Therapeutic Effects of Pharmacologically Induced Hypothermia against Traumatic Brain Injury in Mice

Jin Hwan Lee,¹ Ling Wei,^{1,2} Xiaohuan Gu,¹ Zheng Wei,¹ Thomas A. Dix,^{3,4} and Shan Ping Yu¹

Abstract

Preclinical and clinical studies have shown therapeutic potential of mild-to-moderate hypothermia for treatments of stroke and traumatic brain injury (TBI). Physical cooling in humans, however, is usually slow, cumbersome, and necessitates sedation that prevents early application in clinical settings and causes several side effects. Our recent study showed that pharmacologically induced hypothermia (PIH) using a novel neurotensin receptor 1 (NTR1) agonist, HPI-201 (also known as ABS-201), is efficient and effective in inducing therapeutic hypothermia and protecting the brain from ischemic and hemorrhagic stroke in mice. The present investigation tested another second-generation NTR1 agonist, HPI-363, for its hypothermic and protective effect against TBI. Adult male mice were subjected to controlled cortical impact (CCI) (velocity = 3 m/sec, depth = 1.0 mm, contact time = 150 msec) to the exposed cortex. Intraperitoneal administration of HPI-363 (0.3 mg/kg) reduced body temperature by 3–5°C within 30–60 min without triggering a shivering defensive reaction. An additional two injections sustained the hypothermic effect in conscious mice for up to 6 h. This PIH treatment was initiated 15, 60, or 120 min after the onset of TBI, and significantly reduced the contusion volume measured 3 days after TBI. HPI-363 attenuated caspase-3 activation, Bax expression, and TUNEL-positive cells in the pericontusion region. In blood–brain barrier assessments, HPI-363 ameliorated extravasation of Evans blue dye and immunoglobulin G, attenuated the MMP-9 expression, and decreased the number of microglia cells in the post-TBI brain. HPI-363 decreased the mRNA expression of tumor necrosis factor- α and interleukin-1 β (IL-1 β), but increased IL-6 and IL-10 levels. Compared with TBI control mice, HPI-363 treatments improved sensorimotor functional recovery after TBI. These findings suggest that the second generation NTR-1 agonists, such as HPI-363, are efficient hypothermic-inducing compounds that have a strong potential in the management of TBI.

Key words: blood–brain barrier; hypothermia; inflammation; neurotensin analogue; sensorimotor function; traumatic brain injury

Introduction

TRAUMATIC BRAIN INJURY (TBI) is a leading cause of human death and severe disability in the United States and worldwide, especially in children and young adults.^{1–3} Recent data show that approximately 1.7 million persons die and at least 10 million persons are hospitalized because of TBI in the United States each year. The cost of treatment and rehabilitation for patients with TBI is about 2 billion dollars per year in the United States.⁴ In spite of advancements in understanding the cellular/molecular mechanisms and some successes in pre-clinical studies, however, effective clinical treatment for patients with TBI has yet to be developed or translated from the pre-clinical stage.^{5,6}

TBI-induced brain damage has two pathological phases. Primary damage occurs at the moment of impact, and secondary damage

occurs later because of various cascade events in the brain. Multiple factors are involved in TBI-induced brain injury, including excitotoxicity, oxidative stress, inflammation, ischemia, edema, ionic imbalance, ATP depletion, intracranial pressure (ICP), and proteolysis.⁷ The current therapeutic approach to TBI is aimed at preventing or reducing secondary brain damage during the time window before treatment is available, while identifying cellular/molecular targets during this stage.

The clinical trial failures of these treatments can be multifaceted. The narrow focus, however, on a single target such as one signal pathway or one pathogenic gene is likely the primary reason for the failure of many potential therapies. It has thus been proposed that an effective treatment should target multiple mechanisms for comprehensive brain protection in a complicated brain disorder such as stroke or TBI. This may be achieved by a combination

Departments of ¹Anesthesiology and ²Department of Neurology, Emory University School of Medicine, Atlanta, Georgia.

³Department of Drug Discovery and Biomedical Sciences, Medical University of South Carolina, Charleston, South Carolina.

⁴Halimed Pharmaceuticals, Inc., Mt. Pleasant, South Carolina.

therapy or using a treatment that has broader effects on multiple injurious and/or regenerative mechanisms.

Pre-clinical and clinical studies have demonstrated the efficacy and safety of therapeutic hypothermia in the treatment for patients with TBI and stroke.⁸ In patients with TBI, therapeutic hypothermia for 48 or 72 h via surface cooling or intravascular cooling reduced mortality, improved prognosis, or showed few complications.^{9–11} In addition, therapeutic hypothermia reduced ICP, or improved clinical outcomes in patients with stroke.^{12–14}

Therapeutic hypothermia protects the brain through inhibition of multiple pathways and protection of different cell types including neuronal and non-neuronal cells such as vascular endothelial cells.^{15–17} Hypothermia can decrease excitotoxicity and inflammation, inhibit oxidative stress, and has the profound effect of reducing apoptosis.^{17–19} In addition, physical and pharmacological cooling protects the blood–brain barrier (BBB) and reduces brain edema after ischemic and hemorrhagic stroke.^{20–22}

Thus, therapeutic hypothermia is not only a neuroprotective treatment, but also provides global brain protection of multiple cell types in the gray and white matter. As a therapy that broadly suppresses injurious pathways and cell death events, mild to moderate hypothermia (3–5°C reduction of therapeutic hypothermia) improves histological and functional outcomes in various animal models of TBI and stroke.^{23–25} In clinical trials, therapeutic hypothermia using different physical cooling methods has been shown to improve functional outcomes and regulate ICP.^{26,27}

Despite the beneficial effects of therapeutic hypothermia, clinical application of hypothermia therapy for patients with acute stroke and TBI has been hindered by some disturbing limitations. Physical cooling is generally slow in inducing hypothermia in humans (2–8 h for mild to moderate hypothermia) and is associated with shivering, a defensive metabolic response to cold that severely battles against temperature reduction. In most cases, patients need general anesthesia to combat against this cold defense response, which may lead to possible infection and other side effects during prolonged hypothermia.²⁸

Recently, pharmacological reagent-induced controlled hypothermia targeting the brain thermoregulatory center has emerged as a more efficient and safer treatment for patients with CNS disorders.¹⁸ It was reported that agonists of NT receptor subtype 1 (NTR1) showed a hypothermic effect.¹⁵ These original NTR1 agonists, however, cannot pass through the BBB and therefore have limited clinical applications. The C-terminal hexapeptide of NT, Arg-Arg-Pro-Tyr-Ile-Leu (NT[8–13]), has the essential structural elements for full biological activity.²⁹ NT (8–13) analogs that are biologically stable can penetrate the BBB.³⁰ These NTR1 agonists induce hypothermia via the NTR receptor in the brain.¹⁵

We recently introduced a second generation of novel NT (8–13) analogs. These compounds show high affinity for human NTR1, exhibit BBB permeability, and effectively induce regulated hypothermia in rodents.^{31,32} Therefore, they represent a novel group of hypothermic compounds that decrease the brain and core body temperature by reducing the set-point of the central thermoregulatory center.

In our previous investigations, we showed that, as a representative compound, HPI-201 (also known as ABS-201) can induce mild to moderate hypothermia within 30 min in a dose-dependent manner.²⁰ Acute and delayed administrations of HPI-201 were highly protective against brain injury after both ischemic and hemorrhagic strokes in rodent models.^{20,21} Clinically important, these NTR1 compounds do not trigger the defensive shivering response; therefore, they can be administered without the need of anesthesia or use of anti-shivering drugs.

In this investigation, we tested another NTR1 compound, HPI-363, for its brain protective effect against TBI damage in a mouse model of controlled cortical impact (CCI).

Methods

Chemicals

NTR1 agonist HPI-363 was synthesized using procedures described previously.^{31,32} The chemical structure of HPI-363 is shown in Supplementary Figure 1 (see online supplementary material at ftp.liebertpub.com).

Animals and CCI of TBI model

C57BL/6 male mice (8–12 weeks, 22–28 g) were used in this study. The mice were housed in standard cages in 12-h light/12-h dark cycle and given food and water *ad libitum*. The animal protocol was approved by the Emory University Institutional Animal Care and Use Committee, in compliance with National Institutes of Health (NIH) guidelines.

The experimental TBI method used in this study was performed as described previously with minor modification.^{33,34} Mice 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. During the craniotomy, mice were excluded from the study if dura matter was breached. CCI was induced with an electric impact device using an impact tip. In this study, we used a PCI3000 precision cortical impactor (Hatteras Instruments, Cary, NC) and a 2.0 mm diameter flat fact tip with a slightly rounded edge (velocity = 3 m/sec, depth = 1.0 mm, and contact time = 150 msec). Temperature was monitored by a rectal thermometer in all groups and maintained at 37 ± 0.5°C during surgery, using a heating pad controlled by a homeothermic blanket control unit (Havard Apparatus, Holliston, MA).

