially in neonates and children, have been much less investigated.

Since conventional cooling methods such as the use of ice or surface cooling pads are not efficient (Jacobs, et al., 2013), new methods in hypothermia therapy have been developed. These include epidural placement of cooling catheter (Inoue, et al., 2012), passive heat dissipation (D'Ambrosio, et al., 2013), local cold fluid infusion (Chen, et al., 2013) and extracorporeal veno-venous blood cooling (Kuboi, et al., 2013, Testori, et al., 2013). Recently, pharmacologically induced hypothermia (PIH) or drug-induced hypothermia (DIH) has drawn increased attention due to its target specific, receptor/channel mediated effect and effective inductions of regulated hypothermia (Muzzi, et al., 2013, Tupone, et al., 2013, Zhang, et al., 2013). For example, compounds acting at adenosine A1 receptors, opioid receptors, transient receptor potential (TRP) channels, and dopamine receptors can induce hypothermic effects (Muzzi, et al., 2013, Tupone, et al., 2013, Zhang, et al., 2013). In

mechanisms of pharmacological hypothermia, the hypothalamic thermoregulatory set point or peripheral temperature sensitive channels are affected (Katz, et al., 2012); (Chang, et al., 2013). Using our second generation neurotensin receptor (NTR) agonists we recently demonstrated dose-dependent regulatory hypothermia in the mouse and rat. These PIH compounds showed protective effects against brain injury and improved functional recovery after ischemic or hemorrhage stroke and TBI in adult animals (Choi, et al., 2012, Wei, et al., 2013). The NTR compound-induced neuroprotection is likely due to its hypothermic effect because when the animal body temperature was kept at normal level (36-37°C) the protective effect of NTR compounds disappeared (Choi, et al., 2012, Wei, et al., 2013).

In the present investigation, we tested the hypothesis that pharmacological hypothermia can protect against TBI-induced brain injury in neonatal rats. The brain protective effect was examined at the molecular, cellular and tissue level as well as through functional testing, which provides a foundation for further evaluation of its potential for clinical development.

Materials and methods

Animals and trauma brain injury model

Neonatal Wistar rats at post-natal day 14 (P14) were subjected to controlled cortical impact insult. Pathological and behavioral changes were examined different days after TBI. The rats were housed in standard cages in 12 hr light/12 hr dark cycle. The animal protocol was approved by the Emory University Institutional Animal Care and Use Committee (IACUC), in compliance with National Institutes of Health (NIH) guidelines. The experimental TBI procedures were performed as previously described with minor modifications. P14 rats were anesthetized with 1.5 % isoflurane and placed on a stereotaxic frame. After a midline skin incision, a 3.5 mm circular craniotomy was performed midway between lambda and bregma, 2.0 mm to the right of central suture using an electric drill. Controlled cortical impact was induced with an electric impact device using an impact tip. The PCI3000 precision cortical impactor (Hatteras Instruments, Cary, NC) and a 3.0 mm diameter flat fact tip with a slightly rounded edge (velocity=3m/s, depth=2.0 mm, and contact time=150 ms). Temperature was monitored by a rectal thermometer in all groups and maintained at $36 \pm 0.5^{\circ}\text{C}$ during surgery, using a heating pad controlled by a homeothermic blanket control unit (Harvard Apparatus, Holliston, MA, USA). After the injury, the skin was glued, and rats were allowed to recover in a humidity-controlled incubator (Thermocare, Incline Village, NV, USA). Animals in the sham and TBI groups were injected with saline after TBI, and their body temperature was maintained at 36-37°C in a humidity-controlled incubator for up to 6 hr after TBI. In the hypothermia group, animals were subjected to HPI201 and no other intervention of temperature change was applied.

Drug administration and hypothermia induction

Animals were randomly divided into 3 groups: Sham control, TBI plus saline vehicle control and TBI plus HPI201-induced hypothermia group. HPI201, dissolved in saline, was intraperitoneally (i.p.) injected. The first bolus injection (2 mg/kg) was given 15 min after TBI followed by additional one or two injections at a half of the initial dose (1 mg/kg, 1.5-4 hr interval) to keep a constant mild hypothermia (32-35°C) for 6 hr.

