Published in final edited form as:

Neurotox Res. 2013 August ; 24(2): 176–190. doi:10.1007/s12640-013-9376-5.

PUTATIVE AGMATINASE INHIBITOR FOR HYPOXIC-ISCHEMIC NEW BORN BRAIN DAMAGE

John E. Piletz^{1,3}, Stephanie Klenotich^{1,2}, Ken S. Lee³, Qian Long Zhu¹, Edward Valente⁴, Michael A. Collins⁵, Vyvyca Jones³, Soeb Nam Lee⁶, and Feng Yangzheng⁷

¹Department of Psychiatry, Loyola University Stritch School of Medicine, Maywood, Illinois

²Committee on Neurobiology, University of Chicago, Chicago, Illinois

³Department of Chemistry, Jackson State University, Jackson, Mississippi

⁴Department of Chemistry, Mississippi College, Clinton, Mississippi

⁵Department of Pharmacology, Loyola University Stritch School of Medicine, Maywood, Illinois

⁶Department of Anatomy, College of Medicine, Konyang University, Daejeon, South Korea

⁷Department of Pediatrics, University of Mississippi Medical Center, Jackson, Mississippi

Abstract

Agmatine is an endogenous brain metabolite, decarboxylated arginine, which has neuroprotective properties when injected intraperitoneally (i.p.) into rat pups following hypoxic-ischemia. A previous screen for compounds based on rat brain lysates containing agmatinase with assistance from computational chemistry, led to piperazine-1-carboxamidine as a putative agmatinase inhibitor. Herein, the neuroprotective properties of piperazine-1-carboxamidine are described both *in vitro* and *in vivo*. Organotypic entorhinal-hippocampal slices were firstly prepared from seven-day-old rat pups and exposed *in vitro* to atmospheric oxygen depletion for 3 hrs. Upon reoxygenation the slices were treated with piperazine-1-carboxamidine or agmatine (50 µg/ml agents), or saline, and 15 hours later propidium iodine was used to stain. Piperazine-1-carboxamidine or agmatine produced substantial *in vitro* protection compared to post-reoxygenated saline-treated controls. An *in vivo* model involved surgical right carotid ligation followed by exposure to hypoxic ischemia (8% oxygen) for 2.5 hours. Piperazine-1-carboxamidine at 50 mg/kg i.p. was given 15 minutes post-reoxygenation and continued twice daily for 3 days. Cortical agmatine levels were elevated (+28.5%) following piperazine-1-carboxamidine treatment with no change in arginine or its other major metabolites. Histological staining with anti-Neun monoclonal antibody also revealed neuroprotection of CA1–3 layers of the hippocampus. Until endpoint at 22 days of age, no adverse events were observed in treated pups' body weights, rectal temperatures, or prompted ambulation. Piperazine-1-carboxamidine therefore appears to be a neuroprotective agent of a new category, agmatinase inhibitor.

Keywords

Agmatine; neuroprotection; agmatinase; hypoxia; ischemia

INTRODUCTION

Research on arginine metabolism in mammals has focused predominantly on two biochemical pathways: (1) the arginase-ornithine decarboxylase (ODC) pathway and (2) the nitric oxide synthase (NOS) pathway (Salimuddin et al., 1999, Morris, 2009). Both are well-characterized in the brain. A serendipitous discovery made in 1994 while screening bovine brain extracts for the endogenous clonidine-displacing substance (an antihypertensive agent), led Donald Reis and coworkers to discover a third metabolic pathway (Li et al., 1994). Today, it is known that this third pathway derives from arginine decarboxylase (ADC) to form 4-(aminobutyl)guanidine, also known as agmatine. Mammalian ADC has been described in many cell types (Li et al., 1995, Horyn et al., 2005), its gene has been cloned (Zhu et al., 2004) and its mRNA has been quantified across brain regions (Iyo et al., 2006, Molderings and Haenisch, 2012). Of regulatory interest is a specific ureohydrolase, agmatinase, which has been found in synaptic structures (Madaï et al., 2011). The enzymatic activity of agmatinase across brain regions has been linked to the control of agmatine levels (Sastre et al., 1996). Underlining the importance of agmatinase is a recent study showing massive over-expression of agmatinase protein in postmortem hippocampus sections from suicide victims having documented mood disorders (Bernstein et al., 2011, Bernstein et al., 2012).

Other studies have shown agmatine to be neuroprotective (Gilad et al., 1996, Yu et al., 2000, Kim et al., 2004). Whether studies involved treatment intraperitoneally (i.p.), subcutaneously (s.c.), or intracerebroventricularly (i.c.v.), the ameliorative effects of injected agmatine have appeared robust in animal models. The positive results even extend to neurodegenerative disease (Zarifkar et al., 2010), neuropathic pain (Paszczuk et al., 2007), addiction-related behaviors (Su et al., 2008), antidepressant-like behaviors (Zomkowski et al., 2002), and seizure thresholds (Demehri et al., 2003). Initially, the effects of agmatine were suggested to bear similarity to the antihypertensive agent, clonidine, that is via I₁ imidazoline receptors (Yang et al., 2005). However, agmatine has been found to more potently inhibit the neuroresilience NMDA receptor (NMDAR) and does so through a non-polyamine binding site (Askalany et al., 2005). Besides this, Demady and coworkers (Demady et al., 2001) have emphasized agmatine's noncompetitive inactivation of neuronal NOS (nNOS) in the micromolar range. Agmatine has the additional ability to down-regulate proinflammatory inducible NOS (iNOS) (Regunathan and Piletz, 2003) with little effect on endothelial NOS (eNOS) or blood pressure (Galea et al., 1996). Furthermore, clinical trials of agmatine-sulfate have been appearing recently; the first used agmatine as a dietary supplement – a nutraceutical – to enhance the treatment of sciatica with other pain therapies (Keynan et al., 2010). That double-blind study revealed agmatine's superiority over placebo. The second clinical trial focused on major depression in an open-label manner whereby oral agmatine was revealed as an effective antidepressant (Shopsin, 2012). Such treatment studies, however, remain difficult to extrapolate to endogenous agmatine because ODC largely outpaces ADC in terms of absolute enzyme activity and therefore some agmatine could be metabolized via ODC (Coleman et al., 2004). Thus, the precise role for ADC and agmatinase *in vivo* awaits pharmacological tools such as a selective agmatinase inhibitor.