After the injury, the skin was sutured, and mice were allowed to recover in a humidity-controlled incubator (Thermocare, Incline Village, NV). Animals in the sham and TBI groups were injected with saline after TBI, and their body temperatures maintained at 37°C in a humidity-controlled incubator for up to 6 h after TBI. In the hypothermia group, animals were subjected to HPI-363 injections, and no human intervention of temperature change was applied.

Drug administration and induction of hypothermia

HPI-363 was dissolved in saline and injected intraperitoneally. To determine the dose-response relationship, HPI-363 was first tested at 0.1, 0.3, 0.5, and 1 mg/kg in C57BL/6 male mice without TBI surgery. Mice were randomly divided into five groups: (1) saline control ($n=3$); (2) 0.1 mg/kg HPI-363 group ($n=6$); (3) 0.3 mg/kg HPI-363 group ($n=11$); (4) 0.5 mg/kg HPI-363 group ($n=6$); and (5) 1 mg/kg HPI-363 group ($n=5$). In the investigation of the brain protection after TBI, mice were randomly divided into two groups: (1) TBI plus saline injection ($n=8$); (2) 0.3 mg/kg HPI-363 group ($n=7$).

For the HPI-363 treatment group, the first bolus injection (0.3 mg/kg) was given immediately or at 15, 60, 120, or 180 min after TBI, followed by additional injections at a half of the initial dose (0.15 mg/kg). The interval between the injections was ≥ 1.5 h, with adjustments made to keep a constant mild hypothermia (32–34°C). Anesthesia was applied only during the TBI procedure; animals recovered from the isoflurane anesthesia and received HPI-363 intraperitoneally (i.p.) without other sedation measures.

Physical forced cooling was tested as a hypothermia control. The targeted body temperature was the same as in the pharmacologically induced hypothermia (PIH) experiments (32–34°C). Animal

were placed on ice during the first 10–15 min and then in a $\geq 4^{\circ}\text{C}$ chamber during the maintenance period.

Measurements of body and brain temperature

Rectal temperature was monitored using a rectal probe (Harvard Apparatus) for ≥ 6 h after TBI, 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.^{20,35} The Physitemp system allows simultaneous monitoring and data acquisition from seven animals either during or after anesthesia. The implantable thermocouple probe has a tip diameter of 0.16 mm in diameter. 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). 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 laptop computer.

Physiological parameter measurements

To evaluate the effects of HPI-363 on physiological parameters, blood pH and mean arterial blood pressure (MABP) were monitored. MABP was recorded using the Blood Pressure Analyzer-400 system (Digi-Med, Mobile, AL) after left common-carotid artery catheterization with PE-10 polyethylene tubing (Becton Dickinson, Franklin Lakes, NJ) under anesthesia before, immediately after, and at 30 and 60 min after HPI-363 injection. Animals were awake and freely moving between measurements, and the indwelling catheter was treated with heparin. Whole-blood samples were also collected to evaluate blood pH. Blood serum was separated by centrifugation at 15,000 g for 5 min at 4°C and was then applied on Hydrion pH test paper (Micro Essential Laboratory, Brooklyn, NY) to measure the blood pH value.

Nissl staining and measurement of contusion volume

To measure the contusion volumes, Nissl staining was performed at 3 or 21 days after TBI. 10-mM-thick fresh-frozen brain sections containing target lesions were collected every 200 μm and 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), and the areas of the contusion and the two hemispheres quantified by an investigator blinded to the treatment of the animals ($n=9$ –10 per group). Contusion volume was assessed based on the Cavalieri method of stereology.³⁶ The number of sections and the section thickness (10 μm) 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). In addition, 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 ratio of lesion volume against the corrected hemisphere volume.

Immunohistochemistry

Mice were euthanized with 10% chloral hydrate and perfused with 4% paraformaldehyde. Brains were removed and kept overnight in 4% paraformaldehyde and then transferred to 30% sucrose ($n=6$ –7 per group). Frozen perfused brain tissue was sliced into 10 mm-thick coronal sections using a cryostat vibratome (Leica CM 1950; Leica Microsystems, Buffalo Grove, IL). Sections were dried on a slide warmer for 30 min, fixed with 10% formalin buffer, washed with -20°C precooled ethanol:acetic acid (2:1) solution for 10 min, and permeabilized with 0.2% Triton-X 100 solution for 5 min.

All slides were washed three times with phosphate-buffered saline (PBS) (5 min each) after each step. Tissue sections were blocked with 1% fish gelatin (Sigma) in PBS for 1 h at room temperature, then incubated with the primary antibody occludin (1:200; Millipore, Billerica, MA), ionized calcium binding adaptor molecule 1 (Iba1; 1:200; Biocare Medical, Concord, CA), and NeuN (1:300; Millipore, Billerica, MA) overnight at 4°C . Next day, the slides were washed three times with PBS for 5 min, then reacted with the secondary antibodies Alexa Fluor®488 goat anti-mouse (1:300; Life Technologies, Grand Island, NY) and Cy3-conjugated donkey anti-rabbit (1:300; Jackson ImmunoResearch Laboratories, West Grove, PA) or Cy5-conjugated donkey anti-mouse or rabbit (1:400; Jackson ImmunoResearch Laboratories) for 90 min at room temperature. After three washes with PBS, nuclei were stained with Hoechst 33342 (1:20,000; Molecular Probes) for 5 min as a counterstain, then mounted with Vectashield fluorescent mounting medium (Vector Laboratory, Burlingame, CA), and a coverslip applied for microscopy and image analysis.

Terminal deoxynucleotidyl transferase dUPT nick end labeling (TUNEL) staining

A TUNEL assay kit was used to examine cell death by detecting fragmented DNA in 10-mM-thick coronal fresh frozen sections as described previously ($n=9$ –10 per group).²⁰ After fixation (10% buffered formalin for 10 min and then ethanol:acetic acid (2:1) solution for 5 min) and permeabilization (0.2% Triton X-100 solution), brain sections were incubated in the equilibration buffer for 10 min. Recombinant terminal deoxynucleotidyl transferase (rTdT) and nucleotide mixture were then added on the slide at 37°C for 60 min in the dark. Reactions were terminated by 2X SSC solution for 15 min. Nuclei were counterstained with Hoechst 33342 (1:20,000; Molecular Probes, Eugene, OR) for 5 min.

Quantification of immunostaining positive cells

Cell count was performed as described previously.²⁰ Cell counting was performed following the principles of design based stereology. Systematic random sampling was used to ensure accurate and non-redundant cell counting. Six brain sections per animal were collected at 100 mm distance between sections for non-overlapping multistage random sampling. Six fields were chosen in each section in the penumbra region and viewed at $40\times$ for cell counting. ImageJ (NIH) was used to analyze each picture. The percentage of immunoreactive cells (e.g. TUNEL-positive cells) among total Hoechst 33342-positive cells in the pericontusion area were counted and summarized in a total of six non-overlapping fields.

All analysis was performed in a double-blinded fashion. The researcher who counted the cells was blinded to the treatment group. The number of counted cells was also confirmed by two persons to verify the accuracy of the result.

Extravasation of Evans blue (EB) dye

BBB permeability was investigated by monitoring extravasation of Evans blue (EB) dye as previously described.²⁰ The red

auto-fluorescence of EB dye-albumin conjugate is evident in tissue sections examined by fluorescence microscope. Sterilized 2% EB dye (Sigma, St. Louis, MO) solution was administered intravenously (i.v.) at a dosage of 0.1 mL per mouse 6 h before sacrifice to visualize the BBB leakage. At 6 h after 2% EB injection, and 12 h after TBI, mice were sacrificed with an overdose of pentobarbital (100 mg/kg) and decapitated ($n=6-7$ per group). Brains were removed. Coronal sections were used to examine the EB extravasation by photographing under the TRITC (red) excitation wavelength at $1.25\times$ for fluorescent microscopy (BX51, Olympus, Japan). Photoshop Professional was used to make an image mosaic (Adobe® Photoshop® CS5, San Jose, CA).

Western blot analysis

Western blot analysis was used to detect the expression of apoptotic and BBB disruption markers. Three animals were included in the sham group, five animals in the TBI group, and five animals in the TBI plus HPI-363 group. After sacrifice, animals were subjected to transcardial perfusion using 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 ethylenediaminetetraacetic acid (pH 8.0), 1% Triton, 1 mM ethylene glycol tetracetic acid, 2 mM Na_3VO_4 , and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). 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). Equivalent amounts of total protein were separated by molecular weight on an SDS-polyacrylamide gradient gel, and then transferred to a polyvinyl fluoride membrane. The blot was incubated in 10% nonfat dry milk for 1 h 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) 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-matrix metalloproteinase (MMP)-9 antibody (Millipore, Billerica, MA) at 1:2500, 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) for 2 h at room temperature. After final washing with TBST, the signals were detected with bromochlorodiolylphosphate/nitroblue tetrazolium (BCIP/NBP) solution (Sigma) or film. Signal intensity was measured by ImageJ (NIH) and normalized to the actin signal intensity.