Temperature measurement

Rectal and brain temperatures were measured during and after injury. Rectal temperature was monitored using a rectal probe (Harvard Apparatus) and measurements were repeated every 15 min for the first hour and every 60 min thereafter. Brain temperature was measured using a Physitemp probe (Temperature data acquisition system, Physitemp, Clifton, NJ) placed on the surface of the cerebral cortex. The Physitemp system allows simultaneous monitoring and data acquisition from 7 animals either during or after anesthesia. The implantable thermocouple probe has a tip diameter of 0.16 mm. The microprobe was connected to a guide cannula that attached to the caps of two dummy cannulas (model C313 DC, Plastic Products Co., Roanoke, VA, USA). A small hole was drilled into the caps of a size sufficient to accommodate the shaft of the microprobe. The microprobe was then inserted through caps, positioned, and glued into place with epoxy resin. The cap closer to the needle tip was used to screw the modified probe into an implanted guide cannula; the other cap provided additional mechanical strength. The probe was connected with an extension wire to the data acquisition system controlled by a computer.

Quantification of contusion volume

To measure the contusion volumes, Nissl staining was performed on the brain sections collected at different time points after TBI. The rat brains were harvested and cut into 10 μ M-thick fresh-frozen brain sections with 200 μ m intervals. Sections were then fixed with a 1:1 mixture of 10% formalin and acetic acid for 10 min. After washing with distilled water for 5 min, slices were stained with a working solution containing buffer solution (0.1 M acetate acid and 0.1 M sodium acetate. 94:6) and Cresyl Violet acetate at a ratio of 5:1. The sections were then dehydrated in 100% ethyl alcohol and mounted.

After Nissl staining, the sections were digitized using ImageJ software (NIH, Bethesda, MD, USA), and the areas of the contusion tissue and the two hemispheres quantified by an investigator blinded to the treatment of the animals (n=5-8 per group). Contusion volume was assessed based on the Cavalieri method as previously described (Lee, et al., 2014). The number of sections and the section thickness (10 μ m) were multiplied by the mean area of the remaining cortex. Contusion volumes were calculated based on the contusion areas (C) obtained from 10-12 sections as follows: $C1 \cdot D + 0.2(C1 + C2 + 3 + \dots + C12)$, with D being the distance between two sections (0.2 mm). Additionally, to consider the effects of swelling or edema, hemispheric tissue loss was calculated as a percentage calculated by $[(\text{contralateral hemispheric volume} - \text{ipsilateral hemispheric volume}) / (\text{contralateral hemispheric volume}) \times 100\%]$. The calculated result is reported as the ratio of lesion volume against the corrected hemisphere volume.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

A TUNEL assay kit (DeadEnd Fluorometric TUNEL system; Promega, Madison, WI, USA) was used to assess cell death by detecting fragmented DNA in 10- μ m-thick coronal fresh frozen sections, followed the manufacture's instruction and as described previously (Li, et al., 2013). Nuclei were counterstained with Hoechst 33342 (1:20,000; Molecular Probes, Eugene, OR, USA) for 5 min.

Western blot analysis

Tissues were collected and protein was isolated with RIPA buffer or Trizol. After sacrifice, animals were subjected to transcardial perfusion using phosphate-buffered saline (PBS; pH 7.4). Brain penumbra tissue was lysed in a buffer containing 0.02 M $\text{Na}_4\text{P}_2\text{O}_7$, 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton, 1 mM EGTA, 2 mM Na_3VO_4 , and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The supernatant was collected after centrifugation at 15,000 g for 10 min at 4°C. Protein concentration was determined with a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL, USA). Equivalent amounts of total protein were separated by molecular weight on an SDS-polyacrylamide gradient gel, and then transferred to a PVDF membrane. The blot was incubated in 10% nonfat dry milk for 1 hr and then treated with primary antibodies at 4°C overnight. The primary antibodies used and the dilutions for each were rabbit anti-cleaved caspase-3 antibody (Cell Signaling, Danvers, MA, USA) at 1:400, rabbit anti-B-cell lymphoma-2 (bcl-2; Cell Signaling) at 1:1000, rabbit anti-bcl-2 associated X protein antibody (Cell Signaling) at 1:2000, mouse anti-actin (Sigma) at 1:5000, rabbit anti-MMP-9 antibody (Millipore, Billerica, MA, USA) at 1:2500, and rabbit anti-MMP-2 antibody (Millipore) at 1:2500. After washing with TBST, membranes were incubated with AP-conjugated or HRP-conjugated secondary antibodies (GE Healthcare, Piscataway, NJ, USA) for 2 hr at room temperature. After final washing with TBST, the signals were detected with bromochloroidolylphosphate/nitroblue tetrazolium (BCIP/NBP) solution (Sigma) or film. Signal intensity was measured by ImageJ (NIH) and normalized to the actin signal intensity.