Mounting evidence also supports agmatine as a neurotransmitter or co-transmitter (Reis and Regunathan, 1999). Radiolabeled agmatine was first shown to be uptaken and packaged into synaptic vesicles and released upon membrane depolarization (Goracke-Postle et al., 2006, Goracke-Postle et al., 2007). Immunolabeled agmatine was co-localized with vasopressin and oxytocin in neurons of the rat hypothalamic paraventricular and supraoptic nuclei (Gorbatyuk et al., 2001). Immunolabeled agmatine was also co-localized with glutamate in CA1–CA3 hippocampal pyramidal cell bodies (Seo et al., 2011). Another *in vivo* monitoring study of hippocampal agmatine by microdialysis and capillary electrophoresis proved the

calcium and impulse dependence on increased extracellular agmatine concentration (Betancourt et al., 2012).

By 2003, we had reported (Huang et al., 2003) a preliminary screening study of compounds for the cell-free inhibition of the agmatinase activity found in rat brain lysates. From those findings we generated a series of synthetic analogues and modeled them computationally by a Quantitative Structure Activity Relationship (QSAR) program. The QSAR pointed to aminopropylguanidine as well as to piperazine-1-carboxamidine (PZC) as the leading candidates (Figure 1). Additional computer modeling suggested that PZC should cross the blood-brain-barrier better than aminopropylguanidine (or actually, agmatine). Other studies suggested that derivatives of PZC might be even better selective agmatinase inhibitors than PZC (Halaris and Piletz, 2007, Piletz et al., 2008). Finding no previous neurobiological literature on PZC, we therefore undertook this exploratory research.

In vitro as well *in vivo* studies of PZC are presented using 7-day-old rat pups in hypoxia-ischemia (HI) models (Rice et al., 1981). Seven-day-old rat pups were chosen for study because their blood brain barrier is not fully developed and thus PZC enters freely. We designed our studies around three hypotheses: (1) PZC should be directly neuroprotective, which was assessed by application to brain hippocampal slices after challenging *in vitro* with a brief anoxia + low glucose condition. (2) The second hypothesis stated that injection of PZC *in vivo*, post-HI, should protect intact hippocampal neurons and allow for a degree of normal brain development thereafter. This effect was expected because agmatine, itself, can spare neurons post-HI *in vivo* (Feng et al., 2002). Neuron-specific staining with anti-Neun was used to assess the hippocampal sections from HI pups with or without PZC treatment. (3) Our third hypothesis was that pups injected with PZC would emerge with higher concentrations of agmatine in both cerebral hemispheres which would be above and beyond any changes that emerged due to the hypoxicischemia itself. To test this, agmatine and other arginine metabolites were measured in the left and right cortices of the post-HI pups. The results largely support PZC as a selective agmatinase inhibitor.

Methods

Synthesis and Purification of Piperazine-1-Carboxamidine (PZC)

PZC was prepared following chemical synthesis in the laboratory of coauthor Ken Lee according to the reaction scheme of Figure 1A. This was modified from Calis and colleagues (Calis et al., 1994). The starting materials, piperazine and s-methylisothiourea hemisulfate (2:1 ratio) were combined in water (Sigma-Aldrich Chemicals). The mixture was refluxed at 55°C for 7 hrs while nitrogen purged the byproduct, methylthiol gas, trapped in a flask containing sodium hydroxide in ethanol. Water was removed under reduced pressure with a rotary evaporator. The product, a white solid, was removed from the reaction flask with acetone, rinsed with methanol to remove impurities, and re-crystallized in water for use in experiments. Various analytical procedures were used to ensure the product's identity and purity as PZC: (1) ¹H NMR of product (Figure 1B) based on D₂O, ppm, δ 3.01 (t, 4H, -CH₂-, J=5.1Hz) and 3.52 (t, 4H, -CH₂-, J=5.1Hz). (2) ¹³C NMR of product (Figure 1C) based on D₂O, ppm, δ 46.2 (-CH₂-), 47.9 (-CH₂-), δ 159.3 (guanidine carbon). (3) Liquid chromatography-tandem mass spectroscopy (LC/MS/MS) analysis based on [MH⁺] = 129.0 (protonated PZC), 112 (less NH₃), 87 (protonated piperazine, base peak) (Figure 1D). (4) X-ray diffraction crystallographic properties and data were collected using MoK α radiation and the charge-coupled area detector (CCD) detector on an Oxford Diffraction Systems Gemini diffractometer at 293(2)K. A preliminary set of cell constants was calculated from reflections observed on three sets of 5 frames which were oriented approximately in mutually orthogonal directions of reciprocal space. This initial orientation matrix contained 60 reflections. Data collection was carried out using MoK α radiation (graphite