Isolation of total RNA and real-time-polymerase chain reaction (RT-PCR)

Total RNA from tissues of TBI mice was isolated according to the manufacturer's instruction (Life Technologies, Grand Island, NY). Three animals were included in the sham group ($n=5$), the TBI control group ($n=5$), and the TBI plus HPI-363 group ($n=5$). 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 mL of a reaction mixture containing 2X RT buffer and 20X RT enzyme mix according to the manufacturer's instruction (Life Technologies, Grand Island, NY) 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 mL) 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 mL PCR reaction buffer (New England Biolabs Inc., Ipswich, MA).

PCR primers were used as follows (5'-3'): for tumor necrotizing factor (TNF)- α , GATCTCAAAGACAACCAACTAGTG (forward)

and CTCCAGCTGGAAGACTCCTCCCAG (reverse); for interleukin (IL)-1 β , TCGGCCAAGACAGGTCGCTCA (forward) and TGGTTGCCCATCAGAGGCAAGG (reverse); for IL-6, GAGGATACCCCAACAGACC (forward) and AAGTGCATCATC GTTGTTTCATACA (reverse); for IL-10, CACCCACTTCCAGT CCGCCA (forward) and TGCTTCTCTGCCGGCATCA (reverse); for 18s, GACTCAACACGGGAAACCTC (forward) and ATGC CAGAGTCTCGTTCGTT (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).

Adhesive removal test

The adhesive removal test measures sensorimotor function as described previously.²⁰ A small adhesive dot was placed on each forepaw, and the amount of time (sec) needed to contact and remove the tape from each forepaw was recorded. Recording stopped at 6 min. The test was performed three times per mouse, and the average time was used in the analysis. Mice were trained three times before TBI surgery to ensure that they were able to remove the tape. Mice that showed no response or prolonged time to remove the tape during the training period were excluded from future experiments. In this behavioral study, 9 animals were randomly assigned to the TBI control group, 11 animals for the HPI-363 15 min delay group, 7 animals for the HPI-363 60 min group, 7 animals for the HPI-363 120 min delay group, and 8 animals for the HPI-363 180 min delay group.

Cylinder test

A unilateral injury to the motor cortex results in an asymmetry in the forelimb used for support during rearing, which can be measured using the cylinder test. The mice were placed in a glass cylinder (9.5 cm diameter and 11 cm height), and the number of times each forelimb or both forelimbs were used to support the body on the wall of the cylinder was counted for 5 min. The animals were evaluated at 1, 3, 7, 10, and 14 days after TBI. Two mirrors were placed behind the cylinder to view all directions. The numbers of impaired and non-impaired forelimb contacts were calculated as a percentage of total contacts.³⁷

Home Cage behavioral tests

Behavioral changes of experimental mice were monitored and analyzed using the HomeCageScan (Clever Sys Inc., Reston, VA). The system had four cameras that monitored four cages, with each cage (191 mm $\times$ 292 mm $\times$ 127 mm) containing one mouse. The behavior patterns were continuously recorded for 12 h during nighttime. After finishing the recording, the videos were analyzed by the Home Cage Software 3.0 (Clever Sys Inc.). To perform this behavioral study, we used 3–6 animals per group. The HomeCageScan System was also performed to measure the shivering activities during hypothermia ($n=3-5$ per group). Subtle twitch-like behavior was detected by the monitoring camera and analyzed by the Home Cage Software 3.0 (Clever Sys Inc., Reston, VA) for shivering activity. To distinguish the shivering from twitch behavior, the detection factors in the data acquisition system were modified as the following: (1) animal size threshold (pixel)=160; (2) contrast threshold=24; (3) muscle twitch max length=30; (4) twitch/sleep merge limit=10. Shivering was measured in a 3-h period soon after the hypothermic treatment in normal animals.

FIG. 1. HPI-363 induced hypothermic effect in normal awake and traumatic brain injury (TBI) mice. The hypothermic effect of NTR1 agonist HPI-363 was assessed in normal C67BL/6 mice without anesthesia and in mice subjected to anesthetic and TBI procedures. Body temperature was measured using a rectal probe, and brain temperature was measured using a brain exclusive probe. (A) Dose-response relationship of HPI-363-induced hypothermic effect in normal mice. Intraperitoneal administration of HPI-363 reduces body core temperature in a dose-dependent manner. Mild to moderate temperature reductions were reached within 30 to 60 min after the drug injection, and the hypothermic effect lasted for various durations depending on the HPI-363 dosage. $*p < 0.05$ vs. saline control; $n = 3-11$ /group. (B) HPI-363-induced hypothermic effect in TBI mice. The reduction in body and brain temperature was parallel and consistent. The relatively lower brain temperature was because of the different measurement devices and methods. (C) Using the HomeCageScan system, walking behavior (distance) of sham control mice and hypothermia treated mice were recorded for 3 h. Both HPI-363 and physical cooling treated mice showed reduced locomotion activities. Physical cooling, however, resulted in the most profound effect of reducing walking activity. $*p < 0.05$ vs. normal controls; $n = 3-5$ per group. (D) Shivering behavior in sham control, HPI-363, and physical cooling mice. Shivering was measured during a 3-h period after the treatment in normal animals using the HomeCageScan System. No shivering was triggered by HPI-363-induced hypothermia. $*p < 0.05$ vs. normal controls; $n = 3-5$ per group.

Statistical analysis

GraphPad Prism 5 (GraphPad Software, San Diego, CA) was used for statistical analysis and graphic presentation. Student two-tailed *t* test was used for comparison of two experimental groups, and one-way analysis of variance (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 $\pm$ standard error of the mean.

Results

PIH by HPI-363 in mice

We first tested the hypothermic effect of HPI-363 in normal adult mice without anesthesia. Four doses of HPI-363 (0.1, 0.3, 0.5, and 1.0 mg/kg) were injected i.p. into different mice; core body temperature was measured using a rectal temperature probe. A 3–5°C reduction in body temperature was induced within 30 min after HPI-363 injection in a dose-dependent manner (Fig. 1A). One bolus injection of HPI-363 caused mild to moderate hypothermia for ≥ 3 h. Two additional injections at half of the initial dose maintained the hypothermia for up to 6 h (data not shown). At lower body temperature, locomotor activity of animals decreased (Fig. 1C), yet HPI-363 injection did not trigger shivering responses, which is in sharp contrast to noticeable shivering in animals under physical cooling (Fig. 1D). PHI-363 induced a similar hypothermic effect when administered after TBI (Fig. 1B). Using a brain temperature probe, we confirmed that the brain temperature reduction was parallel with the change in body temperature (Fig. 1B).

These results showed that HPI-363 is an effective hypothermic compound in normal mice as well as after a TBI insult. The dosage needed for producing mild to moderate hypothermia was about 10 times lower than that for HPI-201.²⁰ According to the dose-response relationship of PHI-363, we chose the bolus dosage of 0.3 mg/kg followed by two additional injections to maintain 6 h of hypothermia in the following experiments. Physiological monitoring verified that HPI-363 had no significant effect on MABP and blood pH (Table 1).

PIH reduces cortical contusion volume

In mice that received the TBI insult to the right cortex, HPI-363 or saline control were administered 15, 60, 120, or 180 min after the onset of TBI. All animals with HPI-363 or saline did not show any mortality after TBI. On sacrifice at 3 days after TBI, brain sections with Nissl staining showed cortical tissue loss in the ipsilateral hemisphere (Fig. 2A). The contusion volume was $12.61 \pm 0.69 \text{ mm}^3$ in TBI-saline control mice. In TBI animals that received HPI-363 treatment (0.3 mg/kg followed by two additional injections of 0.15 mg/kg for 6-h hypothermia) with 15, 60, or 120 min delay, the contusion volume was reduced to 6.94 ± 0.32 , 8.98 ± 0.22 , and $9.14 \pm 0.43 \text{ mm}^3$, respectively ($p < 0.05$ vs. saline control for all three treated groups, $n = 9-10$ /group) (Fig. 2B).

TABLE 1. PHYSIOLOGIC PARAMETERS AFTER HPI-363 ADMINISTRATION

Parameter	Time after injection (min)			
	–30	0	30	60
MABP	102.7 $\pm$ 2.637	101.6 $\pm$ 3.604	100.3 $\pm$ 2.565	105.2 $\pm$ 2.835
SABP	126.9 $\pm$ 2.801	121.4 $\pm$ 4.799	126.5 $\pm$ 1.896	121.7 $\pm$ 1.238
DABP	77.39 $\pm$ 3.43	77.4 $\pm$ 1.759	79.94 $\pm$ 2.887	80.4 $\pm$ 2.024
Blood pH	7.5 $\pm$ 0.03	7.2 $\pm$ 0.03	7.2 $\pm$ 0.03	7.3 $\pm$ 0.03

Values are expressed as mean $\pm$ standard error of the mean at each time point.

MABP, mean arterial blood pressure; SABP, systolic arterial blood pressure; anDABP, diastolic blood pressure.

