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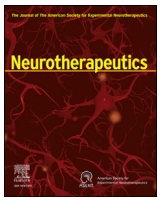

Original Article

Neuroprotective efficacy of hypothermia and Inter-alpha Inhibitor Proteins after hypoxic ischemic brain injury in neonatal rats

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ABSTRACT

Therapeutic hypothermia is the standard of care for hypoxic-ischemic (HI) encephalopathy. Inter-alpha Inhibitor Proteins (IAIPs) attenuate brain injury after HI in neonatal rats. Human (h) IAIPs (60 mg/kg) or placebo (PL) were given 15 min, 24 and 48 h to postnatal (P) day-7 rats after carotid ligation and 8% oxygen for 90 min with (30 °C) and without (36 °C) exposure to hypothermia 1.5 h after HI for 3 h. Hemispheric volume atrophy (P14) and neurobehavioral tests including righting reflex (P8–P10), small open field (P13–P14), and negative geotaxis (P14) were determined. Hemispheric volume atrophy in males was reduced ($P < 0.05$) by 41.9% in the normothermic-IAIP and 28.1% in the hypothermic-IAIP compared with the normothermic-PL group, and in females reduced ($P < 0.05$) by 30.3% in the normothermic-IAIP, 45.7% in hypothermic-PL, and 55.2% in hypothermic-IAIP compared with the normothermic-PL group after HI. Hypothermia improved ($P < 0.05$) the neuroprotective effects of hIAIPs in females. The neuroprotective efficacy of hIAIPs was comparable to hypothermia in female rats ($P = 0.183$). Treatment with hIAIPs, hypothermia, and hIAIPs with hypothermia decreased ($P < 0.05$) the latency to enter the peripheral zone in the small open field test in males. We conclude that hIAIPs provide neuroprotection from HI brain injury that is comparable to the protection by hypothermia, hypothermia increases the effects of hIAIPs in females, and hIAIPs and hypothermia exhibit some sex-related differential effects.

Introduction

Hypoxic-ischemic (HI) brain injury in newborns is a significant cause of morbidity and mortality and can result in long-term neurological abnormalities, such as cerebral palsy, seizure disorders, sensory and/or motor and cognitive impairment, and developmental delay [1,2]. Currently, hypothermia is the standard of care to treat HI encephalopathy (HIE) in full-term infants [3–6]. However, the effectiveness of this therapy is limited as it provides only partial protection and must be initiated within the narrow timeframe of 6 h after birth [3–6]. Therefore, there is a critical need to develop alternative and/or additive therapeutic strategies to the standard therapeutic hypothermic treatment. Although there have been some novel therapies that have been considered to exhibit promise as alternative and/or adjunctive treatments for HI-related brain injury in newborns including erythropoietin [7–10], xenon [11–13], melatonin [14], stem cells [15,16], N-acetylcysteine [17], exendin-4 [18], and phenobarbital [19], these additional additive therapies have not as yet proven efficacious in the treatment of newborns. However, several critical aspects, including dosing regimens, histopathology, neurobehavioral outcomes, mechanisms of action, and potential sex-dependent effects require further investigation to advance the treatment of HIE in newborns.

Inter-alpha inhibitor proteins (IAIPs) are naturally occurring molecules found in relatively high concentrations in human plasma [20,21]. These proteins exhibit important anti-inflammatory and immunomodulatory properties and have gained attention because of their beneficial effects in various conditions including sepsis, HI-related brain injury in newborns, and stroke in adult subjects [22–29]. Recent studies have demonstrated the potential benefits of treatment with human plasma derived IAIPs (hIAIPs) to reduce neuronal cell death and neuroinflammation in male neonatal rats

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after exposure to HI [23,30]. Furthermore, treatment with hIAIPs reduces brain injury determined by MRI analysis and improves short- and long-term behavioral and histopathological outcomes including decreases in the loss of brain tissue in both male and female neonatal rats after exposure to HI brain injury [22,23,25,27,31,32]. Moreover, this treatment is durable because young adult rats at P42 exhibit less parenchymal brain volume loss even 35 days after exposure to HI as neonates, suggesting that not only is this treatment neuroprotective in neonates, but it also exhibits durable neuroprotection in the young adult rats after neonatal exposure to HI [25].

Disruption of the blood-brain barrier (BBB) is well known to exacerbate brain injury because of exposure of the brain parenchyma to multiple inflammatory factors and neurotoxins originating from the systemic circulation [33]. In this context, hIAIPs significantly attenuate lipopolysaccharide-induced BBB disruption in adult male mice and in both male and female neonatal mice [34,35].

Given the above considerations, the objective of the current study was to examine the effects of hypothermia, and of hIAIPs with and without exposure to hypothermia in neonatal rats after exposure to HI-related brain injury. We hypothesized that the neuroprotection provided by hIAIPs was at least as beneficial as treatment with hypothermia alone, and that hIAIPs could be potentially additive to the therapeutic effects of hypothermia. To test these hypotheses, experimental HI was induced in postnatal day 7 (P7) rats by right carotid artery ligation and exposure to 8% oxygen for 90 min. The P7 rat brain is considered somewhat analogous to the brain of late preterm infants (32–34 GW) [36–39].

Materials and Methods

The experimental procedures conducted in this study were approved by the Institutional Animal Care and Use Committees (IACUC) of Brown University and Women & Infants Hospital of Rhode Island (IACUC #22-06-0006). All experimental procedures were performed in accordance with the guidelines described in the National Institutes of Health guide (NIH #8023, 1978) for the care and use of laboratory animals and followed the ARRIVE guidelines.

Preparation of hIAIPs

The hIAIPs were purified according to previously described procedures [23,40,41]. They were extracted from fresh frozen human plasma (Rhode Island Blood Center, RI, USA) or from industrial plasma intermediates (Prometic Biotherapeutics, Rockville, MD). A scalable purification process involving a capture step using anion-exchange chromatography (Toyopearl GigaCap Q650 M, Tosoh Bioscience, King of Prussia, PA) and a polishing step using a proprietary synthetic chemical ligand affinity chromatographic media (Astrea Bioseparations, Cambridge, UK) resulted in highly pure (>90%) and biologically active hIAIPs. Eluted proteins were concentrated and buffer-exchanged using a tangential flow filtration device (Labscale TFF System, MilliporeSigma, Burlington, MA, USA) that utilized a Pellicon XL50 cartridge with 30 kDa cut-off Biomax membrane (MilliporeSigma, Burlington, MA, USA). The purity of the hIAIPs was examined by SDS-PAGE, Western immunoblot, and ELISA [42,43]. The biological activity of the hIAIPs was examined by their ability to inhibit trypsin-mediated hydrolysis of the substrate N-Benzoyl-L-arginine-*p*-nitroaniline HCl (MilliporeSigma, St. Louis, MO, USA) [42,43]. Furthermore, purified hIAIPs were tested for endotoxin using a Limulus amebocyte lysate endotoxin-based chromogenic test (Pierce Biotechnology, Thermo Fisher Scientific, Waltham, MA, USA). In addition, consistency in the purity and quantity of the hIAIPs was further ensured by the use of a single lot of purified hIAIPs for the entire study.

Animals and experimental procedures

Pregnant Wistar rats were acquired from Charles River Laboratories (Wilmington, MA, USA) on embryonic day 15 or 16 and were housed in a temperature-controlled environment with 12 h light/dark cycles and ad libitum access to food and water. The day of delivery of the rat pups was designated as P0. Litters were randomly culled on P1 to ten and balanced such that there were approximately equal numbers of males and females to reduce inter-litter variability. The litters were randomly assigned on P7 to normothermic or hypothermic groups. The randomization process used an online random number generator (https://stattrek.com/statistics/random-number-generator). On P7, the pups of each litter received a unique numerical identifier generated by the random number generator and were then allocated to either the normothermic or hypothermic groups. This approach ensured an unbiased assignment for each pup and litter and maintained masking of the researchers regarding the group allocations. Subsequently, each normothermic and hypothermic group was further subdivided such that the rat pups were randomly assigned into four sub-groups: Sham + Placebo (PL)-treated, Sham + IAIP-treated, HI + PL-treated (HI-PL), and HI + IAIP-treated (HI-IAIP). The Sham + PL and Sham + IAIP groups were combined into one Sham group based upon our previous work showing that differences were not detected between sham treated placebo and sham treated hIAIP groups [22,23,27]. Consequently, the final six experimental groups were: Normothermia + Sham, Normothermia + HI-PL, Normothermia + HI-IAIP, Hypothermia + Sham, Hypothermia + HI-PL, and Hypothermia + HI-IAIP. Sex was determined for each subject.