monochromator) with 7 runs consisting of 308 frames with a frame time of 40 sec, and a crystal-to-CCD distance of 50.000 mm. A randomly oriented region of reciprocal space was surveyed to the extent of one sphere and to a resolution of 0.70 Å. The runs were collected with omega scans of width 1.0 degrees and at detector positions of 28.468 and -29.874 degrees in 2 θ . The intensity data were corrected for absorption and decay. Final cell constants were calculated from 1063 strong reflections from the actual data collection after integration. The structure was solved using SHELXS-86 and refined using SHELXL computer programs. The space group Pnma (#62) was determined based on reflection extinctions and successful refinement. A direct-methods solution was calculated which provided most non-hydrogen atoms from the E-map. Full-matrix least squares/ difference Fourier cycles were performed which located the remaining non-hydrogen atoms. All non-hydrogen atoms were refined with anisotropic displacement parameters. All hydrogen atoms were placed in ideal positions and refined as riding atoms with relative isotropic displacement parameters. The final full-matrix least-squares refinement converged to $R1 = 0.0806$ and $wR2 = 0.2310$ (on F^2 , $F^2 > 2\sigma(F^2)$). The final x-ray crystal structure of the sulfate salt as H⁺ cation is shown in Figure 1E. After our research was completed, piperazine-1-carboxamide hemisulfate became commercially available (Oakwood Products, West Columbia SC), but our study did not use it (the hemisulfate form).

Animals

Timed-pregnant Sprague-Dawley rats were ordered from Harlan Inc. (Indianapolis IN). They arrived at our AALAC-approved animal facility usually two days prior to delivery (i.e., 9 days prior to starting the formal experiments). Housing was one pregnant dam per cage with standard laboratory chow and water provided *ad libitum*. The room was temperature-controlled (25° C) with a 12-hour day-night cycle. Delivery thereafter occurred normally. Each litter was soon monitored by weighing the pups and examining for motility prior to, during, and after experimentation, continuing until 22 days after birth (when considered weaned). At 7-days of age we began our experiments because the blood brain barrier is not yet fully developed. Animal husbandry was in accordance with the guidelines published in the NIH *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 85-23) and principles in the “Guidelines for the Use of Animals in Neuroscience Research” by the Society for Neuroscience.

Organotypic Brain Slice Cultures

Sprague-Dawley rat pups (7 days old) were used to prepare static hippocampal/entorhinal cortex (HEC) slice cultures (Brown et al., 2009). First, the pups were cold-anesthetized and quickly decapitated. The HEC complex was then removed for transverse slicing (350 μ), with slices placed on Millipore 0.4 μ Millicel tissue culture inserts in 6-well Falcon plates with covers. Each well containing 3–4 slices, and sufficient wells were obtained from one litter for a complete set of treatment groups. Standard culture was performed in atmosphere of 95% O₂/5% CO₂ at 37°C on MEM media (Gibco/Invitrogen, 11575-032) containing 25% horse serum (Gibco/Invitrogen, 16050-122), 25% Hanks buffer, 20 mM HEPES and 6.5 mg/ml glucose. The media was changed every 3 days until experimentation.

After 10–15 days in culture, the slices were reexamined by phase contrast microscopy and had to display characteristic hippocampal architecture to be considered for subsequent experiments. Step-1 replaced the medium with low glucose (0.1 $\times$ normal concentration: i.e., 0.65 mg/ml). One plate with 4 wells was placed in a sealed chamber and made hypoxic by substituting 95% O₂/5% CO₂ with 95% argon/5% CO₂ gas at 37°C. A comparator plate with 4 wells remained normoxic at 37°C. Both conditions were incubated for 3 hrs. The medium was then replaced with fresh normoxic medium of normal glucose concentration (6.5 mg/ml) that contained either: (1) no compounds, (2) 50 μ g/ml PZC, or (3) 50 μ g/ml agmatine.

After such manipulations, the slices were re-incubated in the normoxic incubator at 37°C for another 15 hours. By then, degenerating neurons could be detected in HEC slices by staining with propidium iodine (Sigma Chemicals, St. Louis MO), added during the last hour of incubation (5 µg/ml media). Propidium iodine is well-documented to detect and quantify degenerating neurons in hippocampal slice cultures (Noraberg et al., 1999). Measurement of neurodegeneration with propidium iodine is all that is necessary in organotypic slice culture studies with neurotoxins, and with it, neurodamage is readily quantified. Uptake of propidium iodine into each slice was assessed by capturing 8–12 sec exposures with a DS-5M Nikon color camera attached to an Eclipse TS100 inverted microscope with an Epi-fluorescence attachment. Using Image J version 1.36 (NIH, Bethesda MD), the entire slice was outlined and the intensity of the entire slice area that displayed red fluorescence was quantified and used in statistical analyses.