FIG. 2. HPI-363-induced neuroprotection against traumatic brain injury (TBI). Adult mice were subjected to controlled cortical impact and TBI-induced contusion volume, and cell death was measured 12 h or 3 days after the insult. **(A)** Nissl stained brain sections of TBI mice at 3 days after TBI. Images show brain sections from a TBI-saline control mouse and a TBI plus HPI-363 treatment mouse (15 min after TBI). The HPI-363 treatment (0.3 mg/kg bolus followed by two supplemental intraperitoneal injections at 0.15 mg/kg, 6-h hypothermia) resulted in a smaller contusion area (*). **(B, C)** Bar graphs summarize the contusion volume and the loss of hemispheric tissues in the TBI group and the TBI plus HPI-363 group. HPI-363 was administered with 15, 60, 120, or 180 min delay after TBI. The hypothermic treatment with 15 to 120 min delay significantly reduced the contusion volume and the loss of hemispheric tissue. As a control, a group of HPI-363-treated mice was kept in a temperature-controlled incubator to counteract the hypothermic effect. Body temperature in the mice was maintained at 36–37°C during and after TBI. Contusion infarct volumes developed in the mice similar to TBI saline controls. The protective effect disappeared when HPI-363 was administered 180 min after TBI. The contusion volumes and the loss of hemispheric tissues in TBI plus HPI-363 treatment with 15 min delay were still reduced even at 21 days after TBI. * $p < 0.05$ vs. TBI saline group; $n = 9$ –10/group. **(D)** TUNEL staining revealed DNA damage and cell death 12 h after TBI. Total cells were visualized with Hoechst 33342 staining (blue). Massive TUNEL-positive cells (green) were observed in the TBI injured cortical region. HPI-363 treatment resulted in fewer TUNEL-positive cells. **(E)** The percentage of TUNEL-positive cells among total Hoechst 33342-positive cells in the pericontusion area were counted and summarized in the bar graph. HPI-363 treatment with 15 min to 120 min delay after TBI significantly attenuated cell death. Similar to the contusion volume assay, the hypothermia therapy initiated with a 180 min delay lost the protective effect of TBI-induced cell death. * $p < 0.05$ vs. TBI group; $n = 9$ –10/group. Scale bars = 20 μ M. Color image is available online at www.liebertpub.com/neu

The contusion volume was also measured at 21 days after TBI, and HPI-363 treated animals (15 min delay) still showed significantly less brain damage compared with saline controls (Fig. 2B). When HPI-363 administration was further delayed up to 180 min after the TBI onset, its protective effect started to disappear, because no significant reduction in the contusion volume was observed ($11.27 \pm 0.60 \text{ mm}^3$, $p > 0.05$ vs. saline control, $n = 9$). Consistently, mice that received HPI-363 with 15, 60, or 120 min delay showed significant reduction in the loss of hemispheric tissues in the cerebral hemisphere (Fig. 2C). Also, HPI-363 treatment still showed the reduced loss of hemispheric tissues compared with saline controls at 21 days after TBI. Thus, the HPI-363-induced PIH therapy has a therapeutic window of at least 2 h after the acute TBI injury in mice.

As a control, a group of TBI mice received the same HPI-363 injection 15 min after TBI while their body temperature was kept

normal ($36.7 \pm 0.4^\circ\text{C}$) in a temperature controlled warm incubator after TBI. The contusion volume and the loss of hemispheric tissues in these animals showed no reduction compared with TBI animals, suggesting that the protective effect of HPI-363 after TBI is because of the temperature reduction (Fig. 2B, C).

PIH inhibits TBI-induced cell death

To understand the cortical protection of HPI-363 at a cellular level, we examined cell death using TUNEL staining and Western blot analysis at 12 h after TBI. In the pericontusion region, the HPI-363 treatment group showed significantly fewer TUNEL-positive cells compared with the TBI-saline group (Fig. 2D). Consistent with its effect against contusion formation, the cellular protective effect was confirmed with delayed HPI-363 treatments up to 120 min after the onset of TBI (Fig. 2D, E). Conversely, mice that

received HPI-363 but had their body temperature physically kept at a normal level showed no difference from TBI controls (Fig. 2E). In Western blot analysis, cleaved caspase-3 and expression of Bax, both key apoptotic markers, were significantly increased in the TBI brain. These apoptotic markers were suppressed by HPI-363 treatment (Fig. 3A–D). The effect of HPI-363 on Bcl-2 expression in the pericontusion region was not statistically significant (Fig. 3E, F).

PIH prevents the damage of the BBB

Disruption of the BBB can lead to increased brain swelling and increased ICP after TBI, contributing to secondary injury and cell death.³⁸ In the BBB integrity assay, we inspected the extravasation of EB dye in brain sections 12 h after TBI. HPI-363 treatment (15 min delay) significantly reduced the EB leakage ($p < 0.05$; Fig. 4A, B). In addition, Western blot analysis on the expression of immunoglobulin G (IgG) in the cortical tissue also revealed a suppression effect of HPI-363 on the upregulated expression of IgG after TBI ($p < 0.05$; Fig. 4C, D). Also, the expression of occludin, a major tight junction protein and an indication of the endothelial cell tight junction, was assessed using immunohistochemical staining. On the border of TBI damage, the expression of occludin was barely detectable 12 h after TBI. In the HPI-363-treated brain, however, significantly higher expression of occludin was seen compared with TBI-saline controls (Fig. 4E, F). Consistent with the BBB protection, HPI-363 also suppressed the expression of MMP-

9 (Fig. 4G, H), while the MMP-2 expression showed no difference between TBI control and HPI-363 groups (Fig. 4I, J).

PIH reduces inflammatory responses

Microglial activation and inflammatory mediators such as cytokines play important roles in the development of TBI.³⁹ In immunostaining assays of the contusion core and pericontusion regions 12 h after TBI, the number of microglia cells that positively reacted to ionized calcium binding adaptor molecule 1 (Iba1) significantly increased. This increase was attenuated by HPI-363 treatment (15 min delay) (19.83 ± 0.74 vs. 12.29 ± 0.59 cells/field in TBI control and HPI-363 treated, respectively; $n = 6-7$ animals/groups, $p < 0.05$; Fig. 5A, B). Further, increased NeuN-positive cells were observed in the HPI-363 group (28.64 ± 2.25 vs. 46.54 ± 2.16 cells/field; $n = 6-7$ /group, $p < 0.05$; Fig. 5C).

Next we assessed the effect of HPI-363 on the expression of inflammatory mediators. RT-PCR experiments showed increases in the expression of TNF- α and IL-1 β in the TBI brain (Fig. 5D–F). The expression ratios of TNF- α and IL-1 β in the HPI-363 treatment group were significantly decreased toward normal levels. The expression of anti-inflammatory cytokines, IL-6 and IL-10, were markedly increased up to 2.6 and 3.1 fold in HPI-363-treated brains compared with TBI controls (Fig. 5G, H).

PIH improved functional outcome after TBI

To examine functional outcomes after TBI, adhesive dot removal tests and cylinder tests were performed after TBI. In the adhesive dot removal test, the latency times to recognize and remove the sticky dot from the right and left forelimbs were recorded at 3 and 7 days after TBI (Fig. 6A, B). Before TBI, the contact and removal times were similar among all groups. After TBI damage to the right side of the sensorimotor cortical area, injured animals showed prolonged times in response to the sticky dot attached to their left paws, indicating impaired sensorimotor function on the right side of the sensorimotor cortical area.

Animals with TBI that received HPI-363 treatment showed significantly improved performance 3 days after TBI on both time to detect and time to remove sticky dot activities. The functional benefits were achieved with delayed HPI-363 administration up to 120 min after TBI ($p < 0.05$; Fig. 6A, B). By day 7 after TBI, these activities returned to normal levels in both groups. In addition to the benefit shown in the adhesive dot removal test, TBI animals that received HPI-363 (15 min delay) showed significantly better performances in the cylinder test (Fig. 6C). The difference from the TBI controls disappeared because of spontaneous recovery of the motor function in this moderate TBI model (Fig. 6C).

Behavioral improvements monitored under home cage environment

Next, we used a new behavioral study method using the HomeCageScan system (Clever Sys Inc., Reston, VA). Animals in sham control, TBI control, and TBI plus HPI-363 groups were under the surveillance in their home cage environment using a camera monitoring system, which is a video-based platform for automated high-resolution behavior analysis.⁴⁰ The system acquires multiple sensorimotor and behavioral data during extended periods (hours) under unconstrained and non-stressful environment without human intervention. Using this system, data collection and data analysis are unaffected by possible human bias and errors.