The Rice-Vannucci method was used to induce HI in P7 rats as previously described [44]. In brief, the P7 rats were anesthetized with 4% isoflurane and maintained with 2% isoflurane during the surgical procedures. The body temperature was maintained at 36 °C using a heating pad during the surgery. A vertical midline incision of 0.5–1 cm was made on the neck, and the right common carotid artery was double-ligated with 5-0 silk sutures (Ethicon, Raritan, NJ, USA) in the HI-PL and HI-IAIP groups. A similar incision was made in the Sham treated groups, but the right common carotid artery was not ligated. The incision was then closed, and each rat was marked with tail ink injections for identification (Neo-9, Animal Identification & Marking Systems, Inc., Hornell, NY, USA). The carotid occlusion surgery typically lasted 5–8 min per pup, and the animals remain anesthetized for approximately 10–15 min. The rats were returned to the dam for recovery after surgery. The rat pups were then placed in a hypoxia chamber (Biospherix, Parish, NY, USA) containing 8% oxygen and balanced nitrogen for 90 min (Fig. 1) after remaining with the dams for 1.5–3 h. One non-ligated sentinel pup from each litter was selected to have a rectal temperature probe inserted (RET-4, Physitemp, Clifton, NJ, USA) to monitor temperature continuously with a digital thermometer (TH-5, Physitemp Instruments INC, USA). Rectal temperature measurements were obtained every 10 min and maintained as close to 36.0 °C as possible during the HI procedure [5,23,25,27,45]. Rectal temperature provides an accurate reflection of brain temperature in rodents during exposure to HI [46,47]. The sentinel pup was not included in the studies because the rectal probe placement affects the outcomes of the HI studies [45,48]. The Sham subjects were placed in a similar container and exposed to room air for 90 min. A total of 287 pups were used for the study. Three out of the 153 HI exposed pups died during the experiment for a mortality rate of 2%.

Determination of temperature for therapeutic hypothermia in our laboratory setting

Rectal temperatures were systematically determined in separate groups of Sham and HI-treated neonatal rats, which were not included in any of the principal study groups summarized above under the Methods section of animals and experimental procedures. This initial evaluation was critical to determine the appropriate temperature for the studies of therapeutic hypothermia after exposure to HI brain injury in our laboratory as described below in the Methods section for experimental designs for the normothermic and hypothermic studies. A rectal temperature probe was inserted to monitor and record body temperatures with a digital thermometer. Rectal temperatures were

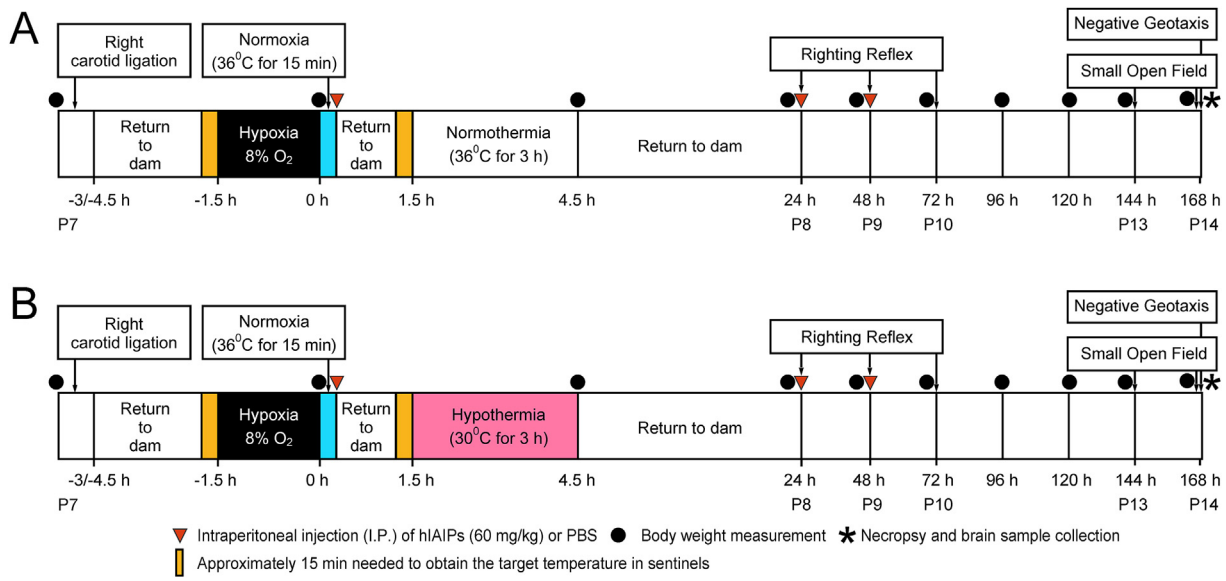

Fig. 1. Study design. (A) After right common carotid artery ligation at P7, the pups were returned to the dams for 1.5–3 h. Subsequently, the pups were exposed to 8% oxygen with balanced nitrogen for 90 min (black colored box) at a constant rectal temperature of 36 °C. After exposure to normoxia for 15 min at 36 °C, the first intraperitoneal (I.P.) injection of 60 mg/kg of hIAIPs or placebo (PBS) was administered (designated by the inverted orange triangle). After an additional 1 h period with the dams to allow for feeding and recovery, the rats were exposed to normothermia for 3 h. Thereafter, the pups were again returned to the dams. Additional doses of 60 mg/kg of hIAIPs or placebo (PBS) were given at 24 and 48 h after the termination of HI (designated by the inverted orange triangles). The righting reflex test was performed on P8–P10, the small open field test on P13 and P14, and the negative geotaxis test on P14. Body weights were obtained as indicated by the closed circles. The study design was identical for the pups exposed to the hypothermic treatment except that they were exposed to hypothermia from 1.5 h after the termination of HI for 3 h (rose color shaded box). A necropsy was performed 168 h after exposure to HI. hIAIP = Human Inter-alpha Inhibitor Proteins, PBS = phosphate buffered saline.

systematically measured daily from P5 to P14, before and after the surgical procedures, before and after exposure to room air in the Sham, and before and after 90 min of hypoxia in the HI exposed pups. Rectal temperatures were also measured at 1, 2, and 6 h after the exposure to room air or the induction of HI at P7, and daily from P8 up until P14 (Fig. 2).

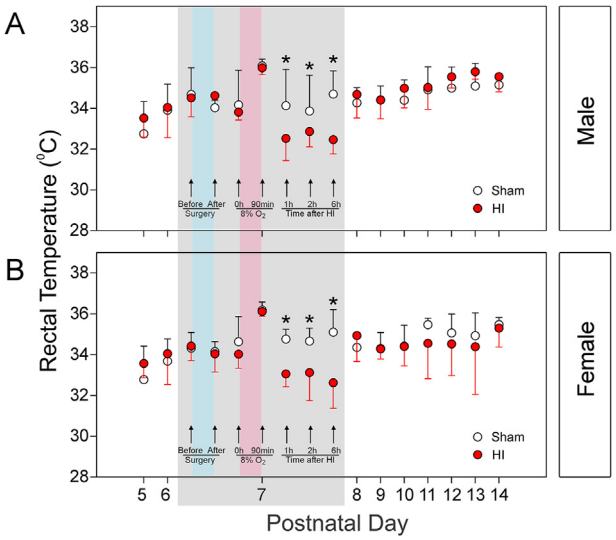

Fig. 2. Rectal temperatures were measured daily from P5 to P14. Rectal temperatures are plotted on the y-axis and postnatal day is plotted on the x-axis. Rectal temperatures were recorded on P7 before and after surgery, at zero h before exposure to the hypoxic procedure, and at zero, 1, 2, and 6 h after the induction of HI injury. Values are mean ± SD. Open circles are Sham treated pups not exposed to HI and the red closed circles are pups exposed to HI. *$P <$ 0.05. Sham: male n = 8–17, female n = 6–15; HI: male n = 5–14, female n = 5–9.

Experimental designs for the normothermic and hypothermic studies

The experimental designs for the study are schematically illustrated in Fig. 1A and B. Fig. 1A illustrates the study design for the groups exposed to the normothermic conditions. The pups were returned to normoxia for 15 min at 36 °C to recover from the 90 min hypoxic exposure. Thereafter, the pups were given an initial 60 mg/kg dose of hIAIPs or an equivalent volume of PL (phosphate-buffered saline, PBS) after the recovery from HI via an intraperitoneal (I.P.) injection. The timing of the initial dose was selected based upon previous work suggesting that, when topiramate was given 15 min after exposure to HI in conjunction with hypothermia, pathological outcomes, and behavioral performances were improved [49]. In addition, we thought that there was potential for the efficacy of hIAIPs to be more effective after an interval of recovery from HI and under normothermic conditions similar to our previous studies [23,27,30]. The selection of the 60 mg/kg dose of hIAIPs was based upon our studies that demonstrated that this dose reduced infarct volumes in the brain even after exposure to severe HI [27]. Although we have also demonstrated that treatment with 30 mg/kg of hIAIPs attenuated pathological brain injury, brain volume loss, and neuroinflammation after exposure to moderate HI [23,30], the higher dose was selected in the current study potentially to augment the neuroprotection afforded by hIAIPs in the setting of delayed hypothermia (Fig. 1B).

Thereafter, the pups were again returned to the dams for 1 h to feed and recover from HI before exposure to the normothermic conditions 1.5 h after the termination of HI. The pups were placed in a specially designed chamber (Supplementary Fig. 1) on a heating pad with the rectal temperature of the sentinel pup continuously maintained at 36 °C for 3 h during exposure to normothermia. After the 3 h exposure to normothermic conditions, the pups were again returned to the dams. The pups were given additional 60 mg/kg doses of hIAIPs or equivalent volumes of PL at 24 h and 48 h after the termination of HI. This treatment schedule of hIAIPs was selected based upon the half-life of hIAIPs that we reported in neonatal rats [40] and the favorable outcomes in our previous

publications [23,27]. Behavioral analyses indicated by the arrows (Fig. 1) included the righting reflex at 24 h (P8), 48 h (P9) and 72 h (P10), small open field tests at 144 h (P13) and 168 h (P14), and negative geotaxis test performed after the completion of the second open field test at 168 h (P14) after the termination of HI.