Hypoxic-Ischemia Model, In Vivo

Except for a few modifications noted below, the *in vivo* hypoxic-ischemia (HI) model was prepared exactly as described in our first study of agmatine neuroprotection (Rice et al., 1981, Feng et al., 2002). To parallel the aforementioned *in vitro* slices, the protocol began with 7-day-old Sprague-Dawley rat pups. Pups were removed from their dam one by one to a sealed plexiglass chamber for 1 min with isoflurane gas flowing with O₂ at 5L/min. Once unconscious, each pup was fitted to a nozzle device flowing with isoflurane (setting 3 mixture flowing), and placed atop a sterile surgical pad (final outflow of isoflurane was charcoal filtered before exhaust hood: modification #1). Sterile surgery ensued. The right carotid artery was doubly ligated (double-knots) to ensure complete lack of blood flow and it was broken between the knots. The wound was sutured with nylon (Ethicon 5–0, 1.0 metric: sterile). Modification #2 was nylon rather than cotton suture. We noted the duration of surgery varied from 4–14 mins per pup. A brief recovery period followed under normal atmosphere in a covered chamber fitted with a 37°C heating pad (modification #3 was the heating pad). Once awake, the pups remained without their dam on the heating pad until the entire litter had awakened from anesthesia. Only *en masse* were the pups returned to the dam. Some 2–3 hrs later, fully mobile and now suckled, they were placed into pre-warmed (37° C), dimly lit, brown glass jars which were the hypoxia chambers. The jars were sealed except for pass-through humidified gas (8% oxygen: balance N₂) monitored at 4 cc/min with a regulator on a CGA-590 tank. Each jar contained no more than 6 pups. For large litter sizes there were thus 3 jars interconnected to a common hypoxia gas flow. The jars were floating in a 37° C water bath during the entire hypoxia period, which lasted 2.5 hrs, keeping the pups warm - but hypoxic. Upon opening, the pups were placed on the 37°C heating pad (modification #4 was warmed recovery). Shortly thereafter, injections occurred of the test compounds listed below. A specific ordering was followed that staggered so that each jar yielded the same overall duration time (re-oxygenation to injection). The protocol specified maximum duration from reoxygenation until injection of no more than 15 min. Then, shortly after injection, each pup was weighed, ear-marked, and monitored for rectal temperatures with a 1.7 mm o.d. thermistor (Vestavia Scientific, Vestavia Hills, AL). The pups, in home cage with their dam were then returned to the animal room for the next 15 hrs (overnight). Before subsequent procedures (per schedule below) the pups would always be taken from the dam to a nearby covered cage for the procedure. They were injected per the regimen, monitored for body weights, assessed for rectal temperatures, and visually inspected for signs after a single tail pinch. Each time, as soon as these steps were accomplished, they went promptly to the nursing dam.

Injections In Vivo

Stock solutions of PZC (synthesized powder) and agmatine-sulfate (AGM; Sigma-Aldrich) were prepared at 30 mg/40 ml sterile saline, and stored in 4 ml aliquots at –20° C. A single

aliquot was thawed for each experiment (i.e., one tube sufficient per one litter). Dosing of PZC was based on calculated equivalency to the in vitro study and was also the same as previously optimized for AGM in vivo (Feng et al., 2002). This dose, 100 mg/kg/day i.p., was given as 50 mg/kg b.i.d. for three days. The injection volume was 0.1 ml/20 g body weight. Depending on litter size, a typical experiment consisted of the following groups: 4–6 pups injected with active substance (usually PZC; sometimes AGM), 4–6 pups injected with sterile saline, and 1 sham-operated pup injected with sterile saline. Sham pups had undergone right carotid-exposing surgery (lasting exactly 10 min under anesthesia), but had not been ligated. The first injection occurred just minutes after HI with subsequent injections occurring for 2.5 more days b.i.d., at 8:30 hours and 20:30 hours each day. The last injection occurred at 8:30 hours on day-3 post-HI. By 12:30 hrs on day-3, Endpoint #1 occurred. A subset from each litter was taken beyond, until Endpoint #2 (described below).

Biochemical Processing

Before processing the brains, they were placed on ice-cold Petri dishes and photographed. Firstly, the cerebral hemispheres were dissected to be free without meninges and weighed for determination of the right:left ratio. Each cerebral hemisphere was wrapped in labeled aluminum foil, frozen on dry ice, and stored at -80°C . Various types of biochemical analyses later proceeded from the thawed cortices, each beginning by dilution in ice-cold four-volumes (by weight) of freshly prepared buffer (20 mM Tris-HCl, 20 mM EDTA, pH 7.4) which contained the following protease inhibitors: bestatin (0.0138 mg/ml), aprotinin (0.00345 mg/mL), PMSF (fresh 0.348 mg/ml), leupeptin (0.0238 mg/mL), and pepstatin (0.001 mg/ml). For HPLC purposes, an internal standard, 1-(3-aminopropyl) 2-pipecoline (APP: Sigma-Aldrich), was spiked to the slurry to a final concentration of 50 μM . The slurry was homogenized with a Polytron (KINEMATICA POLYTRON® PT 1600E) at setting #10 for 5 sec at 4°C , and it was centrifuged at $20,000 \times g$ (12,750 rpm in a Sorvall RC5 rotor) for 30 min at 4°C . An aliquot of supernatant (0.4 ml) was removed and mixed with equal volume of ice-cold ethanol. This was allowed to precipitate on ice for 10 mins and re-centrifuged at $10,000 \times g$ for 10 min at 4°C . The identical ethanol extraction procedure was repeated by another equal volume of ice-cold ethanol and this was re-centrifuged at $10,000 \times g$ at 4°C . The final de-proteinized extract contained 75% ethanol. In exploratory studies we compared this with our previously published extraction method for agmatine (Feng et al., 2002). The old method had used trifluoroacetic acid rather than ethanol precipitation. The peaks by gradient HPLC were found identical in either case, however arginine was better separated with the ethanol extraction procedure. Besides this, the remaining first volume of supernatant was saved (unextracted by ethanol), and the pellet also resuspended by vortexing in 1 volume of the above ice-cold buffer ($v = \text{tissue weight}$). These fractions were frozen at -80°C . No evidence of loss (i.e., declining agmatine levels) was observed over the freezer storage times.