FIG. 3. HPI-363-induced hypothermia attenuated apoptotic genes in mice after traumatic brain injury (TBI). Expressions of proteins associated with apoptotic cell death were measured using Western blot analysis in the penumbra region 12 h after TBI. (A–D) TBI significantly enhanced expression of the pro-apoptotic genes caspase-3 (A and B) and Bax (C and D). In TBI mice that received HPI-363 (15 min delay), the increases in these pro-apoptotic factors were effectively reduced. (E, F) The expression of bcl-2 in the TBI brain was not significantly altered in the TBI brains although HPI-363 showed a trend of increasing this anti-apoptotic gene. * $p < 0.05$ vs. sham control; # $p < 0.05$ vs. TBI control; $n = 3$ in sham group, $n = 5$ in TBI and TBI plus HPI-363 groups, respectively.

FIG. 4. HPI-363-induced hypothermia attenuated traumatic brain injury (TBI)-induced blood-brain barrier (BBB) disruption. The functional and morphological integrities of the BBB were measured 12 h after TBI. (A, B) The leakage of Evans blue (EB) was photographed under the TRITC (red) excitation wavelength at $1.25\times$ under a fluorescent microscope. The EB dye positive area (red) was markedly decreased in the HPI-363 treatment group. $*p < 0.05$ vs. TBI group; $n = 6-7$ per group. Scale bars = $400\ \mu\text{M}$. (C, D.) Western blot analysis showed marked immunoglobulin G (IgG) extravasation in the TBI group compared with the sham group. HPI-363 treatment significantly reduced IgG extravasation compared with TBI controls. $*p < 0.05$ vs. sham group; $\#p < 0.05$ vs. TBI group; $n = 3$ in sham control, $n = 5$ in TBI group, and $n = 4$ in TBI plus HPI-363 group. (E, F) Immunohistochemical staining of occludin (green), Glut-1 (red), and Hoechst (blue). TBI dramatically reduced occludin expression, indicative of severe BBB damage. HPI-363 treatment showed significant protective effect on occludin expression. $*p < 0.05$ vs. sham group; $\#p < 0.05$ vs. TBI group; $n = 9-10$ per group. Scale bars = $20\ \mu\text{M}$. (G, H) Western blot analysis of MMP-9 showed that TBI increased MMP-9 expression while HPI-363 significantly prevented the increase. (I, J) The expression of matrix metalloproteinase (MMP)-2 was not changed in the TBI brain; HPI-363 showed an inhibitory effect on MMP-2 expression. $*p < 0.05$ vs. sham group; $\#p < 0.05$ vs. TBI group; $n = 3-5$ per group in G to J assays. Color image is available online at www.liebertpub.com/neu

FIG. 5. HPI-363-induced hypothermia reduced inflammatory response after traumatic brain injury (TBI). TBI-induced inflammation was evaluated in the pericontusion region 12 h after TBI insult. (A-C) Activated microglia and neuronal cells were detected using immunostaining of Iba1 (green) and NeuN (red) immunofluorescent staining (A). Hoechst 33342 (blue) staining revealed all cells in the region. TBI injury drastically increased Iba1-positive microglia cells while HPI-363 significantly prevented this microglia activation (B). In the TBI brain in the pericontusion region, the number of NeuN-positive neurons was decreased by more than 50%. This neuronal damage was lessened in the HPI-363-treated TBI brain (C). $*p < 0.05$ vs. sham group; $\#p < 0.05$ vs. TBI group; $n = 6-7$ /group. Scale bars = $20\ \mu\text{M}$. (D) Real-time-polymerase chain reaction (RT-PCR) analysis of inflammatory cytokines. (E-H) Summarized RT-PCR assays. TBI increased proinflammatory cytokines including tumor necrotizing factor (TNF)- α (E) and interleukin (IL)- 1β (F), while HPI-363 largely prevented these cytokine increases. Decreased anti-inflammation factor IL-6 was seen in the TBI brain, and HPI-363 maintained the IL-6 mRNA expression near the normal level (G). There was no change in the anti-inflammation cytokine IL-10 after TBI, while HPI-363 treatment substantially enhanced IL-10 mRNA expression (H). $*p < 0.05$ vs. sham group; $\#p < 0.05$ vs. TBI group; $n = 3-5$ per group. Color image is available online at www.liebertpub.com/neu

FIG. 6. HPI-363-induced hypothermia improved sensorimotor functional recovery after traumatic brain injury (TBI). Adhesive dot removal tests and cylinder tests were used to evaluate the sensorimotor functional recovery after TBI. (A, B) The latency to recognize the sticky dot and removal time for the right and left forelimb was recorded at 3 and 7 days after TBI. After TBI damage to the right side of the sensorimotor cortex, both time to feel and time to remove the sticky dot were increased. Mice that received HPI-363 treatment showed significantly improved performance on the removal test even when HPI-363 was administered 2 h after TBI. (C) The cylinder test showed that forelimb activities of mice with TBI were impaired during the first few days after TBI. Mice that received HPI-363 performed significantly better than TBI controls. The difference disappeared 10 days after TBI because of spontaneous recovery of this moderate TBI models. * $p < 0.05$ vs. sham group; # $p < 0.05$ vs. TBI group; $n = 3-7$ per group.

In this test, traveled distance, rearing, turning, and jumping during a 12-h monitoring period were used to measure motor functions 3 days after TBI. TBI can induce depression and anxiety behaviors such as low grooming time, finally leading to prolonged inactive time.^{41,42} Therefore, time spent sleeping and being stationary were used to verify the depression-like behaviors after TBI. After TBI damage, injured animals showed short travel distance (Fig. 7A), prolonged inactive time such as time spent sleeping and being stationary (Fig. 7B, C), and reduced active time such as time spent rearing, grooming, hanging, and jumping, compared with sham animals (Fig. 7E-H). In addition, animals with TBI made more turns than sham control animals, while mice with TBI that received HPI-363 behaved normally (Fig. 7D). In general, animals with TBI in the HPI-363 treatment group behaved similarly to sham control mice, which was a strong indication that the hypothermia therapy minimized TBI brain damage and endorsed better functional recovery in these animals (Fig. 7I).

Discussion

The present investigation provides new evidence for PIH in normal and TBI mice using the NT [8-13] analog HPI-363. Our data show that HPI-363 is about 10 times more potent in inducing therapeutic hypothermia compared with the previously reported HPI-201.²⁰ HPI-363 reduces the body and brain temperature by 3–5°C within 30–60 min in a dosage-dependent manner. This onset

of hypothermic action of HPI-363 is slower than HPI-201 that induces the same temperature reduction within 15–30 min.²⁰ Although the HPI-363 effect is relatively slower, it still shows the brain protective effect of therapeutic hypothermia. Delayed initiation of HPI-363 treatment (15 min to 2 h delay) after the TBI insult shows protective effects of reducing contusion volume, attenuating inflammation, apoptotic cell death, and BBB damage. HPI-363 treatment promotes functional recovery after TBI. The brain protection of HPI-363 is sustainable long after the cessation of the PIH treatment. Significant reduction in brain contusion volume was detected 3 weeks after TBI in the rodent model. This report appears to be the first to demonstrate the mechanism and therapeutic benefits of a PIH after TBI.

Growing evidence suggests that hypothermia is a proven treatment for patients with neurological diseases such as stroke and TBI.^{25,43} Mild to moderate hypothermia improves neurological functional recovery and reduces neuronal death and mortality in animal models of stroke.^{20,44} Consistently, moderate cooling showed reduction in contusion volume and improved behavioral and pathological outcomes after TBI.^{8,45,46}

Therapeutic hypothermia with body temperature reduced to 32–35°C has also been used for management of ICP after TBI in human patients.⁴⁷ In a clinical trial, lowering the body temperature to 33°C had a beneficial effect against secondary neurological injuries after TBI.⁴⁸ Neurological recovery was improved significantly in several clinical trials that included 81–392 patients with severe brain injury.^{43,49}

On the other hand, there are conflicting reports on clinical efficacy of hypothermia in treatments of patients with TBI. Some trials saw no beneficial effect of hypothermia in the management of TBI.^{50,51} These conflicting results may be a reflection of the slow process of physical cooling, side effects from long-term sedation, and the difficulties in controlling the body/brain temperature because of the cold defense response triggered by forced cooling.^{28,52,53}

To improve the efficiency and efficacy of hypothermia therapy, new approaches such as cooling helmets/baskets and cold solution infusion have been developed. These methods, however, still necessitate general anesthesia, involve invasive procedures, and are not available to patients with acute TBI soon after the injury. Our previous and current investigations demonstrated that treatment with NTR1 agonists does not trigger shivering activity and does not cause significant alterations in blood glucose, blood pH, and local cerebral blood flow.²⁰ On cessation of drug administration, the hypothermic effect subsides at a reasonably slow pace (~1°C/h in the case with HPI-363). This reasonable pace of rewarming is important because fast rewarming may lead to harmful consequences and even the mortality of patients.²⁰ Besides, the rewarming speed should be controllable by a protocol of multiple injections of gradually reduced dosages of the hypothermic drug.