The experimental design (Fig. 1B) was identical in the neonatal rats that were exposed to hypothermia to those exposed to the normothermic conditions (Fig. 1A) except that hypothermia was initiated 1.5 h after the termination of HI. The exposure to whole-body hypothermia was accomplished by placing the rats within the specially designed chamber (Supplementary Fig. 1A) with the temperature meticulously maintained at 30 °C for the 3 h exposure using the TECOtherm NEO Hypothermia System (Inspiration Healthcare Ltd, West Sussex, UK). The selection of 30 °C for the hypothermic treatment was based upon preliminary findings in our laboratory (Fig. 2) and the 3-h duration of exposure to hypothermia was based upon previous studies of therapeutic hypothermia in neonatal rats [49,50]. The 15-min delayed administration of hIAIPs after termination of HI and the delayed exposure to hypothermia 1.5 h were designed to simulate potential conditions in infants exposed to HIE because the timing of injury before birth is not known and often delayed as a result of logistical circumstances such as transport [51,52]. Although these time frames are considerably shorter than those that are likely relevant in human infants, the life span of a rodent is substantially shorter than in a human [53]. The unit of time in the rat is approximately five times faster compared with that of humans based upon interspecies scaling between humans and rats [53].

Each pup was weighed before surgery, after exposure to normoxia or HI, before each dose of hIAIPs or PL, at the end of normothermia or hypothermia and before each of the behavioral studies, and also at 96 h and 120 h after exposure to normoxia or HI (Fig. 1A and B, closed circles). The pups were sacrificed 168 h after exposure to HI on P14 (Fig. 1A and B). The subjects were weighed and sedated with a cocktail of ketamine (74 mg/kg, I.P.) and xylazine (4 mg/kg, I.P.). Blood samples (0.1–0.3 ml) were individually collected via cardiac puncture and immediately centrifuged at 1200×*g* at room temperature for 5 min. After the removal of the fibrin clot, the serum was collected by centrifugation again at 1200×*g* for 5 min and stored at −80 °C until analysis [54,55]. The brain was perfused by cardiac puncture using PBS and 4% paraformaldehyde (PFA) at a flow rate of 3 ml/min. Subsequently, the brains were removed, weighed, post-fixed in PFA for 24 h, and stored in 0.1 M phosphate buffer (MilliporeSigma, Burlington, MA, USA) containing 30% sucrose (MilliporeSigma, Burlington, MA, USA) at 4 °C until they were cryosectioned for hemispheric tissue volume atrophy analysis [23,25,27,56].

Hemispheric tissue volume atrophy measurements

The brains were sliced into five 2 mm coronal sections using a brain slicer matrix to examine the hemispheric tissue volume atrophy after HI (Zivic Instruments, Pittsburgh, PA, USA). These sections were then immersed in Tissue-Tek optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA) and frozen by placing them in a metal beaker containing isopentane (MilliporeSigma, Burlington, MA, USA) surrounded by crushed dry ice [23,27,56]. Five cryosections (20 μm) were obtained from each 2 mm section and mounted on Superfrost™ Plus microscope slides coated with gelatin (Fisher Scientific International, Inc., Hampton, NH, USA). Every second cryosection (20 μm) was randomly selected and stained with cresyl violet (MilliporeSigma, Burlington, MA, USA) to evaluate HI-induced cell injury [23,27,56]. Images of the cresyl violet-stained brain sections were captured using a Micropublisher 6 CCD Camera (Qimaging, Surrey, British Columbia, Canada) to quantify hemispheric tissue volume atrophy in the entire hemispheres and damaged areas of the brains across the study groups. The resulting images were analyzed using ImageJ software (NIH, Bethesda, MD, USA) by two examiners who were not aware of the group assignments. Tissue area atrophy was calculated as a percentage of the damaged hemisphere compared to the total contralateral hemisphere, taking into account corrections for hemispheric edema, according to the following formula: tissue area atrophy (%) = [1 - (total ipsilateral hemisphere - ipsilateral hemisphere damage)/total contralateral hemisphere)] x 100% [23,27,57–59]. The respective volumes were determined by multiplying the measured area by the distance (2 mm) between the sections. The values calculated by the two examiners were averaged and used for the final data analysis.

Enzyme-linked immunosorbent assay (ELISA) for IAIP concentrations

Rat serum samples were collected as described above. Two separate ELISA protocols were employed to detect changes in exogenous human IAIPs (hIAIPs) and endogenous rat IAIPs, according to detailed procedures from previous publications [24,34]. A monoclonal antibody (MAb 69.26, ProThera Biologics Inc., Providence, RI, USA) highly specific for hIAIPs was used for the competitive hIAIP ELISA. This assay was developed to quantify the measurement of hIAIPs in biological fluids. The MAb 69.26 antibody specifically binds to hIAIPs and does not cross-react with endogenous rat IAIPs. A competitive rodent IAIP ELISA was performed to measure endogenous rat IAIP concentrations using a rabbit polyclonal antibody against rodent IAIPs (PAb-R22C, ProThera Biologics Inc., Providence, RI, USA). This polyclonal antibody was generated by immunizing rabbits with highly purified rat IAIPs. Biotinylated PAb-R22C was utilized in the competitive rodent IAIP ELISA to compete with both exogenous and endogenous IAIPs present in the serum samples against the purified rat IAIP immobilized on the microplates. The binding of biotinylated molecules was visualized using streptavidin-conjugated HRP, and the subsequent color change after the addition of tetramethylbenzidine substrate (Enhanced K-Blue TMB substrate, Neogen Corp., Lansing, MI) was measured spectrometrically at 450 nm.

Behavioral analyses

All of the behavioral studies were consistently conducted starting at approximately 10 a.m. The rats were given a minimum of 15 min to acclimate to the testing area before initiating each of the behavioral procedures. The behavioral tests were recorded using a monochrome GigE camera (Noldus, Wageningen, Netherlands) and subsequent analysis was conducted without the knowledge of the treatment group assignments.

The righting reflex test was conducted on P8, P9, and P10 to examine early motor coordination after exposure to HI. The procedure involved placing the rat in a supine position on a flat surface and measuring the time required for the rat to right itself to a prone position with all four paws in contact with the surface [25,26]. The latency to achieve the prone position was recorded in seconds (sec). The test was repeated for five consecutive trials to obtain reliable data.

The small open field test was conducted on P13 and P14 to assess early locomotor activity, exploratory, and anxiety-like behaviors in neonatal rats after exposure to HI-related brain injury [60–62]. Each rat was individually placed in the center of a small empty arena measuring 28 cm × 28 cm and allowed to freely explore the environment for approximately 10 min. The arena was divided into 16 squares, including a peripheral zone (S1, S2, S3, S4, S5, S8, S9, S12, S13, S14, S15, and S16) and a central zone (S6, S7, S10, and S11) (Supplementary Fig. 1B). The behavior of the rats during the test was recorded using video files and analyzed using Ethovision XT software (Noldus). The dependent measures included the total distance traveled divided by the observation time (cm/sec), the latency to the first entry into the peripheral zone (sec), which was used as the variable to evaluate anxiety-like behavior [63]. The percentage of postural changes involving body extension or contraction, and the mobility. High-frequency postural changes to extended or contracted positions can also indicate anxiety or distress [64,65].

The negative geotaxis performance of the pups was evaluated on P14 to examine their vestibular, early motor development, and coordination

ability [66,67]. The examination was conducted using a 30° inclined wire mesh (26 × 20 cm) with the pups placed in the center and their heads facing downwards. The experimenter gently released their hands once the pup firmly grasped the wire mesh and could support its body weight, permitting the pup to turn and face upwards on the inclined platform. The dependent measures included the total distance traveled (cm) on the wire mesh, the total cumulative duration (sec) for the pups to reach the top of the incline, and the percentage of postural changes involving body extension or contraction. Each pup performed the task three times, and all experiments were video-recorded and analyzed using EthoVision XT software (Noldus) without knowledge of the study groups.

Statistical analyses

All data were first examined for outliers using the ROUT test [68] in GraphPad Prism software (GraphPad Software, San Diego, CA, USA). The normality of the data distribution was then examined using the Shapiro-Wilk normality test. Analysis of variance (ANOVA) was used for normally distributed data, whereas the Kruskal-Wallis test was used for non-normally distributed data. The determination of the hypothermic temperature and its sex-dependence was analyzed using factorial ANOVA. Body weight changes over time and the righting reflexes between study groups were compared using factorial ANOVA with repeated measures. Additionally, the results of brain weights, hemispheric tissue volume atrophy, small open fields, and negative geotaxis, and other categorical independent variables such as sex versus drug treatment (sham, placebo, or hIAIPs), sex versus temperature conditions (normothermia or hypothermia), and sex versus drug treatment versus temperature conditions were analyzed using factorial ANOVA. Post-hoc tests, such as Fisher's least significant difference (LSD) test or Dunn's test for multiple comparisons, were employed if a significant difference was observed in the ANOVA or Kruskal-Wallis's test, respectively.