High Performance Liquid Chromatography (HPLC) of Arginine, Agmatine and Metabolites

The method to detect arginine and its metabolites is based on Zhang and Kaye (Zhang and Kaye, 2004). The cortical ethanol extracts were firstly dried in a speed-vac (Savant Instruments, Farmingdale, NY). These resuspended in 100 μl of HPLC mobile phase A (acetonitrile/ methanol/ water at 4/3/3, v/v/v). From this, 10 μl was mixed with 70 μl of 200 mM potassium-Borate buffer pH 9.5 (final pH 9.5). From this, 36 μl was mixed with 9 μl of *O*-phthaldehyde/3-mercaptopropionic acid (Sigma-Aldrich), allowing for OPA derivatization reaction. Two min later, 20 μl was injected into a HPLC gradient system (Shimadzu, Kyoto, Japan). A column 150 mm $\times$ 3.9 mm, 4 micron C18 (Nova-Pak, Waters, Milford, MA) was used to separate substances flowing at 0.75 ml/min at temperature of 25°C . The gradient started at 14% mobile phase A with 86% mobile phase B (20 mM potassium phosphate buffer, pH 6.0). The Shimadzu HPLC control unit (SCL-10 AVP)

regulated the gradient to 100% mobile phase A by 50 min. Substances were detected using a Perkin-Elmer LS-2 fluorimeter (Waltham, MA). Peaks eluted in the following order: citrulline, N(G)-hydroxy-L-arginine (NOHA), L-arginine, agmatine, APP (the internal standard), ornithine, and putresine. The integrated areas under the peaks were assessed relative to APP. Standard curves for each purified substance were found to be linear.

Nitric Oxide (NOx) Assay

Total nitrite was assayed in the cortical brain supernatants (not the ethanol extracts). This involved subsequent conversion of nitrate to nitrite by the enzyme nitrate reductase. Detection of the total nitrite level (called “NOx”) was based on the azo-dye product of Greiss Reaction that absorbs light at 540 – 570 nm (kit from R&D Systems Inc., Minneapolis, MN). The lowest detection limit of the NOx assay was 1.35 $\mu\text{mol/L}$. The intra-assay and inter-assay CVs for NOx were 5.3% and 7.0%, respectively (Piletz et al., 2009).

Quantitative Histopathology

At Endpoint-1 the pups were lethally dosed with sodium pentobarbital (50 mg/kg i.p.) prior to transcardial perfusion. Whole body fixation was achieved by perfusion from the heart with 0.1M phosphate buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde/0.1M PBS solution (Sigma). Following this, the brains were extracted and stored briefly in jars of 4% paraformaldehyde/0.1M PBS at 4°C. The brains were cryoprotected in 30% sucrose solution overnight and stored for up to one week at –20°C prior to sectioning. Brain sectioning was done on a cryostat (Leica, CM1850). Every 10th coronal section (25 μm) was placed onto a 3% gelatin-subbed slide (Sigma) for immunodetection. These slides were washed in 0.1M PBS and blocked for 1 hour in 10% normal horse serum (NHS) (Sigma). Anti-Neun monoclonal antibody (Millipore, Billerica, MA) at 1:1,000 dilution was added for overnight incubation at 4°C in 5% NHS/0.3% TritonX-100/PBS. Slides were washed with 0.1% TritonX-100/PBS (Sigma), followed by 0.1M PBS, and incubated for 1 hr with biotinylated horse anti-mouse secondary antibody at 25°C in 5% NHS/0.3% TritonX-100/PBS. After another wash in 0.1% TritonX-100/PBS, the slides were rewashed in 0.3% H_2O_2 in methanol and again washed in 0.1% TritonX-100/PBS and 0.1M PBS. The slides were finally prepared for visualization using the avidin-biotin reaction (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA) as enhanced with 0.05% 3,3' diaminobenzidine tetra-hydrochloride (DAB)/PBS and H_2O_2 (Sigma). For preservation, the slides were stored up to four days in distilled water. Slides were serially dehydrated in increasing concentrations of ethanol solutions (70%, 90%, 95%, 100%) and soaked in xylene (Sigma) prior to cover slipping (Permount Mounting Medium, Fisher Scientific). The slides from four comparable brain levels were selected from each animal for photography/densitometry: (1) forebrain containing fornix but no hippocampal formation, (2) mid-hippocampal formation, (3) late hippocampal formation, and (4) hind brain without hippocampal formation. Microscopic images were photographed in scale and analyzed by ImageJ software (NIH, Bethesda, MD).

Weanlings Assessments

Pups at 22 days of age were at Endpoint #2 (i.e., weaning age and 15 days post-HI). Since 10 days of age (3 days post-HI) these juveniles had not been treated. The brains were expected to display some gross evidence of right-side cortical damage in the absence of an effective agmatinergetic treatment (Feng et al., 2002). To prepare for sacrifice, the pups were injected with pentobarbital (50 mg/kg i.p.) inducing deep anesthesia. Once unconscious to tail pinch, a sharp guillotine was used for decapitation. Brains were removed, weighed, and photographed. Photos were used to score the extent of right cortical brain damage according to previously described criteria (Feng et al., 2002).

Statistics

Data are expressed as mean $\pm$ SEM. Statistical tests were performed with InStat (GraphPad Software, La Jolla, CA), and the test for significance was achieved by first performing a one-way analysis of variance (ANOVA). If appropriate, repeated measures ANOVA was performed. Subsequent pair-wise comparisons were performed using Tukey-Kramer and/or Bonferroni post-hoc tests. Experiments were replicated at least three times.