Results from pre-clinical and clinical studies suggest that BBB breakdown follows TBI and lasts from a few days to even years after the early event.^{54–56} In the early phase after TBI, BBB breakdown leads to the leakage of large molecules and toxic substances, a change in blood flow, and metabolic imbalance. All these events may result in secondary damage and complications. In addition, long-term disruption of the BBB is associated with induction of inflammation and altered tight junction protein expression.^{57–59}

It is suggested that inhibition of BBB impairment after TBI is an important therapeutic target. We showed that HPI-363 treatment dramatically reduced the area and volume of EB dye leakage. The Western blot results on IgG levels also showed the protective effect

FIG. 7. HPI-363 treatment improved home cage behavior after traumatic brain injury (TBI). A 24-h continuous monitoring and comprehensive analysis of the behavioral changes of animals in different groups (sham control, TBI control, and TBI plus HPI-363) were performed using the HomeCageSys system (Clever Sys Inc.) Three days after sham and TBI procedures. (A–H) Changes of animal home cage activities quantified from the video recordings. HPI-363 largely corrected many dysfunctional behaviors in animals with TBI. (I) Summary of the percentages of time spent on each behavior during a 12-h monitoring period. * $p < 0.05$ vs. sham group; # $p < 0.05$ vs. TBI group; $n = 3–6$ per group.

of HPI-363 against BBB breakdown. Recent studies have shown that MMP plays a central role in the BBB breakdown by degrading tight junction proteins.⁶⁰ HPI-363-induced hypothermia dramatically reduced the increased expression of MMP-9 after TBI. The regulation time course of different MMPs after an insult may be different. For example, a previous investigation showed that up-regulation of MMP-2 was observed at 2 h after ischemic injury while MMP-9 expression increased at 24 h after ischemic injury.⁶¹ This may explain why we detected the MMP-9 increase 12 h after TBI while MMP-2 was unchanged.

Loss of occludin is closely correlated with BBB breakdown.⁶² Immunofluorescence revealed that disrupted and decreased expression of occludin was observed after TBI, indicating the damage of the endothelial cell tight junction. The expression of occludin was increased in the HPI-363 treatment group compared with the TBI group. This result suggests that HPI-363 prevents BBB disruption through inhibition of MMP-9 and the maintenance of the integrity of tight junctions after TBI.

There are multiple pathways in the cascade of brain injury after TBI such as apoptosis, oxidative stress, and excitotoxicity.^{63–65} Clinical and experimental research has indicated that inflammatory responses, endoplasmic reticulum stress, and autophagy could play important roles in the development of TBI.^{66,67} Among them, inflammation is a main contributor to secondary injury after TBI, leading to neural death and increased brain edema. Post-TBI inflammation is characterized by the production of pro- and anti-inflammatory cytokines, the accumulation of neutrophils, and the activation of microglia in the injured brain.^{39,68,69} Inflammatory responses can also exacerbate the breakdown of the BBB after TBI.⁵⁵

Our immunohistochemical results showed increased Iba1 immunoreactivity after TBI. Iba1 immunoreactivity is lower in the HPI-363 treatment group than in the TBI group. We also found that TBI resulted in the increased expression of TNF- α and IL-1 β mRNA after TBI by RT-PCR analysis. TNF- α and IL-1 β are proinflammatory cytokines produced by activated microglia that are involved in mediating various cell signaling such as apoptosis.^{39,70,71} Expression of TNF- α and IL-1 β mRNA were reduced by HPI-363 treatment after TBI, which is consistent with previous reports using hypothermia as a treatment.⁷² We also observed that HPI-363 treatment increased the expression IL-6 and IL-10 mRNA, which can act as anti-inflammatory cytokines.⁷² It might be important to demonstrate potential effects of HPI-363 on IL-10 expression because IL-10 has been shown to have a cellular protection effect after brain injury by inhibiting microglial activation and macrophage infiltration.⁷³ We noted controversial results on IL-10 expression in previous reports. In some reports, decreased IL-10 production was seen during induced hypothermia.^{74–76} These results could be explained by differences in species, injury model, depth, and method of hypothermia, or measurement time.

Functional outcomes, which have close clinical relevance, were significantly improved in the HPI-363 group with up to 2 h delay after TBI. This is consistent with our results measuring contusion volume, cell death, and BBB leakage. It should be noted that the time course revealed in rodent models may not be directly applied to humans. The basal metabolic rate per gram of body weight in mice is seven times higher than that in humans.⁷⁷ Considering the different metabolic rate and species difference, it is possible that HPI-363-mediated PIH therapy could have a longer therapeutic

time window in humans. Accurate estimation of the PIH therapeutic time window needs to be performed in nonhuman primates and in human patients.

Isoflurane (1.5%) was used as the anesthetic for TBI procedures in this investigation. It was a concern whether isoflurane may interact with HPI-363, resulting in additive effects on hypothermia. First, HPI-363 and our other hypothermic compounds show similar hypothermic effects in normal animals without anesthesia as well as after isoflurane administration. To address this question further, we tested chloral hydrate instead of isoflurane in a few more animals. The hypothermic effect of HPI-363 under chloral hydrate-induced anesthesia (1 h after injection: $32.4 \pm 0.5^\circ\text{C}$, $n=4$) was similar to that under isoflurane-induced anesthesia (1 h after injection: $32.8 \pm 0.6^\circ\text{C}$, $n=8$). According to our experimental design, HPI-363 was administered 15 min to 3 h after TBI. At these time points, animals have already awakened from the short anesthesia, and their body temperature reached the normal level ($36.8 \pm 0.4^\circ\text{C}$). These results suggest that the temperature interference from isoflurane in our experiments was minimum.

Previous studies showed the beneficial effect of physical cooling in TBI induced by the fluid percussion and CCI animal models.^{78,79} Among these models, CCI can cause a different degree or distribution of cortical edema than other models. For example, our CCI model leads to intense damage in most cortical areas, but some investigations have used a severe CCI model that causes hippocampal injury. Further research is needed to determine whether PIH therapy has cellular protective effects against more severe injury and/or other tissues damage such as in the hippocampus after TBI. In this study, functional deficits recovered at 7 or 10 days after TBI in the adhesive removal and cylinder tests. Longer evaluation of the sustained benefits of PIH therapy was not performed because of the spontaneous recovery of sensorimotor functions in this relatively moderate TBI model. In a more severe TBI model, long-term functional benefits may be evaluated for sustained brain protection.

The present findings support that HPI-363-induced PIH can be a promising therapeutic approach in patients with TBI through its effective induction of therapeutic hypothermia and inhibitory actions on multifaceted injurious mechanisms and pathways in the damaged brain.

Acknowledgments

This work was supported by the NIH grants NS073378 (TAD and SPY), NS0458710 (SPY), NS075338 (LW), NS062097 (LW), a VA Merit grant (SPY), the American Heart Association (AHA) grant 12GRNT12060222 (SPY), and the AHA Established Investigator Award (LW).

Author Disclosure Statement

No competing financial interests exist.