A linear regression model was employed to estimate the relationship between residual exogenous hIAIP values in the rat serum and hemispheric tissue volume atrophy in both the Normothermia + HI-IAIP and Hypothermia + HI-IAIP groups. The strength and direction of this association were assessed using Spearman's correlation coefficient (ρ). Analysis of covariance (ANCOVA) with an equal slopes model was used to compare the two regression lines between the Normothermia + HI-IAIP and Hypothermia + HI-IAIP groups.

The results were presented as mean ± standard deviation (SD), and statistical significance was set at $P < 0.05$. The outlier identification and normality assessment were carried out using GraphPad Prism software. The linear regression, Spearman rank, and ANCOVA analyses were conducted using SigmaPlot (Inpixon–systatsoftware, Palo Alto, CA, USA). All other analyses were performed utilizing the STATISTICA package (TIBCO Software Inc., Palo Alto, CA, USA).

Results

Preliminary temperature examination for the studies of therapeutic hypothermia in our laboratory setting

The rectal temperature values from P5 up to P14 in Sham control and HI-exposed neonatal rats were examined in order to select the optimal temperature for the study of therapeutic hypothermia in our laboratory. These initial rectal temperature measurements served the purpose of elucidating the baseline and fluctuations in body temperature during the experimental procedures (Fig. 2: Sham: open circles, n = 14–32, HI: closed red circles, n = 10–23).

Fig. 2A contains the rectal temperatures (°C) in the male group plotted against postnatal age in days. The rectal temperature remained relatively stable in the Sham pups and those destined for exposure to HI, before and after the surgical procedures and before and immediately after exposure to the Sham and HI treatments. However, the male rats exposed to HI (n = 5–14) exhibited lower body temperatures at 1 h (HI: 32.5 ± 1.1 °C, Sham: 34.1 ± 1.8 °C; factorial ANOVA, $P < 0.001$), 2 h (HI: 32.9 ± 0.8 °C, Sham: 33.9 ± 1.8 °C; factorial ANOVA, $P = 0.024$), and 6 h (HI: 32.5 ± 0.7 °C, Sham: 34.7 ± 1.1 °C; factorial ANOVA, $P < 0.001$) compared with the Sham (n = 8–17) group. Additionally, the rectal temperature patterns were also similar in the female rats (2B) at 1 h (HI: 33.1 ± 0.6 °C, Sham: 34.8 ± 0.5 °C; factorial ANOVA, $P < 0.001$), 2 h (HI: 33.1 ± 1.4 °C, Sham: 34.7 ± 0.6 °C; factorial ANOVA, $P < 0.001$), and 6 h (HI: 32.6 ± 1.3 °C, Sham: 35.1 ± 1.1 °C; factorial ANOVA, $P < 0.001$) after HI (n = 5–9) compared with the Sham (n = 6–15) group. Significant differences were not observed between the Sham and HI exposed groups at the other time points examined (factorial ANOVA, all $P > 0.05$). Consequently, the body temperatures were reduced in the neonatal rats for at least 6 h after exposure to HI.

Previous work has suggested that decreases of 4 °C in core body temperature for 5 h have detrimental effects on the brain of late-preterm rats after exposure to HI [69]. In contrast, more moderate reductions in body temperature of 1.5 °C for 2 h resulted in substantial decreases in brain injury in male and female rats after exposure to HI injury at P7 [37,70]. Consequently, a temperature of 30 °C was selected for our subsequent hypothermic studies. Thirty degrees centigrade is approximately 2.5 °C below the normothermic rectal temperatures determined after HI in our preliminary results (Fig. 2: 1 h, 2 h and 6 h after HI). Therefore, therapeutic hypothermia was induced at 30 °C and continued for 3 h in our experimental protocol.

Body and brain weights in the experimental study groups

Fig. 3A contains the percentage change in body weight plotted against the study time in hours for the male (top panel) and female (bottom panel) rats. There were significant increases in body weight in the Sham, HI-PL, and HI-IAIP groups under normothermic and hypothermic conditions throughout the study period in the male and female rats (factorial ANOVA with repeated measures, $P < 0.001$). The Sham group exhibited a greater increase in body weight over time in males and females compared with both the Normothermia + HI-PL (factorial ANOVA, $P < 0.001$) and Normothermia + HI-IAIP (factorial ANOVA with repeated measures, $P < 0.001$) groups. Similarly, hypothermia alone did not alter body weight changes in the neonatal rats exposed to HI injury (factorial ANOVA with repeated measures, Male: $P = 0.915$; Female: $P = 0.647$). Differences were also not detected between the hypothermic hIAIP-treated HI (Hypothermia + HI-IAIP) and the other groups [Normothermia + HI-PL (factorial ANOVA with repeated measures, male: $P = 0.551$; female: $P = 0.924$), Normothermia + HI-IAIP (factorial ANOVA with repeated measures, male: $P = 0.322$; female: $P = 0.565$), Hypothermia + HI-PL (factorial ANOVA with repeated measures, male: $P = 0.625$; female: $P = 0.744$)].

Fig. 3B contains the total brain weights of the Sham, HI-PL, and HI-IAIP groups in males and females at the end of the study (P14). Significant differences were not observed between the brain weights of the Sham, HI-PL, or HI-IAIP groups in the male (factorial ANOVA, $F(2, 86) = 0.218$, $P = 0.805$), or female rats (factorial ANOVA, $F(2, 81) = 0.664$, $P = 0.517$) after exposure to the normothermic or hypothermic conditions.

Therapeutic effects of Inter-alpha Inhibitor Proteins and hypothermia after exposure to HI-related brain injury in neonatal rats

Fig. 4A contains representative cresyl violet images of coronal brain sections from male and female rats in the Sham, HI-PL, and HI-IAIP groups after exposure to normothermic and hypothermic conditions. The HI-PL male and female neonatal rats exhibited increased ipsilateral hemispheric pallor compared with the Sham-treated groups under normothermic conditions. Treatment of the male rats with hIAIPs with and without exposure to hypothermia attenuated the extent of pallor compared with the HI-PL group under normothermic conditions. Similarly, treatment with hIAIPs in the female rats with and without exposure to hypothermia also attenuated the extent of pallor compared with the

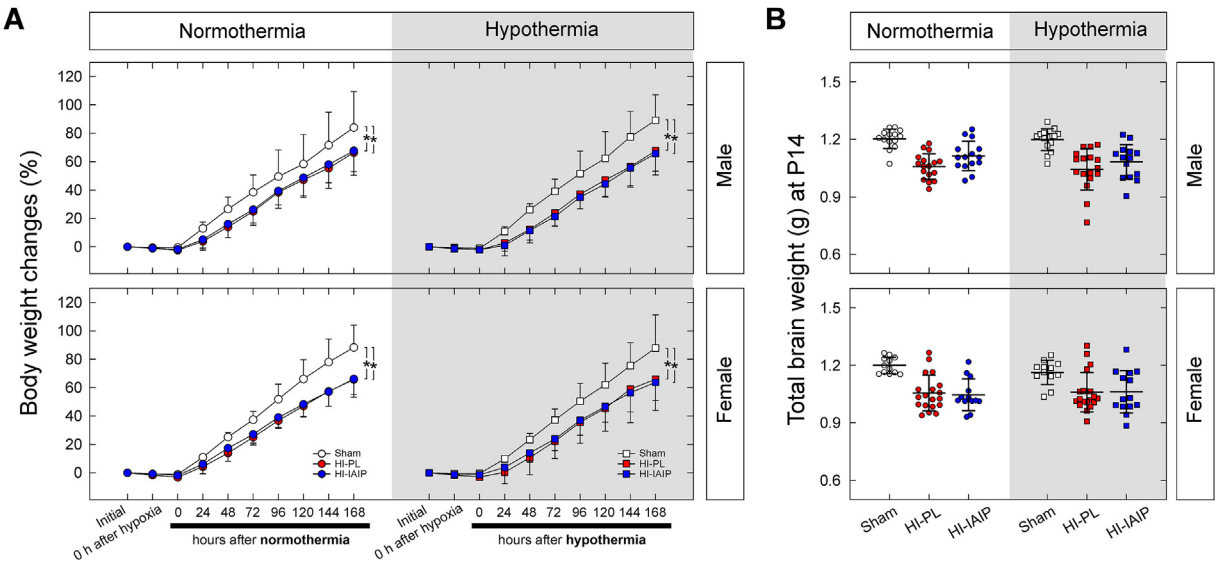

Fig. 3. A. Body weight changes (%) are plotted on the y-axis and hours after exposure to hypoxia are plotted on the x-axis. Groups are separated based on exposure to normothermia (areas not shaded) and hypothermia (gray shaded area) for the males (top row), and females (bottom row). Open circles = Sham, red closed circles = HI-PL, and blue closed circles = HI-IAIP groups exposed to normothermia; Open squares = Sham, red closed squares = HI-PL, and blue closed squares = HI-IAIP groups exposed to hypothermia. Values are mean ± SD. *$P < 0.05$. Male: Normothermia + Sham n = 14, Normothermia + HI-PL n = 18, Normothermia + HI-IAIP n = 15; Female: Normothermia + Sham n = 12, Normothermia + HI-PL n = 19, Normothermia + HI-IAIP n = 13; Male: Hypothermia + Sham n = 15, Hypothermia + HI-PL n = 17, Hypothermia + HI-IAIP n = 14; Female: Hypothermia + Sham n = 12, Hypothermia + HI-PL n = 19, Hypothermia + HI-IAIP n = 14. B. Total brain weights (g) in the males and females are plotted on the y axis and the experimental groups are plotted on the x-axis. Symbol legends as for A. Male: Normothermia + Sham n = 14, Normothermia + HI-PL n = 17, Normothermia + HI-IAIP n = 15; Female: Normothermia + Sham n = 12, Normothermia + HI-PL n = 19, HI- Normothermia + IAIP n = 13; Male: Hypothermia + Sham n = 15, Hypothermia + HI-PL n = 17, Hypothermia + HI-IAIP n = 14; Female: Hypothermia + Sham n = 12, Hypothermia + HI-PL n = 19, Hypothermia + HI-IAIP n = 14.