RESULTS

Various analytical techniques were used to confirm the purity of PZC (Fig. 1A–E). For instance, two different methylene hydrogens in the ^1H NMR observed as triplets are indicative that the piperazine ring had been attached to a guanidine carbon. The peak at 159.3 ppm in the ^{13}C NMR also represents the carbon of the guanidine group. In the mass spectrum, the parent peak or molecular ion came out at m/z 129 because it is the $M+1$ peak. The calculated formula weight was 128.19 g/mol, but this slight weight increase can be contributed to an additional atom: the hydrogen atom gives the entire molecule a positive charge. The peak at m/z 112 corresponds to the amine group and the peak at m/z 87 corresponds to the loss of the guanidine group. Also, the structure of the compound, which was crystallized from acidic aqueous solution was confirmed by X-ray crystallography. The crystal structure was composed of a single cation, a water molecule, and half of a sulfate anion, which was from the recrystallization step. One of two nitrogens of the guanidine group was protonated and the sulfate ion was used for the crystallization with water molecule. The secondary nitrogen on the piperazine ring formed a hydrogen bond with water and the sulfate anion.

Preliminary experiments ensured animal safety. For instance, we undertook a few injections of PZC alone and observed the behaviors of normal “naïve” 7-day-old rat pups. Seeing no harm in them, even more pups were injected - up to 100 mg PZC /kg/day i.p. (b.i.d. at 50 mg/kg in the morning and evening) on three consecutive days - and assessments were made of body temperatures, weights, and mobility to a tail pinch throughout and beyond. No adverse events from PZC were noted at any time point. Since nothing adverse emerged from these preliminary injections, the experiments proceeded to hypoxia paradigms.

Hypoxia was first studied *in vitro* using the brain HEC slice paradigm. Immediately after exposure to combined hypoxia + low glucose condition, the HEC slices were treated with compounds. The next day they were stained with propidium iodine, yielding a red color if there had been damage. The presence of propidium iodine stain in the post-hypoxic saline-treated slices is indicative of neuronal damage due to the hypoxia. By contrast, the companion slices treated post-hypoxia with AGM or PZC showed almost no red color, i.e. nearly complete neuroprotection (Fig. 2A, top row). Control normoxic slices treated with AGM, itself, showed no neuronal damage of the brain slices even under the low glucose medium (shown in lower panels of Fig. 2A). While there was a hint that PZC, itself, might have produced an elevated staining of the dentate gyrus of the control normoxic slice (lower panel of Fig. 2A), this was an anomaly due to higher background staining across the entire entorhinal cortex and it was not replicated in the other set of triplicates. Taken together, the quantitative data are shown as bar graphs in Fig. 2B. Note that post-hoc saline (SAL) comparisons between hypoxia-low glucose and normoxia was statistically significant: $p=0.02$. Under hypoxia-low glucose the following treatments were also statistically significant: +AGM, $p=0.02$; +PZC, $p=0.02$. No other post-hoc (Bonferroni or Tukey-Kramer) comparisons approached statistical significance. Treatments with AGM (50 $\mu\text{g/ml}$) or PZC (50 $\mu\text{g/ml}$) thus produced clear and approximately equal neuroprotection when given during the post hypoxia/low glucose period.

By various standards, our *in vivo* HI paradigm appeared to be consistent with earlier reports (Feng et al., 2002). That is, (1) there were no deaths due to the HI period itself, nor were there any significant detriments in rectal temperatures, body weights, or overall mobility after recovery in the surgically-ligated or sham control HI pups; and (2) the tail pinch test after HI almost invariably identified those pups which had right-carotid-ligations since they tended to circle rightwards after the tail pinch. Yet, despite the outward appearances, we weren't able to reproduce the dramatic cortical damage seen in our previous study of the same HI model at a different site (Feng et al., 2002). Specifically, the cortex of the right-carotid-ligated pups this time appeared virtually undamaged whether we'd induced HI or not. To the naked eye there was no obvious cortical damage at Endpoint #1 (3 days post HI) or at Endpoint #2 (15 days post HI). Right-side cortical weights were however some 5% less than the left cortical weights in the right-carotid-ligated HI pups (not in controls) (Feng et al., 2002). These findings are described in context under the Discussion section.

Fortunately, there was substantial HI damage in the hippocampus to make our HI model useful for testing (Fig. 3A). The damage was clear in the uninjected saline-treated HI pups showing substantial size loss of neuronal region stained with anti-Neun antibody in the right hippocampus (-25% versus left side) compared to sham HI controls. Importantly, the HI damage was statistically more across CA1-3 hippocampal regions (Fig. 3B). By contrast, the forebrain sections labeled with anti-Neun antibody, as above, showed no pathological damage in the HI pups (data not shown). Thus, the findings overall indicated that our HI model, this time, was selective to the hippocampal CA1-3 neurons. While selectivity can be highly desirable, it represented a difference from our previous study where massive cortical damage had been a predominant HI feature (Feng et al., 2002). Again, we defer this to the Discussion section.

PZC injections at 50 mg/kg i.p. for three days b.i.d. were chosen mainly because the dose approximated the concentration used successfully in slice cultures (50 µg/ml assuming the pup's body to be a fully-accessible volume). In the right-carotid ligated HI pups, injections with PZC proved able to preserve the vulnerable hippocampal regions (CA1, CA2 and CA3) after HI (Fig. 3A). In fact, the right-side hippocampal regions of PZC-treated HI pup brains were statistically indistinguishable from sham controls or naïve pups which had not undergone HI damage (Fig. 3B). Therefore, three days of PZC treatments beginning immediately post-HI, seemed fully sufficient to completely spare the right hippocampus from HI neuronal loss in the present *in vivo* model.