RT-PCR analysis

Total RNA from tissues of TBI rats were isolated according to the manufacturer's instruction (Life Technologies, Grand Island, NY, USA). RNA integrity was confirmed by detection of a 28S and 18S rRNA band. RNA was confirmed to be free of genomic DNA contamination by PCR in the absence of reverse transcriptase. The RNA samples were reverse transcribed in 20 μl of a reaction mixture containing 2X RT buffer and 20X RT enzyme mix according to the manufacturer's instruction (Life Technologies, Grand Island, NY, USA) at 37°C for 60 min. The samples were then incubated at 95°C for 5 min and transferred to 4°C. The RT product (1 μl) was subjected to PCR amplification with 10 pmole primer, 10X standard Taq reaction buffer, 10 mM dNTP, and 0.625 unit Taq polymerase in 20 μl PCR reaction buffer (New England Biolabs Inc., Ipswich, MA, USA). PCR primers were used as follows (5'-3'): for TNF- α , GATCTCAAAGACAACCACTAGTG (forward) and CTCCAGCTGGAAGACTCCTCCCAG (reverse); for IL-1 β , TCGGCCAAGACAGGTCGCTCA (forward) and TGGTTGCCCATCAGAGGCAAGG (reverse); for IL-6, GAGGATACCCCCAACAGACC (forward) and AAGTGCATCATCGTTGTTTCATACA (reverse); for 18s, GACTCAACACGGGAAACCTC (forward) and ATGCCAGAGTCTCGTTCGTT (reverse). PCR mixtures were heated to 95°C for 10 min and cycled 30-37 times for each primer; cycles consisted of 95°C for 15 sec, 60°C for 1 min, and 72°C for 30 sec. After additional incubation at 72°C for 10 min, the PCR samples were transferred to 4°C. PCR products were subjected to electrophoresis in 2% agarose gel with ethidium bromide.

Relative intensity of a PCR band was analyzed using InGenius3 manual gel documentation systems (Syngene, Frederick, MD, USA).

Extravasation of Evans blue (EB) dye

Sterilized 2% Evans Blue (EB) solution was administered intravenously (i.v.) at a dosage of 0.08 ml per rat at 6 hr before sacrifice to visualize the BBB leakage. BBB permeability was investigated by monitoring extravasation of EB dye as previously described (Lee, et al., 2014). The red auto-fluorescence of EB dye-albumin conjugate is evident in tissue sections examined by fluorescence microscope. Sterilized 2% EB dye (Sigma) solution was administered i.v. at a dosage of 0.08 ml per mouse 6 hr before sacrifice to visualize the BBB leakage. Six hours after 2% EB injection, and 12 hr after TBI, rats were sacrificed with an overdose of pentobarbital (100 mg/kg), decapitated and brains removed. Coronal sections were used to examine the EB extravasation by photographing under the TRITC (red) excitation wavelength at 1.25x for fluorescent microscopy (BX51, Olympus, Japan). Photoshop Professional was used to make an image mosaic (Adobe® Photoshop® CS5, San Jose, CA, USA).

Statistical analysis

Student's 2-tailed t-test or 1-way ANOVA was used to compare different groups. Significant differences between groups were identified by a value of $p < 0.05$. Prism 5 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis and graphic presentation. Student's two-tailed t-test was used for comparison of 2 experimental groups, and One-way ANOVA followed by Bonferroni correction was used for multiple-group comparisons. Two-way ANOVA followed by Bonferroni correction was used for repeated measurements. Significant differences between groups were identified by a P value of < 0.05 . All data are presented as Mean $\pm$ SEM.

Results

Pharmacologically induced hypothermia after brain trauma in neonatal rats

In the present study, HPI201 was used to generate the mild-to-moderate hypothermia in postnatal day 14 (P14) rats. P14 rats were subjected to sham surgery or the controlled cortical impact (CCI) as previously described (Lee, et al., 2014). This impact resulted in a focal and repeatable contusion in the sensorimotor cortex of the right hemisphere. We tested three dosages of HPI201 (2, 4 and 8 mg/kg) in control and TBI animals. Body (rectal) and brain cortical temperatures were monitored at different time after injection to confirm the efficacy of PIH in normal pups and after trauma. We observed a 2-5°C reduction from 36°C in pups after a single bonus of HPI201 (Fig. 1A). After an initial injection of 2 mg/kg of HPI201 followed by two injections of 1 mg/kg, hypothermia was maintained for at least 6 hr after TBI (Fig. 1B). We performed additional experiment to compare the brain and body temperatures and observed similar trends in temperature changes (Fig. 1C).