HI-PL group that had not been exposed to either treatment. Quantification of the percent of hemispheric tissue atrophy corroborated the observations of the cresyl violet stained images in the neonatal rats exposed to HI injury illustrated in Fig. 4B.

The normothermic HI-PL (Normothermia + HI-PL, 48.9 ± 11.0%) hemispheric tissue atrophy was extensive compared with the Sham (Normothermia + Sham, 3.3 ± 1.8%) in the males (factorial ANOVA, $P < 0.001$). Treatment with hIAIPs (Normothermia + HI-IAIP) reduced the hemispheric tissue atrophy from 48.9 ± 11.0% to 28.4 ± 22.9% (factorial ANOVA, $P = 0.003$) and treatment with hIAIPs during hypothermia (Hypothermia + HI-IAIP) reduced tissue atrophy to 35.1 ± 21.4% (factorial ANOVA, $P = 0.047$) resulting in reductions of the hemispheric tissue volume atrophy by 41.9 ± 46.9% and 28.1 ± 43.8% during normothermia and hypothermia in the males, respectively. However, differences were not observed between the Normothermia + HI-PL and Hypothermia + HI-PL (factorial ANOVA, $P = 0.051$), or between the Normothermia + HI-IAIP and Hypothermia + HI-IAIP (factorial ANOVA, $P = 0.278$), or the Hypothermia + HI-PL and Hypothermia + HI-IAIP male groups (factorial ANOVA, $P = 0.970$).

The ipsilateral hemispheric tissue atrophy was also higher in the Normothermia + HI-PL (52.6 ± 16.0%) than in the Normothermia + Sham, (3.4 ± 2.3%) female group (factorial ANOVA, $P < 0.001$). Treatment with hIAIPs under normothermic conditions (Normothermia + HI-IAIP, factorial ANOVA, $P = 0.019$), hypothermic conditions (Hypothermia + HI-PL, factorial ANOVA, $P < 0.001$), and treatment with hIAIPs during hypothermia (Hypothermia + HI-IAIP, factorial ANOVA, $P < 0.001$) significantly reduced hemispheric tissue volume atrophy from 52.6 ± 16.0% to 36.6 ± 17.4%, 28.5 ± 21.0%, and 23.5 ± 19.2%, respectively, resulting in reductions of the hemispheric tissue volume atrophy by 30.3 ± 33.0%, 45.7 ± 39.9%, and 55.2 ± 36.5%. Moreover, the hemispheric tissue volume atrophy was significantly lower in the Hypothermia + HI-IAIP (23.5 ± 19.2%) compared with the Normothermia + HI-IAIP (36.6 ± 17.4%) in the females (factorial ANOVA, $P = 0.033$). This finding suggests that hIAIP treatment during hypothermia is more effective than hIAIP treatment during normothermia in females. However, significant differences were not observed in hemispheric tissue loss between the Hypothermia + HI-PL and the Hypothermia + HI-IAIP females (factorial ANOVA, $P = 0.408$).

Taken together, these findings can be interpreted to suggest that treatment of neonatal rats with hIAIPs after exposure to HI is at least as effective as therapeutic hypothermia alone in the male (factorial ANOVA, $P = 0.262$) and female treated groups (factorial ANOVA, $P = 0.183$). Moreover, treatment with hIAIPs during hypothermia augments the effects of therapeutic hIAIPs alone in the group of female rats (factorial ANOVA, $P = 0.033$).

Residual exogenous hIAIP levels show inverse linear correlations with tissue volume atrophy after exposure to HI during normothermic and hypothermic conditions

Competitive ELISAs were conducted on the serum samples obtained at sacrifice on P14 and five days after the final dose of hIAIPs had been given to the rats in order to measure the residual exogenous hIAIPs and total circulating IAIPs levels. Total IAIPs comprise both the exogenous administered hIAIPs and endogenous rat IAIPs. Significant differences were not detected in the residual exogenous hIAIP protein concentrations in the rat serum of the male or female rats that had been treated with hIAIPs (Normothermia + Sham-IAIP, Normothermia + HI-IAIP, Hypothermia + Sham-IAIP, or Hypothermia + HI-IAIP) (factorial ANOVA, all $P > 0.05$; data not shown). Similarly, differences in the concentrations of total circulating IAIPs were also not observed in the male or female groups (factorial ANOVA, all $P > 0.05$; data not shown). These findings can be interpreted to suggest that exposure to hypothermia and HI may not significantly affect the metabolism of exogenous hIAIPs or circulating IAIPs in neonatal rats at least when they are measured 5 days after the final dose of hIAIPs.

On the other hand, significant inverse linear correlations (Fig. 5) were observed between the percentage of tissue volume atrophy and the residual exogenous serum concentrations of hIAIPs after exposure to HI and treatment with hIAIPs under normothermic conditions in the male

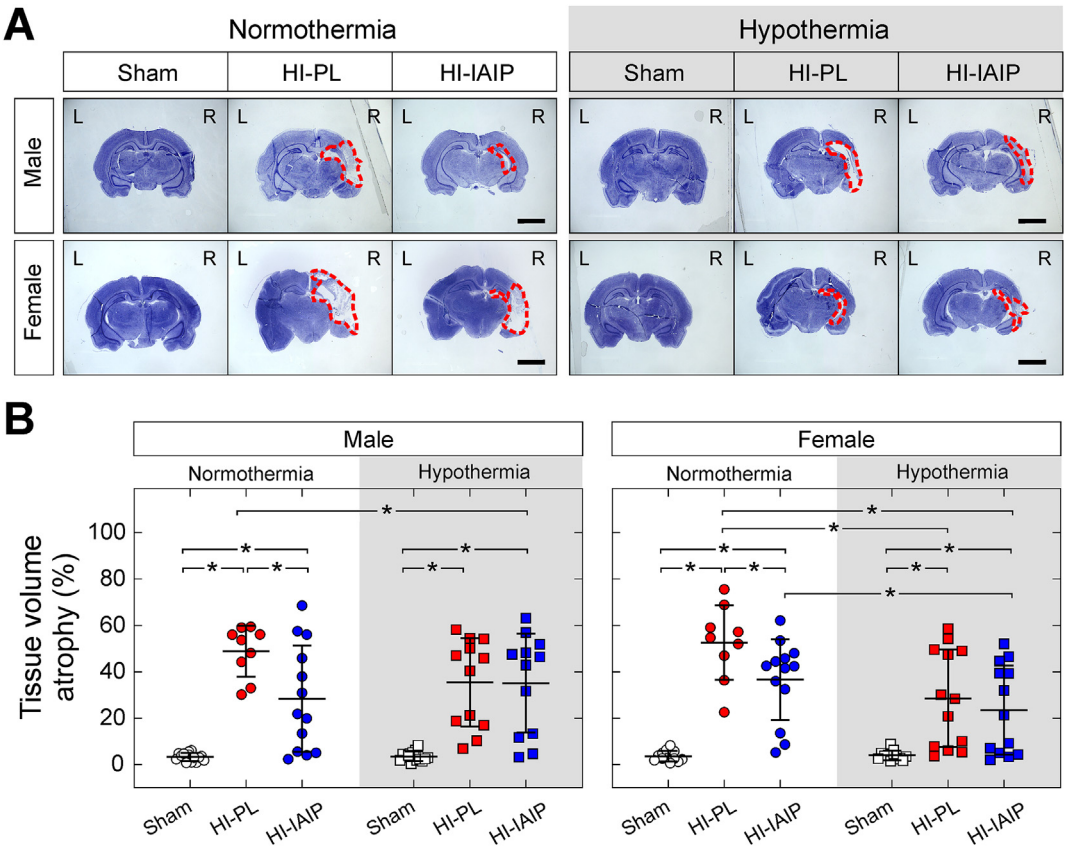

Fig. 4. Hemispheric tissue volume atrophy measured after placebo and hIAIP treatment under normothermia and hypothermia conditions. (A) Representative images of brain sections stained with cresyl violet 7 days after HI exposure are shown for males (top row) and females (bottom row). Scale bar = 3 mm. Normothermia treated: Areas not shaded; Hypothermia treated: shaded gray areas. (B) The percentage of hemispheric tissue volume atrophy plotted on the y-axis for the study groups on the x-axis. Group legends as for Fig. 3. Values are mean ± SD. *$P < 0.05$. Male: Normothermia + Sham n = 14, Normothermia + HI-PL n = 9, Normothermia + HI-IAIP n = 13; Female: Normothermia + Sham n = 12, Normothermia + HI-PL n = 9, Normothermia + HI-IAIP n = 13; Male: Hypothermia + Sham n = 15, Hypothermia + HI-PL n = 17, Hypothermia + HI-IAIP n = 14; Female: Hypothermia + Sham n = 12, Hypothermia + HI-PL n = 19, Hypothermia + HI-IAIP n = 14.