HPLC was performed on the cortical extracts from the HI pups in order to measure metabolite concentrations which eluted in order of citrulline, N(G)-hydroxy-L-arginine (NOHA), L-arginine, agmatine, APP (the internal standard), ornithine, and putresine. The key comparisons were right-side versus left-side concentrations of each of the endogenous substances; especially right-side ligated HI pups and sham controls as a function of the injectant (PZC, AGM, or saline). An elevation of agmatine (+28.5%) was apparent in the right cortices following PZC as compared to the saline-injected controls (Table 1). The elevation was comparable in amount on both sides of the PZC-treated brain, although slightly more pronounced in the ischemic right cortex (Table 1). It was suggested that this elevation in agmatine due to PZC is selective to agmatinase inhibition because L-arginine and its other metabolites were unchanged by PZC, except for a tendency (non-significant) to reduce citrulline (Table 1). Also intriguing was the selective elevation in agmatine after three days' injection of PZC was roughly similar in magnitude to that seen after three days of AGM injection, itself (+29.3%; Table 1). In contrast to PZC, the regimen of AGM injections led to higher levels of cortical L-arginine and NOHA, as well as agmatine (Table 1).

The final experimental variable of this study was cortical NO_x. Based on previous literature values (Tsuji et al., 2000), we had anticipated seeing an elevation in right cortical NO_x that would have been a consequence of the HI itself. The question for which no literature existed was whether PZC would inhibit iNOS and thereby change NO_x in any manner. We observed the saline-treated cortical concentration of NO_x (μmol/g wet weight) 3-days post-HI to be 3.4 ± 0.4 on the normoxic left-side. This compared to 2.9 ± 0.3 μmol/g on the hypoxic right side (n=15 each; N.S.). These were not statistically different than the PZC-treated cortical concentration of NO_x at 3-days post-HI: 2.8 ± 0.3 on normoxic left-side versus 3.2 ± 0.4 on hypoxic right side (n=10 each, p =0.65 by one-way ANOVA) (data not shown as figures). Thus, cortical NO_x was not significantly elevated in the right-side cortex at 3-days post-HI (the paradigm, itself), nor did treatment with PZC have any statistical effect on NO_x concentrations.

DISCUSSION

There remains a major unmet need for a neuroprotective medicine to treat hypoxic brain injuries. According to the available literature (Legido et al., 2007, Liu and Levine, 2008), more than 300 compounds have so far been studied. The list can be categorized according to three prominent strategies: (A) improving cerebral blood flow, (B) inducing hypothermia, or (C) interfering with the cytotoxic cascade. Under the strategy of cytotoxic cascade inhibitors have been NMDAr antagonists, inhibitors of iNOS, anti-apoptosis agents, free radical scavengers, neurotrophic factors, ion channel modulators, and gene-based therapies. Unfortunately, none of the compounds so far tested have shown satisfactory results in the clinic – often owing to unwanted side effects. Our present investigation of PZC can be viewed as another attempt to interfere with the cytotoxic cascade (Wang et al., 2006, Hong et al., 2009).

Following extensive screening of analogues, we reported (Huang et al., 2003) the first evidence that PZC might be an agmatinase inhibitor which was clarified by a subsequent QSAR study (Piletz et al., 2008). That evidence, however, remained grounded in lysate biochemical assays. We searched the literature for PZC and found that its derivative, carboxypiperazine-phosphonate, acts as an NMDRr antagonist (Holmes et al., 1990). Another study of a PZC-like compound, 3-methylpyridyl-n-methylguanidine, showed it crosses the blood brain barrier and displays analgesic properties in rats by an unknown mechanism (He et al., 2006). However, we could find no relevant literature on PZC, itself. Because there was no previous literature on PZC, the present study obligated to be an exploratory study on the singular question of neuroprotection. Many other important questions, of course, need to be answered such as whether PZC acts as a competitive agmatinase inhibitor and whether PZC has similar activities in non-brain tissues or other models of cytotoxicity.

After assuring the purity of chemically-synthesized PZC (Fig. 1), we settled on addressing three core hypotheses posited of an agmatinase inhibitor. The first hypothesis was that PZC would be directly neuroprotective, which was addressed by showing favorable sustenance of neurons much as agmatine provides alone when applied to brain slices after exposure to hypoxia+low glucose *in vitro* (Fig. 2). The second hypothesis was that PZC would be neuroprotective *in vivo*, which was addressed by showing protection of hippocampal neurons and a degree of normal development after HI *in vivo* (Fig. 3). We then hypothesized that rat pups injected with PZC would emerge with high concentrations of cortical agmatine in both cerebral hemispheres above and beyond any changes that might emerge due to HI itself. To gain further insight, we quantified the cortical concentrations of endogenous agmatine elicited by PZC injections as well as its precursor (l-arginine) and a panel of arginine metabolites, using HPLC (Table 1). Tissue NO_x was measured as a potential signal

of the hypoxia and PZC effects. Pup vitality was also monitored in several ways to determine if PZC treatment causes obvious adverse events.