Pharmacological hypothermia attenuated trauma-induced neuronal injury

To test whether pharmacological hypothermia can attenuate neonatal traumatic brain injury, HPI201-induced hypothermia was initiated at 15 min after TBI. At 6, 12 and 24 hr post-

trauma, Nissl staining revealed that the contusion volumes were smaller in HPI201-treated animals than that in the saline group (Fig. 2A-C). The reduced contusion volumes were also observed with 2 hr delayed administration of HPI201 (Fig. 2D).

To understand the cellular event of this protective effect, TUNEL staining was performed in brain sections at 6, 12 and 24 hr after trauma. At each time point, significantly fewer TUNEL-positive cells were found in the ipsilateral cortex of animals from the HPI201 treatment group (Fig. 3A-C). The number of dead cells decreased in both traumatic contusion area and peri-contusion regions. Confocal imaging was used to confirm the TUNEL colabeling with NeuN and/or Hoechst 33342 in these regions (Fig. 3D). Double staining of TUNEL-positive and neuronal marker NeuN-positive cells were counted, showing reduced neuronal death after TBI in HPI201-treated animals (Fig. 3E and 3F).

Activation of the apoptotic gene caspase-3 was detected in the TBI brain (Fig. 4A and 4B). At 24 hr post-TBI, the caspase-3 levels declined to the sham control levels in the HPI201 group. We also observed a significant increase of the anti-apoptotic gene Bcl-2 in HPI201-treated animals (Fig. 4C).

Pharmacological hypothermia inhibited brain inflammation in the post-TBI brain

RT-PCR and Western blot analysis showed increased expressions of IL-1 β , IL-6 and TNF- α in peri-contusion regions at early time points (Fig. 5A). HPI201 treatment suppressed the IL-1 β and TNF- α levels in the hypothermia group (Fig. 5B and 5C). On the other hand, IL-6 showed a delayed increase at 24 hr after TBI. The HPI201 treatment reduced IL-6 expression 3 days after TBI (Fig. 5D).

Pharmacological hypothermia protected blood-brain barrier after TBI

The blood-brain barrier (BBB) integrity was evaluated in traumatized brains at 24 hr post-TBI. The protein levels of MMP-9 were upregulated by TBI, suggesting an increased hemorrhagic potential. This MMP-9 increase was inhibited in HPI201-treated animals (Fig. 6A and 6B). EB leakage from the cerebral arteries and vessels was measured in TBI animals received saline vehicle or HPI201 treatment (Fig. 6C and 6D). Compared to the saline group, HPI201 significantly reduced EB leakage at 6, 12 and 24 hr after TBI (Fig. 6E). The BBB protective effect persisted when HPI201-initiated hypothermia is delayed to 2 hr after TBI (Fig. 6F).

Delayed PIH treatment promotes functional recovery after TBI

To determine whether the brain protective effects facilitated functional recovery, sensorimotor functions were evaluated. Animals received saline or HPI201 treatment 1 or 2 hr after TBI. TBI impaired sensorimotor functions detected by adhesive removal test performed at 7 days after TBI. Even with the 2 hr delayed treatment, animals showed significantly better sensorimotor functional behaviors compared to animals in the TBI control group (Fig. 7A, B). At 14 days after TBI, corner test indicated a functional deficit of left paws and left whiskers in TBI controls; HPI201 treatment normalized the turn behavior of the animals (Fig. 7C).

Discussion

The present investigation evaluated the therapeutic benefits of brain protection and functional recovery of the hypothermic compound NTR agonist HPI201 after neonatal TBI. Although many investigations have examined hypothermic therapy using physical cooling in adult TBI models, few studies have explored the hypothermic effect against TBI in neonates. Moreover, we examined the novel pharmacologically induced hypothermia that shows greater feasibility and efficiency for clinical translation. We demonstrated that administration of HPI201 with 15 or 120 min delay after TBI was effective to prevent trauma-induced tissue damage and cell death, attenuated inflammatory response, reduced detrimental cellular signaling, and promoted brain repair and functional recovery after TBI in neonates.