References

- Coronado, V.G., Xu, L., Basavaraju, S.V., McGuire, L.C., Wald, M.M., Faul, M.D., Guzman, B.R., and Hemphill, J.D.; Centers for Disease Control and Prevention. (2011). Surveillance for traumatic brain injury-related deaths—United States, 1997–2007. *MMWR Surveill. Summ.* 60, 1–32.
- Runyan, D.K. (2008). The challenges of assessing the incidence of inflicted traumatic brain injury: a world perspective. *Am. J. Prev. Med.* 34, Suppl, S112–S115.
- Hyder, A.A., Wunderlich, C.A., Puvanachandra, P., Gururaj, G., and Kobusingye, O.C. (2007). The impact of traumatic brain injuries: A global perspective. *NeuroRehabilitation* 22, 341–353.
- McGarry, L.J., Thompson, D., Millham, F.H., Cowell, L., Snyder, P.J., Lenderking, W.R., and Weinstein, M.C. (2002). Outcomes and costs of acute treatment of traumatic brain injury. *J. Trauma* 53, 1152–1159.
- Janowitz, T., and Menon, D.K. (2010). Exploring new routes for neuroprotective drug development in traumatic brain injury. *Sci. Transl. Med.* 2, 27rv21.
- Loane, D.J., and Faden, A.I. (2010). Neuroprotection for traumatic brain injury: Translational challenges and emerging therapeutic strategies. *Trends Pharmacol. Sci.* 31, 596–604.
- Polderman, K.H. (2004). Application of therapeutic hypothermia in the ICU: opportunities and pitfalls of a promising treatment modality. Part 1: Indications and evidence. *Intensive Care Med.* 30, 556–575.
- Dietrich, W.D., and Bramlett, H.M. (2010). The evidence for hypothermia as a neuroprotectant in traumatic brain injury. *Neurotherapeutics* 7, 43–50.
- Adelson, P.D., Ragheb, J., Kanev, P., Brockmeyer, D., Beers, S.R., Brown, S.D., Cassidy, L.D., Chang, Y., and Levin, H. (2005). Phase II clinical trial of moderate hypothermia after severe traumatic brain injury in children. *Neurosurgery* 56, 740–754.
- Zhi, D., Zhang, S., and Lin, X. (2003). Study on therapeutic mechanism and clinical effect of mild hypothermia in patients with severe head injury. *Surg. Neurol.* 59, 381–385.
- Clifton, G.L., Allen, S., Barrodale, P., Plenger, P., Berry, J., Koch, S., Fletcher, J., Hayes, R.L., and Choi, S.C. (1993). A phase II study of moderate hypothermia in severe brain injury. *J. Neurotrauma* 10, 263–271.
- Kammersgaard, L.P., Rasmussen, B.H., Jorgensen, H.S., Reith, J., Weber, U., and Olsen, T.S. (2000). Feasibility and safety of inducing modest hypothermia in awake patients with acute stroke through surface cooling: A case-control study: The Copenhagen Stroke Study. *Stroke* 31, 2251–2256.
- Guluma, K.Z., Oh, H., Yu, S.W., Meyer, B.C., Rapp, K., and Lyden, P.D. (2008). Effect of endovascular hypothermia on acute ischemic edema: Morphometric analysis of the ICTuS trial. *Neurocrit. Care* 8, 42–47.
- Schwab, S., Georgiadis, D., Berrouschot, J., Schellinger, P.D., Grafagnino, C., and Mayer, S.A. (2001). Feasibility and safety of moderate hypothermia after massive hemispheric infarction. *Stroke* 2033–2035.
- Dubuc, I., Sarret, P., Labbe-Jullie, C., Botto, J.M., Honore, E., Bourdel, E., Martinez, J., Costentin, J., Vincent, J.P., Kitabgi, P., and Mazella, J. (1999). Identification of the receptor subtype involved in the analgesic effect of neurotensin. *J. Neurosci.* 19, 503–510.
- Gibbons, H., Sato, T.A., and Draganow, M. (2003). Hypothermia suppresses inducible nitric oxide synthase and stimulates cyclooxygenase-2 in lipopolysaccharide stimulated BV-2 cells. *Brain Res. Mol. Brain Res.* 110, 63–75.
- Popovic, R., Liniger, R., and Bickler, P.E. (2000). Anesthetics and mild hypothermia similarly prevent hippocampal neuron death in an in vitro model of cerebral ischemia. *Anesthesiology* 92, 1343–1349.
- Katz, L.M., Young, A.S., Frank, J.E., Wang, Y., and Park, K. (2004). Regulated hypothermia reduces brain oxidative stress after hypoxic-ischemia. *Brain Res.* 1017, 85–91.
- Truettner, J.S., Suzuki, T., and Dietrich, W.D. (2005). The effect of therapeutic hypothermia on the expression of inflammatory response genes following moderate traumatic brain injury in the rat. *Brain Res. Mol. Brain Res.* 138, 124–134.
- Choi, K.E., Hall, C.L., Sun, J.M., Wei, L., Mohamad, O., Dix, T.A., and Yu, S.P. (2012). A novel stroke therapy of pharmacologically induced hypothermia after focal cerebral ischemia in mice. *FASEB J.* 26, 2799–2810.
- Wei, S., Sun, J., Li, J., Wang, L., Hall, C.L., Dix, T.A., Mohamad, O., Wei, L., and Yu, S.P. (2013). Acute and delayed protective effects of pharmacologically induced hypothermia in an intracerebral hemorrhage stroke model of mice. *Neuroscience* 252, 489–500.
- Kida, H., Nomura, S., Shinoyama, M., Ideguchi, M., Owada, Y., and Suzuki, M. (2013). The effect of hypothermia therapy on cortical laminar disruption following ischemic injury in neonatal mice. *PLoS One* 8, e68877.
- Tomura, S., de Rivero Vaccari, J.P., Keane, R.W., Bramlett, H.M., and Dietrich, W.D. (2012). Effects of therapeutic hypothermia on inflammation signaling after traumatic brain injury. *J. Cereb. Blood Flow Metab.* 32, 1939–1947.