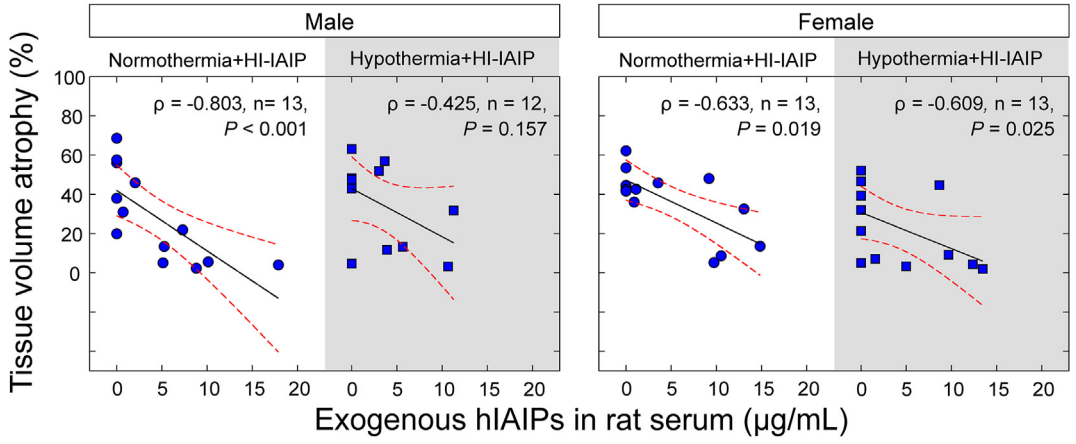

Fig. 5. Tissue volume atrophy plotted as a percentage (%) on the y-axis for the residual exogenous serum hIAIPs in rat serum (μg/mL) on the x-axis. Normothermic and hypothermic conditions as indicated in Fig. 2. Symbol legends as for Fig. 2. Linear regression model and the Spearman's rank test are shown. Male: Normothermia + HI-IAIP n = 13; Female: Normothermia + HI-IAIP n = 13; Male: Hypothermia + HI-IAIP n = 12, Female: Hypothermia + HI-IAIP n = 13 and Hypothermia + HI-IAIP n = 13. The Spearman's rank correlation coefficient (ρ) was used to assess these relationships. The results are indicated by a solid black regression line, and red dashed lines represent the 95% confidence intervals.

(Normothermia + HI-IAIP: $\rho = -0.803$, n = 13, $P < 0.001$) but not the hypothermic condition (Hypothermia + HI-IAIP: $\rho = -0.425$, n = 12, $P = 0.157$), and the normothermic and hypothermic conditions in the female (Normothermia + HI-IAIP: $\rho = -0.633$, n = 13, $P = 0.019$; Hypothermia + HI-IAIP: $\rho = -0.609$, n = 13, $P = 0.025$) groups. The inverse linear correlations suggest that changes in the residual exogenous hIAIPs protein levels were inversely related to the changes in tissue volume atrophy in rats exposed to HI brain injury even at P14 and 5 days after the last doses of hIAIPs were administered. The findings also suggest that higher residual concentrations of hIAIPs could have had a greater impact

on reductions in tissue volume atrophy in the female normothermic and hypothermic, and normothermic male exposed groups. Sham groups were not included in this analysis because they did not exhibit hemispheric tissue volume atrophy.

There was a significant difference in the intercepts of the hemispheric tissue volume atrophy as the dependent variable in the regression equation of the females because the adjusted means were significantly higher in the Normothermia–HI–IAIP than in the Hypothermia–HI–IAIP group (ANCOVA: Equal slopes model, F (1, 23) = 6.559, $P = 0.017$). The differences are substantial enough to rule out the possibility that they resulted from random sample variability. Therefore, this finding suggests that residual exogenous serum hIAIP levels were associated with less tissue volume atrophy during the hypothermic than the normothermic condition in the females. This finding strengthens the contention that hIAIPs were more effective during hypothermia than normothermia in female neonatal rats. However, similar differences were not observed in the male group (equal slopes model, F (1, 22) = 0.207, $P = 0.654$).

Inter-alpha Inhibitor Proteins and hypothermia after exposure to HI improve HI-related anxiety-like behavioral responses in male neonatal rats

A series of neurobehavioral tests were conducted to evaluate the impact of treatment with hypothermia and hIAIPs with and without exposure to hypothermia on functional outcomes after exposure to moderate HI.

The righting reflex was used to test early motor coordination in the neonatal rats after exposure to HI-related brain injury. Significant differences in the response times required for the rat pups to right themselves from a supine to a prone position with all four paws placed on a flat surface did not differ in the males or females between the Sham, Normothermia + HI-PL, Normothermia + HI-IAIP, Hypothermia + HI-PL or Hypothermia + HI-IAIP treated groups at 24, 48, or 72 h (Supplementary Table 1; factorial ANOVA, all $P > 0.05$).

Small open field tasks were used to determine general activity levels, gross locomotor activity, exploration behavior, and anxiety-like behaviors of the rats during the pre-weaning period on P13 and P14 after exposure to moderate HI to determine potentially beneficial effects of treatment with hypothermia and hIAIPs with and without exposure to hypothermia in the male and female neonatal rats. Dependent variables measured included the latency to the first entry into the peripheral zone (sec), the total distance traveled per observation time (cm/sec), the percentage of body postural changes (%), and mobility (mean of % changes in surface area). Statistically significant differences were not observed for the dependent variables in males or females between the Sham, Normothermia + HI-PL, Normothermia + HI-IAIP, Hypothermia + HI-PL or Hypothermia + HI-IAIP treated groups on P13 (Fig. 6 upper panel, Supplementary Tables 2 and 3; factorial ANOVA, all $P > 0.05$).

Nonetheless, the male rats in the Normothermia + HI-PL, group exhibited a greater latency for entry into the peripheral zone compared with the Normothermia + Sham. (factorial ANOVA, $P < 0.001$), Normothermia + HI-IAIP (factorial ANOVA, P = 0.002), Hypothermia + HI-PL, (factorial ANOVA, $P = 0.005$), and Hypothermia + HI-IAIP (factorial ANOVA, $P = 0.043$) groups on P14 (Fig. 6). Therefore, hypothermia, and hIAIPs with and without exposure to hypothermia appeared to attenuate the HI-related anxiety-like behavioral performance in male neonatal rats. However, significant differences were not observed between the Normothermia + HI-IAIP and Hypothermia + HI-PL (factorial ANOVA, P = 0.582), or the Normothermia + HI-IAIP and Hypothermia + HI-IAIP (factorial ANOVA, P = 0.383), or the Hypothermia + HI-PL and Hypothermia + HI-IAIP (factorial ANOVA, P = 0.683) treated males.