Reducing the intracranial temperature is a critical step for head cooling therapy. Recent reports indicate that insufficient brain cooling may be related to the lack of significant benefits (Adelson, et al., 2013, Georgiou and Manara, 2013, Harris, et al., 2012). Although physical cooling had been used in pre-clinical and clinical practice, challenges hinder clinical feasibilities may include disruptions of coagulation system (Mohr, et al., 2013), cardiac function (Egginton, et al., 2013), and fluid pH (Groger, et al., 2013). Moreover, conventional methods in physical cooling processes are costly and time-consuming (Alexander, et al., 2012). We show in previous and current investigations that our NTR compounds effectively reduce the brain temperature and do not show the adverse effects seen with physical cooling (Choi, et al., 2012, Lee, et al., 2014, Wei, et al., 2013). With the hypothermic drug, no post-rewarming rebound was seen in our experiments. Moreover, the lack of shivering is a unique benefit associated with NTR compounds so general anesthesia is not needed for the PIH therapy.

In the investigation of hypothermia therapy against ischemic stroke, it was shown that mild hypothermia conferred significant degrees of neuroprotection in terms of survival, behavioral deficits, and histopathological changes, even when its induction was delayed by 2 hours after onset of MCA occlusion. In a previous investigation, the neuroprotective effect of mild hypothermia (2-hour duration) that was induced during the ischemia period was sustained over 2 months. This observation supports that hypothermia can induce long-term neuroprotective effect (Maier, et al., 2001). In case with TBI, lasting benefits after the PIH therapy should be verified in future pre-clinical and clinical investigations. In the current study, we showed that the PIH treatment was effective with 2 hrs delayed administration. This time window should be long enough for most TBI cases. Even more delayed treatment may happen in some clinical cases and remains to be tested.

In the fluid percussion TBI rat model, it was shown that the apoptosis-regulating protein TIMP-3 increased in normothermic group and reduced in hypothermic group (Jia, et al., 2012). This is consistent with our observation that HPI201 suppresses pro-apoptosis genes and increasing anti-apoptotic genes. The low metabolic rates after hypothermia induction may contribute to inhibitory responses to apoptotic signals and retard secondary injury in the acute phase of traumatic injury. Recent results also revealed that therapeutic hypothermia reduced opening of mitochondrial permeability transition pore (mPTP) in cortex (Gong, et

al., 2013). In endothelin-1-induced transient focal cerebral ischemia in rats, 2 hr physical cooling treatment reduced caspase-3 activation. This neuroprotective effect lasted for 1 week (Zgavc, et al., 2013), suggesting again that hypothermia therapy can provide beneficial effects for many days.

Therapeutic hypothermia attenuated brain edema and intracranial hypertension, which directly constituted a short-term benefit for brain under cooling conditions (See, et al., 2012). We show that pharmacological hypothermia enhanced the integrity of the BBB and decreased brain swelling. In the adult brain, the expression of occludin, a major tight junction protein, was significantly higher after hypothermia (Wei, Sun et al. 2013). We observed in the present investigation that the hemorrhage risk factor MMP-9 was upregulated after TBI and this increase was inhibited by HPI201-induced hypothermia. All these effects should help to reduce the ischemic or hemorrhage risk, maintain intracranial pressure and prevent secondary injury after TBI. Hypothermia modulates the stress response of astrocytes in ischemic brain (Kim, et al., 2013, Seo, et al., 2012). Hypothermia could also decrease microglial inflammatory production of interferon-beta (IFN- β), nitric oxide (NO) and many other pro-inflammatory cytokines (Matsui, et al., 2013, Zhao, et al., 2013). This is consistent with our observation that pharmacological hypothermia attenuated expression of pro-inflammatory factors.

Clinical evidence shows that therapeutic hypothermia reduced neurological morbidity of infants that survived hypoxic ischemic encephalopathy (HIE) (Garfinkle, et al., 2013). Hypothermia was also shown to protect cardiac arrest survivors against cognitive disability (Fugate, et al., 2013). In animal studies, rats under hypothermia combined with erythropoietin (EPO) treatment against hypoxia-ischemia showed an improved sensorimotor function detected by cylinder-rearing test (Fan, et al., 2013). In our investigation, greater improvements were observed at 7 and 14 days later in the sensorimotor functions of neonatal rats that received delayed hypothermia therapy after trauma. This suggests that long-term functional benefits can be achieved after pharmacological hypothermia. The current study sets the stage for further evaluation of the potential use of HPI201-induced PIH on trauma patients.