24. Liu, L., and Yenari, M.A. (2007). Therapeutic hypothermia: neuroprotective mechanisms. *Front. Biosci.* 12, 816–825.
25. van der Worp, H.B., Macleod, M.R., and Kollmar, R. (2010). Therapeutic hypothermia for acute ischemic stroke: Ready to start large randomized trials? *J. Cereb. Blood Flow Metab.* 30, 1079–1093.
26. Jiang, J., Yu, M., and Zhu, C. (2000). Effect of long-term mild hypothermia therapy in patients with severe traumatic brain injury: 1-year follow-up review of 87 cases. *J. Neurosurg.* 93, 546–549.
27. Polderman, K.H., Tjong Tjin Joe, R., Peerdeman, S.M., Vandertop, W.P., and Girbes, A.R. (2002). Effects of therapeutic hypothermia on intracranial pressure and outcome in patients with severe head injury. *Intensive Care Med.* 28, 1563–1573.
28. Schwab, S., Schwarz, S., Aschoff, A., Keller, E., and Hacke, W. (1998). Moderate hypothermia and brain temperature in patients with severe middle cerebral artery infarction. *Acta Neurochir. Suppl* 71, 131–134.
29. Carraway, R., and Leeman, S.E. (1975). The synthesis of neurotensin. *J. Biol. Chem.* 250, 1912–1918.
30. Kokko, K.P., Hadden, M.K., Price, K.L., Orwig, K.S., See, R.E., and Dix, T.A. (2005). In vivo behavioral effects of stable, receptor-selective neurotensin[8–13] analogues that cross the blood-brain barrier. *Neuropharmacology* 48, 417–425.
31. Hadden, M.K., Orwig, K.S., Kokko, K.P., Mazella, J., and Dix, T.A. (2005). Design, synthesis, and evaluation of the antipsychotic potential of orally bioavailable neurotensin (8–13) analogues containing non-natural arginine and lysine residues. *Neuropharmacology* 49, 1149–1159.
32. Orwig, K.S., Lassetter, M.R., Hadden, M.K., and Dix, T.A. (2009). Comparison of N-terminal modifications on neurotensin(8–13) analogues correlates peptide stability but not binding affinity with in vivo efficacy. *J. Med. Chem.* 52, 1803–1813.
33. Mac Donald, C.L., Dikranian, K., Bayly, P., Holtzman, D., and Brody, D. (2007). Diffusion tensor imaging reliably detects experimental traumatic axonal injury and indicates approximate time of injury. *J. Neurosci.* 27, 11869–11876.
34. Onyszchuk, G., Al-Hafez, B., He, Y.Y., Bilgen, M., Berman, N.E., and Brooks, W.M. (2007). A mouse model of sensorimotor controlled cortical impact: Characterization using longitudinal magnetic resonance imaging, behavioral assessments and histology. *J. Neurosci. Methods* 160, 187–196.
35. Miyazawa, T., and Hossmann, K.A. (1992). Methodological requirements for accurate measurements of brain and body temperature during global forebrain ischemia of rat. *J. Cereb. Blood Flow Metab.* 12, 817–822.
36. Coggeshall, R.E. (1992). A consideration of neural counting methods. *Trends Neurosci.* 15, 9–13.
37. Hanell, A., Clausen, F., Djupsjo, A., Vallstedt, A., Patra, K., Israels-son, C., Larhammar, M., Bjork, M., Paixao, S., Kullander, K., and Marklund, N. (2012). Functional and histological outcome after focal traumatic brain injury is not improved in conditional EphA4 knockout mice. *J. Neurotrauma* 29, 2660–2671.
38. Unterberg, A.W., Stover, J., Kress, B., and Kiening, K.L. (2004). Edema and brain trauma. *Neuroscience* 129, 1021–1029.
39. Morganti-Kossmann, M.C., Rancan, M., Otto, V.I., Stahel, P.F., and Kossmann, T. (2001). Role of cerebral inflammation after traumatic brain injury: A revisited concept. *Shock* 16, 165–177.
40. Steele, A.D., Jackson, W.S., King, O.D., and Lindquist, S. (2007). The power of automated high-resolution behavior analysis revealed by its application to mouse models of Huntington's and prion diseases. *Proc Natl Acad Sci U S A* 104, 1983–1988.
41. Jorge, R.E., Robinson, R.G., Moser, D., Tateno, A., Crespo-Facorro, B., and Arndt, S. (2004). Major depression following traumatic brain injury. *Arch. Gen. Psychiatry* 61, 42–50.
42. Wroblewski, B.A., Joseph, A.B., and Cornblatt, R.R. (1996). Anti-depressant pharmacotherapy and the treatment of depression in patients with severe traumatic brain injury: a controlled, prospective study. *J. Clin. Psychiatry* 57, 582–587.
43. Zhao, Q.J., Zhang, X.G., and Wang, L.X. (2011). Mild hypothermia therapy reduces blood glucose and lactate and improves neurologic outcomes in patients with severe traumatic brain injury. *J. Crit Care.* 26, 311–315.
44. Tsuchiya, D., Hong, S., Suh, S.W., Kayama, T., Panter, S.S., and Weinstein, P.R. (2002). Mild hypothermia reduces zinc translocation, neuronal cell death, and mortality after transient global ischemia in mice. *J. Cereb. Blood Flow Metab.* 22, 1231–1238.
45. Lotocki, G., de Rivero Vaccari, J.P., Perez, E.R., Sanchez-Molano, J., Furones-Alonso, O., Bramlett, H.M., and Dietrich, W.D. (2009). Alterations in blood-brain barrier permeability to large and small molecules and leukocyte accumulation after traumatic brain injury: effects of post-traumatic hypothermia. *J. Neurotrauma* 26, 1123–1134.
46. Dietrich, W.D., Alonso, O., and Halley, M. (1994). Early microvascular and neuronal consequences of traumatic brain injury: a light and electron microscopic study in rats. *J. Neurotrauma* 11, 289–301.
47. (2007). Guidelines for the management of severe traumatic brain injury. *J. Neurotrauma* 24 Suppl 1, S1–S106.
48. Arcure, J., and Harrison, E.E. (2009). A review of the use of early hypothermia in the treatment of traumatic brain injuries. *J. Spec. Oper. Med.* 9, 22–25.
49. Clifton, G.L., Coffey, C.S., Fourwinds, S., Zygun, D., Valadka, A., Smith, K.R., Jr., Frisby, M.L., Bucholz, R.D., Wilde, E.A., Levin, H.S., and Okonkwo, D.O. (2012). Early induction of hypothermia for evacuated intracranial hematomas: a post hoc analysis of two clinical trials. *J. Neurosurg.* 117, 714–720.
50. Lundberg, G.D. (2008). Hypothermia for children after severe brain injury does not work. *Medscape J. Med.* 10, 212.
51. Sydenham, E., Roberts, I., and Alderson, P. (2009). Hypothermia for traumatic head injury. *Cochrane Database Syst. Rev.* CD001048.
52. Maier, C.M., Ahem, K., Cheng, M.L., Lee, J.E., Yenari, M.A., and Steinberg, G.K. (1998). Optimal depth and duration of mild hypothermia in a focal model of transient cerebral ischemia: effects on neurologic outcome, infarct size, apoptosis, and inflammation. *Stroke* 29, 2171–2180.
53. Erecinska, M., Thoresen, M., and Silver, I.A. (2003). Effects of hypothermia on energy metabolism in Mammalian central nervous system. *J. Cereb. Blood Flow Metab.* 23, 513–530.
54. Chodobski, A., Zink, B.J., and Szymdynger-Chodobska, J. (2011). Blood-brain barrier pathophysiology in traumatic brain injury. *Transl. Stroke Res.* 2, 492–516.
55. Shlosberg, D., Benifla, M., Kaufer, D., and Friedman, A. (2010). Blood-brain barrier breakdown as a therapeutic target in traumatic brain injury. *Nat. Rev. Neurol.* 6, 393–403.
56. Beaumont, A., Marmarou, A., Hayasaki, K., Barzo, P., Fatouros, P., Corwin, F., Marmarou, C., and Dunbar, J. (2000). The permissive nature of blood brain barrier (BBB) opening in edema formation following traumatic brain injury. *Acta Neurochir. Suppl* 76, 125–129.
57. Nag, S., Venugopalan, R., and Stewart, D.J. (2007). Increased caveolin-1 expression precedes decreased expression of occludin and claudin-5 during blood-brain barrier breakdown. *Acta Neuropathol.* 114, 459–469.
58. Yeung, D., Manias, J.L., Stewart, D.J., and Nag, S. (2008). Decreased junctional adhesion molecule-A expression during blood-brain barrier breakdown. *Acta Neuropathol.* 115, 635–642.
59. Won, S.M., Lee, J.H., Park, U.J., Gwag, J., Gwag, B.J., and Lee, Y.B. (2011). Iron mediates endothelial cell damage and blood-brain barrier opening in the hippocampus after transient forebrain ischemia in rats. *Exp. Mol. Med.* 43, 121–128.
60. Vilalta, A., Sahuquillo, J., Rosell, A., Poca, M.A., Riveiro, M., and Montaner, J. (2008). Moderate and severe traumatic brain injury induce early overexpression of systemic and brain gelatinases. *Intensive Care Med.* 34, 1384–1392.
61. Yang, Y., Estrada, E.Y., Thompson, J.F., Liu, W., and Rosenberg, G.A. (2007). Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat. *J. Cereb. Blood Flow Metab.* 27, 697–709.
62. Kiesel, U., and Wolburg, H. (2000). Tight junctions of the blood-brain barrier. *Cell Mol. Neurobiol.* 20, 57–76.
63. Bullock, R., Zauner, A., Woodward, J.J., Myseros, J., Choi, S.C., Ward, J.D., Marmarou, A., and Young, H.F. (1998). Factors affecting excitatory amino acid release following severe human head injury. *J. Neurosurg.* 89, 507–518.
64. Chong, Z.Z., Li, F., and Maiese, K. (2005). Oxidative stress in the brain: novel cellular targets that govern survival during neurodegenerative disease. *Prog. Neurobiol.* 75, 207–246.
65. Raghupathi, R., Graham, D.I., and McIntosh, T.K. (2000). Apoptosis after traumatic brain injury. *J. Neurotrauma* 17, 927–938.
66. Liu, C.L., Chen, S., Dietrich, D., and Hu, B.R. (2008). Changes in autophagy after traumatic brain injury. *J. Cereb. Blood Flow Metab.* 28, 674–683.
67. Shohami, E., Gallily, R., Mechoulam, R., Bass, R., and Ben-Hur, T. (1997). Cytokine production in the brain following closed head injury:

- dexanabinol (HU-211) is a novel TNF- α inhibitor and an effective neuroprotectant. *J. Neuroimmunol.* 72, 169–177.
68. Clark, R.S., Schiding, J.K., Kaczorowski, S.L., Marion, D.W., and Kochanek, P.M. (1994). Neutrophil accumulation after traumatic brain injury in rats: comparison of weight drop and controlled cortical impact models. *J. Neurotrauma* 11, 499–506.
 69. Scheff, S.W., Ansari, M.A., and Roberts, K.N. (2013). Neuroprotective effect of Pycnogenol® following traumatic brain injury. *Exp. Neurol.* 239, 183–191.
 70. Namas, R., Ghuma, A., Hermus, L., Zamora, R., Okonkwo, D.O., Billiar, T.R., and Vodovotz, Y. (2009). The acute inflammatory response in trauma/hemorrhage and traumatic brain injury: current state and emerging prospects. *Libyan J. Med.* 4, 97–103.
 71. Harting, M.T., Jimenez, F., Adams, S.D., Mercer, D.W., and Cox, C.S., Jr. (2008). Acute, regional inflammatory response after traumatic brain injury: Implications for cellular therapy. *Surgery* 144, 803–813.
 72. Frink, M., Flohe, S., van Griensven, M., Mommsen, P., and Hildebrand, F. (2012). Facts and fiction: the impact of hypothermia on molecular mechanisms following major challenge. *Mediators Inflamm.* 2012, 762840.
 73. Dietrich, W.D., Busto, R., and Bethea, J.R. (1999). Postischemic hypothermia and IL-10 treatment provide long-lasting neuroprotection of CA1 hippocampus following transient global ischemia in rats. *Exp. Neurol.* 158, 444–450.
 74. Stewart, C.R., Landseadel, J.P., Gurka, M.J., and Fairchild, K.D. (2010). Hypothermia increases interleukin-6 and interleukin-10 in juvenile endotoxemic mice. *Pediatr. Crit. Care Med.* 11, 109–116.
 75. Leon, L.R., Blaha, M.D., and DuBose, D.A. (2006). Time course of cytokine, corticosterone, and tissue injury responses in mice during heat strain recovery. *J. Appl. Physiol.* 100, 1400–1409.
 76. Kline, A.E., Bolinger, B.D., Kochanek, P.M., Carlos, T.M., Yan, H.Q., Jenkins, L.W., Marion, D.W., and Dixon, C.E. (2002). Acute systemic administration of interleukin-10 suppresses the beneficial effects of moderate hypothermia following traumatic brain injury in rats. *Brain Res.* 937, 22–31.
 77. Demetrius, L. (2005). Of mice and men. When it comes to studying ageing and the means to slow it down, mice are not just small humans. *EMBO Rep.* 6 Spec. No. S39–S44.
 78. Heegaard, W., Biros, M., and Zink, J. (1997). Effect of hypothermia, dichloroacetate, and deferoxamine in the treatment for cortical edema and functional recovery after experimental cortical impact in the rat. *Acad. Emerg. Med.* 4, 33–39.
 79. Jiang, J.Y., Liang, Y.M., Luo, Q.Z. and Zhu, C. (2004). Effect of mild hypothermia on brain dialysate lactate after fluid percussion brain injury in rodents. *Neurosurgery* 54, 713–718.

Address correspondence to:

Shan Ping Yu, MD, PhD
101 Woodruff Circle, WMB Suite 620
Emory University School of Medicine
Atlanta, GA 30322

E-mail: spyu@emory.edu