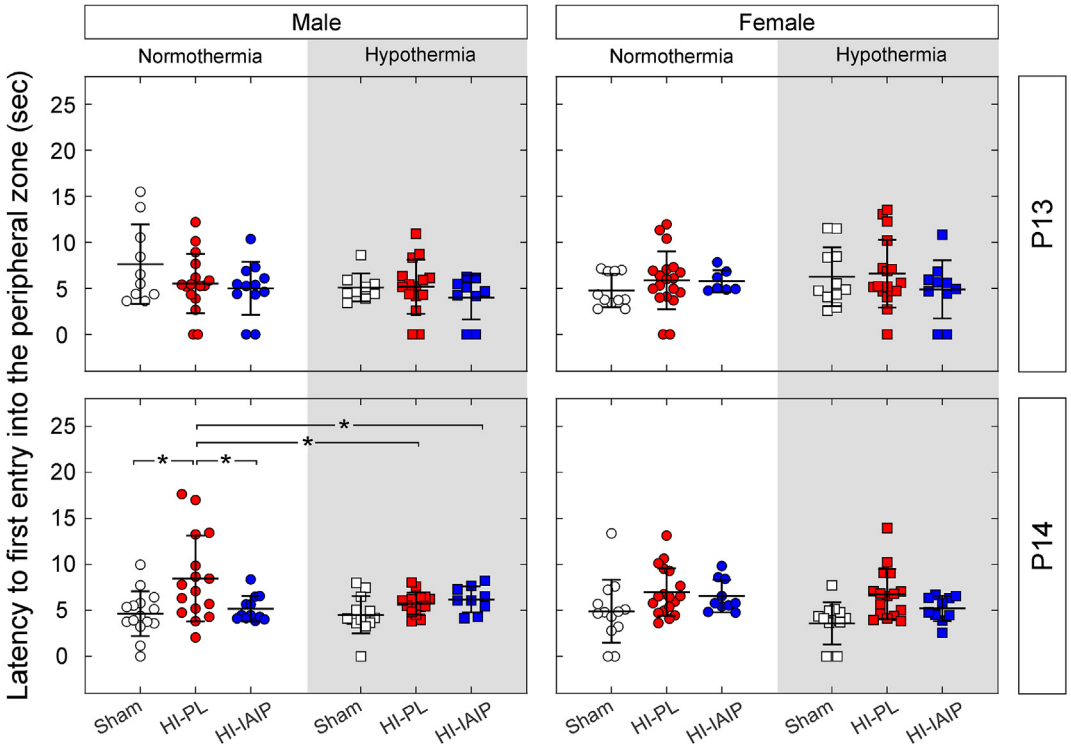

Fig. 6. The effect of hIAIPs and hypothermia with and without hIAIPs on anxiety-like behavioral outcomes in neonatal rats exposed to HI related brain injury. The experimental system utilized a small empty arena measuring 28 cm × 28 cm, which was divided into 16 squares comprising a peripheral zone (S1, S2, S3, S4, S5, S8, S9, S12, S13, S14, S15, and S16) and a central zone (S6, S7, S10, and S11); see Supplementary Fig. 1B. The latency to first enter the peripheral zone (sec) was measured on P13 (upper panel) and P14 (lower panel). Latency to the first entry into the peripheral zone in seconds plotted on the y-axis for the study groups on the x-axis. Normothermia and hypothermia conditions and symbol legends as designated for Fig. 2. Results summarized for males and females. Values are mean ± SD. *$P <$ 0.05. Male: Normothermia + Sham n = 15, Normothermia + HI-PL n = 16, Normothermia + HI-IAIP n = 12; Female: Normothermia + Sham n = 13, Normothermia + HI-PL = 18, Normothermia + HI-IAIP n = 8; Male: Hypothermia + Sham n = 13, Hypothermia + HI-PL = 15, Hypothermia + HI-IAIP n = 9; Female: Hypothermia + Sham n = 13, Hypothermia + HI-PL = 16, Hypothermia + HI-IAIP n = 12.

Therefore, we cannot discern from these studies a specific treatment with superior beneficial effects on this behavioral task. These differences were not observed in the female rats after HI-related brain injury (factorial ANOVA, all $P > 0.05$, Fig. 6 lower panel). Differences were also not observed in the measured dependent variables including the total distance traveled per observation time (cm/sec), the percentage of body postural changes (%), or mobility (mean of % changes in surface area) in the male or female groups treated with hypothermia and hIAIPs with or without exposure to hypothermia.

The negative geotaxis test was performed to evaluate vestibular reflexes, strength, and coordination in neonatal rats exposed to HI injury on P14. However, significant differences were not observed (Supplementary Table 4, factorial ANOVA, all $P > 0.05$) in distance traveled on the inclined wire mesh (cm), total cumulative time to reach the top of the incline (sec) or the percentage of postural changes involving extension or contraction among the study groups treated with hypothermia or hIAIPs with and without exposure to hypothermia. These results may suggest that the negative geotaxis test was not sensitive enough to distinguish behavioral differences among the different treatment groups in neonatal rats after exposure to HI injury [26].

Discussion

The primary objective of the current study was to determine the potential of treatment with hIAIPs and delayed hypothermia to attenuate neuropathological brain injury and possible behavioral deficits resulting from exposure to moderate HI in neonatal rats. Therapeutic hypothermia is the standard care used to treat newborns exposed to HIE [52,71–73]. However, this therapeutic modality is only partially protective, has a narrow therapeutic window, can only be used to treat full-term infants who were exposed to HIE, and cannot be used to treat premature infants exposed to HI-related brain injury [52,71–73]. Consequently, additional therapeutic modalities are needed to further reduce the effects of HI-related brain injury. Pharmacological agents are currently not available to attenuate brain injury in newborns [74]. We have previously shown that hIAIPs exert a wide range of neuroprotective effects in neonatal rodents after exposure to HI and inflammatory agents including reductions in neuropathological injury, neuronal loss, anti-inflammatory effects, attenuation in blood-brain barrier disruption, and beneficial behavioral effects [22,23,25–27,30–32,34,35]. The current study complements our previous work by suggesting that treatment with hIAIPs is at least as efficacious as therapeutic hypothermia to attenuate histopathological brain injury (Fig. 4), treatment with hypothermia appears to augment the beneficial effects of hIAIPs in female neonatal rats (Fig. 4), and that residual serum levels of hIAIPs, even 5 days after administration of the final dose of hIAIPs, correlate with decreased parenchymal brain atrophy during normothermia and hypothermia (Fig. 5).

Treatment of HIE with therapeutic hypothermia is recommended to be initiated within 6 h of birth with whole-body cooling at 33.5 ± 0.5 °C or selective head cooling at 34.5 ± 0.5 °C for 72 h followed by rewarming over 6 h in full-term infants [73,75]. Consequently, it is important to standardize the selected target temperature, time of initiation, duration of hypothermia, and severity of the HI injury for translational studies of hypothermia in rodents [76–78]. Hence, precise systematic rectal temperature monitoring is critical for rodent studies of hypothermia [79]. Although a target temperature of 32 °C has commonly been used in many rodent studies, we elected to carefully monitor the rectal temperature values in Sham control and HI-exposed neonatal rats between P5 to P14 in our laboratory before initiating the main study of hypothermic treatment with and without IAIPs (Fig. 2). The temperatures were measured before surgery, before hypoxia, and after HI in P7 rat pups. Rectal temperatures of the male and female rats were 1–3 °C lower at 1 h, 2 h, and 6 h after exposure to HI compared with those not exposed to HI. These findings are consistent with previous work that reported lower rectal temperatures after HI [79,80]. The decision to use the 30 °C was based upon our monitoring of the temperatures in the neonatal rats in our laboratory along with the important consideration of precise systematic rectal temperature monitoring in rodent studies of hypothermia [79], as well as the potential for variations in the baseline and fluctuations in body temperature during the HI procedures in different laboratory settings. The 30 °C value was 1.8–3.8 °C below the average rectal temperature (32.8 ± 1.0 °C) observed at the one-to 6-h time points after exposure of neonatal rats to HI (Fig. 2).

Hypothermia was initiated 1.5 h after the termination of HI because therapeutic hypothermia is often delayed after exposure to HIE in infants [51,52] and the potential exists that protein components of hIAIPs might be more effective in the normothermic than hypothermic temperature ranges based upon findings of modifications in other proteins during hypothermia [81,82]. The 3-h treatment with hypothermia was based upon previous studies in neonatal rats [49,50]. Although we have demonstrated that hIAIPs exert neuroprotective effects even after exposure to severe HI [27], moderate exposure (90 min of 8% oxygen and balanced nitrogen) was utilized in the current study because previous work has shown that hypothermia may not be protective after exposure to severe HI in neonatal rats [83,84].

Body weight gain in the Sham treated group was greater over time (Fig. 3) compared with those of the HI-PL and HI-IAIP groups, analogous to our previous findings [23,25,27]. However, exposure to hIAIPs and hypothermia with and without treatment with hIAIPs did not modify the pattern of weight gain or brain weights determined on P14. These findings differ from our previous work showing improved body weight gain over time and decreased injury-related reductions in brain weights after treatment with hIAIPs [23,25,27]. These discrepancies could be attributed impart to the age over which the observations were obtained up to P10 or P42 in our former studies and P7 to P14 in the current study [23,25,27]. The observation period of the current study corresponds to approximately 6 months (pre-weaning) in human infants and represents a more advanced stage of development compared with P10 [36,85–87]. Furthermore, multiple doses of hIAIPs were given when body weights and brain weights were examined up to P42 [25]. Moreover, the Rice-Vannucci model of HI is well known to exhibit considerable variability in outcomes that could also contribute to differences in the results [83,88–90]. Nonetheless, we are not able to discern the origin of differences between the findings in our current and former studies [23,25,27].

Histopathological analysis is a widely accepted approach to quantify brain tissue injury [91]. Findings in our previous work suggested that quantified parenchymal volume loss is more accurate than semi-quantitative histopathological examination [23,25]. Consistent with our previous findings, treatment with hIAIPs during normothermic conditions significantly reduced (Fig. 4) the extent of histopathological hemispheric tissue volume loss in male and female groups after HI exposure [23,25,27]. Although hypothermia reduced tissue volume atrophy in the females by 45.7% compared to the normothermic group, it did not reduce tissue atrophy significantly in the male rats. These findings are consistent with previous work showing more significant hypothermic neuroprotection in female than male rodents [45,92–94]. On the other hand, hIAIPs reduced tissue atrophy in males by 41.9% and in females by 30.3% compared with the placebo-treated group exposed to HI. Therefore, it appears that treatment with hIAIPs is at least as effective as therapeutic hypothermia in the female group, and potentially more effective in the males than hypothermia. Even though hIAIPs did not appear to augment the neuroprotective effects of hypothermia, treatment with hypothermia did appear to enhance the effects of hIAIPs in female neonatal rats by 35.7%. This finding suggests that there could be the possibility for treatment with hIAIPs to supplement the protection provided by hypothermia.