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Abbreviations

BBB	Blood brain barrier
CCI	Controlled cortical impact
EB	Evans blue
EPO	Erythropoietin

HIE	Hypoxic ischemic encephalopathy
IACUC	Institutional Animal Care and Use Committee
IFN-β	Interferon-beta
IL-1β	Interleukin-1 β
IL-6	Interleukin-6
I.p.	Intraperitoneal
I.v.	Intravenously
MMP-9	Matrix metalloproteinase-9
mPTP	Mitochondrial permeability transition pore
NO	Nitric oxide
PIH	Pharmacologically induced hypothermia
P14	Post-natal day 14
TBI	Traumatic brain injury
TNF-α	Tumor necrosis factor- α
TRP	Transient receptor potential
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

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Highlight

- The neurotensin receptor agonist HPI201 induced hypothermia in neonatal rats.
- HPI201 attenuated traumatic brain injury (TBI)-induced neuronal damage.
- HPI201 is protective against TBI-induced blood brain barrier damage
- HPI201 prevented the up-regulation of pro-inflammatory factors
- HPI201 improved functional recovery after TBI.

Figure 1. HPI201-induced hypothermic effect

A. Dose-response curve of HPI201-induced hypothermic effect. Core body (rectal) temperature in normal neonatal rats. **B.** Time course of the hypothermic effect in TBI animals. A bolus injection of HPI201 (2 mg/kg, i.p) either at the onset of TBI (time zero) or 90 min after TBI decreased body temperature to the range between 32-35°C. Consequent injections maintained the hypothermic effect for at least 6 hr. **C.** Brain and rectal temperatures measured after HPI201 treatment. Data presented as Mean $\pm$ SEM. N = 6-8 per group.

Figure 2. Hypothermia reduces the contusion volume after TBI

A. and **B.** Representative cresyl violet stained brain sections of TBI rats that received different treatments. **C.** Bar graphs demonstrate the contusion volume in saline group and HPI201 treatment group (15 min delay after TBI) measured at different time points after TBI. **D.** Contusion volume in saline group and 2 hr delay HPI201 treatment group measured at 12 hr after TBI. * $P < 0.05$ versus Saline group. Mean $\pm$ SEM. $N = 6-8$ per group.

Figure 3. HPI201-induced hypothermia reduced TUNEL+ cells after TBI

A and B. Cell death measured by TUNEL+/Hoechst+ double staining at 6 hr in the contusion area. Representative fields and quantified bar graph (B) are provided. **C.** NeuN+/TUNEL+/Hoechst staining in different groups at 12 hr in the peri-contusion region. **D.** Confocal image confirming the colabeling of NeuN+/TUNEL+/Hoechst. **E.** and **F.** Bar graphs showing total cell death and neuronal cell death at different time points after TBI. * $P < 0.05$ versus Saline. Mean $\pm$ SEM. $N = 6-8$ per group.

Figure 4. HPI201-induced hypothermia attenuates apoptosis

Hypothermia treatment increased Bcl-2 expressions and decreased cleaved caspase-3. #

P<0.05 versus Sham; * P<0.05 versus Saline. Mean ± SEM. N = 6-8 per group.

Figure 5. HPI201-induced PIH inhibits inflammatory response

Expression of proinflammatory cytokines at 24 hr measured by RT-PCR. Representative bands (**A**) and bar graphs for the mRNA levels of TNFα (**B**), IL-1β (**C**), and IL-6 (**D**) at different time points in different groups. * $P < 0.05$ compared to the Saline group. Mean $\pm$ SEM. N = 6-8 per group.

Figure 6. Hypothermia preserved blood-brain barrier integrity

A. and **B.** Western blot analysis on MMP-9. **C.** and **D.** Representative brain sections showing Evans Blue dye positive area. **E.** and **F.** Quantitative bar graph indicating the volume of EB leakage at different time points. HPI201 delayed treatment given at 15 min (**E**) or 2 hr (**F**) after TBI. * $P < 0.05$ versus Saline group. Mean $\pm$ SEM. $N = 6-8$ per group.

Figure 7. Hypothermia promotes functional recovery after TBI

A. and **B.** Sticky dot test at 7 days after TBI. 1 and 2 hr delayed HPI201 administration both showed significant improvement in contacting and removing the sticky dots. **C.** Corner test showed a recovery of left paws at 14 days post-TBI after HPI201 delayed treatment. # $P < 0.05$ vs. Sham; * $P < 0.05$ vs. Saline. Mean $\pm$ SEM. $N = 12-16$ per group.