Treatment with hIAIPs has multiple potential properties that could render them beneficial adjuncts to therapeutic hypothermia including their anti-inflammatory effects by attenuating the HI-related upregulation of astrocytes, microglia, infiltrating neutrophils, and MMP9-positive-myeloperoxidase cells, reducing apoptosis, modifying cytokine levels, binding to the high mobility group box-1 (HMGB1) protein,

attenuating inflammatory disruption of the blood-brain barrier, and regulating the shape of neutrophils, facilitating neutrophilic passage through capillaries, and suppressing the release of reactive oxygen species [23,30,34,35,95,96]. Nonetheless, treatment with hIAIPs 15 min after exposure to HI and 1.5 h before treatment with hypothermia did not definitively serve as a therapeutic adjunct to hypothermia. Consequently, it might be more beneficial to delay treatment with hIAIPs until after completion of the hypothermic exposure during the late secondary or tertiary phase after HI, when cell loss/injury and inflammation persist for weeks to months after HI and/or to consider additional doses of hIAIPs [25,97–99].

Serum hIAIP levels were measured by a competitive ELISA on P14, seven days after exposure to HI, and five days after the last dose of hIAIPs was given. Differences were not observed in residual exogenous hIAIP protein concentrations or in the total circulating IAIP levels in rat serum among the hIAIP-treated study groups with or without exposure to hypothermia. Considering that the half-life of circulating hIAIPs is 23.1 h and 16.2 h in HI-exposed male and female neonatal rats, respectively [40], it is not surprising that differences were not detected in residual hIAIP levels among the study groups. Given the lack of differences in the residual hIAIPs among the groups, it was somewhat remarkable that the residual hIAIP levels demonstrated inverse correlations with the percent tissue volume atrophy, suggesting that even these low residual levels were associated with decreased volume atrophy in the female rats under Normothermia + HI and Hypothermia + HI conditions, and under the Normothermia + HI condition in the male rats. In addition, the findings that residual exogenous serum hIAIP levels were associated with less volume loss during hypothermia than normothermia further supports the contention that hypothermia could enhance the effects of hIAIPs to attenuate brain injury in females.

Examination of neurobehavioral outcomes is important because prolonged motor, cognitive, and neurosensory impairment are common in children even after having received treatment with therapeutic hypothermia for HIE as newborns [100,101]. The Rice-Vannucci model has been widely used to examine neurodevelopmental outcomes after exposure to HI-related brain injury in rodents [22,31,32,87,102–106]. The neurobehavioral tests examined in the current study included the righting reflex test to evaluate motor coordination, the small open field test to examine early general locomotor activity and anxiety-like behavior, and the negative geotaxis test to evaluate the vestibular reflex, strength, and coordination in the neonatal rats after exposure to HI. We have previously shown that treatment with hIAIPs improves neurodevelopmental outcomes after exposure to HI, including early improvement in motor outcomes [25], improvement in Morris water maze spatial and non-spatial learning [22], amelioration of complex auditory discrimination deficits [32], and as an adjunct to enhance early behavioral learning improvement in working memory [31]. However, treatment with hIAIPs with and without exposure to hypothermia did not improve the latency response times in the righting reflex test (Supplementary Table 1), some components of the open field tests (Supplementary Tables 2 and 3) or of the negative geotaxis tests (Supplementary Table 4) in the current study. Discrepancies between our earlier work and the current study could in part relate to the duration of exposure to the hypoxic component of HI because the exposure to hypoxia was 90 min in 8% oxygen in the current study compared with 120 min in 8% oxygen in our previous work [22,31,32]. In general, greater neurobehavioral deficits have been reported in neonatal rats after exposure to longer durations of hypoxia [22,32,106]. Other factors that could contribute to the differences include the age at which the behavioral studies were performed and/or more frequent administration of hIAIPs [25].

We measured the latency time (sec.) until the first entry into the peripheral zone as a variable to evaluate anxiety-like behavior in the neonatal rats [63]. The more time spent in the center is suggestive of less anxiety [107]. We found that PL-treated normothermic-HI exposed male rats displayed less anxiety-like behavior when exposed to an unfamiliar environment because they exhibited a greater latency to enter the peripheral zone compared with the Normothermia + Sham treated rats. Consequently, their time to reach the peripheral zone in the open field test was longer than the Sham-treated rats, suggestive of less anxiety-like behavior after HI injury [108,109]. In contrast, hypothermia and hIAIPs with and without exposure to hypothermia attenuated the HI-related anxiety-like behaviors in male neonatal rats. Although beneficial effects of hypothermia and hIAIPs with or without exposure to hypothermia were not detected in the small open field test on P13, anxiety-like behaviors were improved on P14 in the males but not females after exposure to hIAIPs and hIAIPs with or without exposure to hypothermia (Fig. 6).

Significant developmental changes can occur between P13 and P14 in neonatal rats. These neurodevelopmental changes could be accompanied by alterations in behavior [36,110]. To the best of our knowledge, the current study is one of a few studies to show sex-dependent effects on anxiety-like behaviors after treatment with a potential pharmacological neuroprotective agent with and without exposure to hypothermia after HI in neonatal rats. Nonetheless the use of the open field as a single measure to evaluate anxiety-like behavior has several limitations, including the severity of the brain damage, stage of development, stress responses of open field exposure itself, ability to comprehend risk-taking behaviors after HI, and/or sexual dimorphism in neonatal HI. Moreover, we cannot be certain that more time in the central zone in fact denotes less anxiety in neonatal rats after HI. Future studies are required to standardize the experimental protocol and integrate the open field test with other measures such as the elevated plus maze, light-dark test, and ultrasonic vocalization analysis to elucidate a comprehensive understanding of anxiety-like behaviors after HI with and without drug and hypothermia treatments.

Even though individual studies have reported variable findings in sex-related outcomes after neuroprotective strategies for HI, the overall tendency suggests more favorable responses in female than male neonatal rodents [45,106,111]. Consistent with these findings, hypothermia improved neuropathological outcomes in the females but not in the males, whereas hIAIPs with and without exposure to hypothermia improved the neuropathological outcomes in both the male and female neonatal rats (Fig. 6). Furthermore, hypothermia appeared to enhance the neuroprotective effects of hIAIPs in the female, but not male rats after exposure to brain injury. The discrepancy in the treatment effects between neuropathological and behavioral outcomes emphasizes the importance of considering sex as a significant variable regarding the response of the brain to HI injury and neuroprotective strategies. Consequently, males and females might benefit from differential neuroprotective strategies [94,112,113]. Moreover, we have previously reported differential responses between male and female pups in response to treatment with hIAIPs after exposure to HI [23,25,27]. Nonetheless, although differential effects in males and females have been observed in neonatal rats with and without exposure to neuroprotective strategies for HI-related brain injury [37,70,94,106,114,115], post-hoc analysis of the whole-body therapeutic National Institute of Child Health and Human Development Neonatal Research Network Induced Hypothermia trial by sex suggests that the outcomes did not differ between the male and female infants [116,117]. Similarly, studies in larger animals including fetal sheep and pigs do not support differential effects by sex like those observed in rodents [37,70,94,106,114,115].

There are several opportunities for future research and limitations of our study. Although we carefully selected the temperature for the hypothermia studies based upon observed values in our laboratory after HI, there are other important aspects of treatment with hypothermia that need to be considered. Previous work has suggested that hypothermic therapy for 5 h was effective to treat neonatal rats exposed to moderate HI-related brain injury [83]. Therefore, we cannot rule out the possibility that the selection of a target brain temperature of 30 °C for the 3 h duration could have affected the outcomes in the animals treated with hypothermia alone and a longer duration of hypothermia at a slightly higher temperature might have enhanced the effects of hypothermia and

the potential for adjunctive effects of hIAIPs to hypothermia. We will consider using a target temperature of 32 °C for a duration of 5 h in our future studies [118]. In addition, more frequent administration of hIAIPs could potentially improve their efficacy. Finally, delaying hIAIP treatment until after completion of hypothermic exposure during the late secondary or early tertiary phases of recovery from HI-related brain injury could potentially prove more efficacious [98].

Human IAIPs provide neuroprotection from HI-related brain injury that is similar to the protection afforded by hypothermia and the neuroprotective effects of hIAIPs and hypothermia exhibit sex-related differential effects. In addition, hypothermia increased the protective effects of hIAIP treatment on hemispheric tissue loss in females. These findings, in conjunction with our previous studies [22,23,27,30], suggest that hIAIPs potentially represent an important neuroprotective strategy and a potential alternative to hypothermia treatment for neonatal HI brain injury, especially when treatment with hypothermia is not feasible.

Author Contributions

Xiaodi F. Chen designed the experiments, performed the in vivo neonatal rat experiments and the graphical and statistical analyses, and wrote the first draft of the manuscript.

Yuqi Wu, and Boram Kim performed the animal experiments, conducted the immunohistochemical studies, microscopy observation, and quantification analyses.

Kevin Nguyen and Ainuo Chen performed the behavioral analyses and revised the manuscript.

Yow-Pin Lim, Joseph Qiu, and Andre R. Santoso produced the IAIPs, helped to design the studies, and revised the manuscript.

Clemence Disdier helped to design the hypothermic experiments and revised the manuscript.

Barbara S. Stonestreet supervised the project, designed the experiments, and revised the manuscript.

All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Barbara S. Stonestreet reports financial support, administrative support, article publishing charges, and equipment, drugs, or supplies were provided by Women & Infants Hospital of Rhode Island. Barbara S Stonestreet reports a relationship with Women & Infants Hospital of Rhode Island that includes: employment. Barbara S Stonestreet has patent #9,572,872 issued to Y.-P. Lim, MD, PhD. None If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neurot.2024.e00341.

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