Like other known neurotransmitters, this neurotransmitter (agmatine) should be under the control of a specific degradation enzyme: namely, agmatinase (Sastre et al., 1998). Agmatinase activity is somewhat less prominent in the periphery where diamine oxidase (DAO) predominates in the metabolism of peripheral agmatine (Satriano, 2004). Agmatinase is also more substrate-specific than DAO (Sastre et al., 1998, Uribe et al., 2007). Other studies have indicated that brain agmatine rises and falls in line with glucocorticoid hormone treatments (Zhu et al., 2007) as well as with specific stressful learning paradigms (Liu and Bergin, 2009, Liu et al., 2009). In both paradigms, the levels of endogenous brain agmatine have been explained by a combination of glucocorticoid-regulated ADC and agmatinase activities (Zhu et al., 2007, Liu and Bergin, 2009, Liu et al., 2009). Agmatinase has further been revealed to exist in dendritic spines, spine and non-spine terminals, and dendritic profiles (Madaï et al., 2011). The importance of agmatinase may best be revealed by a recent report (Bernstein et al., 2012) showing massive over-expression of agmatinase protein in postmortem hippocampus sections from suicide victims having documented mood disorders.

The techniques of microdialysis and capillary zone electrophoresis plus laser-induced fluorescence detection have revealed mid-nanomolar concentrations of agmatine in the adult rat hippocampal extracellular space (Betancourt et al., 2012). Furthermore, extracellular agmatine was shown to be calcium- and impulse-dependent; and depolarization of the hippocampal neurons increased the agmatine concentrations (Betancourt et al., 2012). In homogenates of naïve rat pup cortex, we previously observed a baseline of agmatine at 605 nM which elevated to 1.76 μ M in damaged pup cortex shortly after the HI procedure (this done in the absence of PZC)(Feng et al., 2002). Taken with the results of Table 1, it suggests that brain agmatine levels remain in the low micromolar range for at least 3 days post-HI. Furthermore, Demady et al reported (Demady et al., 2001) that agmatine acts as a time-dependent irreversible inhibitor of intracellular NOS, with the $K_i = 29 \mu$ M being a pseudo first-order inactivation rate constant at 0.01 min⁻¹. There is also evidence for an accumulating effect over time, because iNOS is inactivated by agmatine through a decrease in iNOS protein over a matter of 6 hours (Regunathan and Piletz, 2003). A strong case can thus be made for agmatinergic neurotransmission (Halaris and Piletz, 2007), but there is no reason to limit agmatine's neuroprotective properties to neuronal release which could also be due to agmatine's intracellular inhibition of iNOS and lowered glial activation. It is therefore plausible for a micromolar rise in agmatine to be neuroprotective in a multi-mechanistic, time-dependent, and model-specific manner.

One might also consider the well-known relative of agmatine, aminoguanidine (Tsuiji et al., 2000). As an iNOS inhibitor, the unfortunate shortcoming of aminoguanidine in clinical studies has been its systemic actions. Aminoguanidine inhibits iNOS in healthy adjacent tissues, as well, and thereby causes unwanted systemic effects that include sedation, confusion, hallucination, and even catatonia (Tsuiji et al., 2000). Based on similarity of structure, it is little wonder that the systemic administration of mega doses of agmatine, itself, could be questioned. To get around this, we are proposing that an agmatinase inhibitor might preferentially target only those tissues where agmatine is induced by the stressor, thereby minimizing the unwanted side effects of high systemic agmatine. We hope that PZC “zeroes-in” wherever agmatinase levels are high (i.e., induced), which presumably is restricted to just HI vulnerable brain regions. The present study unfortunately fails to address the multi-tissue issue, but it is fundamental to the question of “why not just treat with mega doses of agmatine?”

Other models, of course, could have been chosen for this study (Lee et al., 2009). We deemed the HEC slice culture model the best place to start because the hippocampal neurons in these slices are known to be sensitive to hypoxia+low glucose as found *in vivo* (Kreisman et al., 2000). In the hypoxia+low glucose slices we monitored for degeneration of neurons by propidium iodine staining before and after PZC or AGM treatments. A first finding was no observable adverse effects of PZC or AGM alone leading to propidium iodine entry into the slices during normoxic culture. Neither compound appeared in any way detrimental by itself. Moreover, there were positive findings of neuroprotection of the slices afforded by PZC and AGM after the hypoxia–low glucose period (Fig. 2). Although it remained unclear whether the PZC treatment had actually led to higher agmatine levels in the slices – slices being too small for HPLC measurement of agmatine – it would not seem unreasonable to assume such because PZC appeared identical in effect to that of AGM alone (Fig. 2B).

Our *in vivo* model of HI also utilized 7-day-old rat pups and ended with a focus on hippocampal neurons. To induce HI, pups underwent unilateral right-carotid ligation followed by 2 hours in a hypoxic-ischemic chamber. In most regards, this was identically conducted as previously used to study the neuroprotective capacity of AGM itself (Rice et al., 1981, Feng et al., 2002). The main focus was the ratio of neuronal staining in the right:left hippocampal regions (hypoxia:non-hypoxia) in the pups treated with PZC or with saline (Fig. 3). The right:left ratio of neuron density was appropriately ≈ 1.0 in PZC-treated pup's hippocampal regions (Fig. 3B) which indicated the drug treatment had seemingly spared the HI damage found in the saline-treated control pups.

The final key hypothesis was whether PZC as an agmatinase inhibitor would lead to a rise in endogenous brain agmatine. The corollary was that if PZC is selective to agmatinase, the level of l-arginine and its other metabolites would either be unchanged or, if changed, only to a degree explainable by the downstream effects known of elevated agmatine. In support of this, we found a selective elevation in only agmatine concentration following three days of PZC treatment of post-HI animals (Table 1). This elevation was observed in both the left and right cortices, consistent with a peripheral injection source of PZC, but it was slightly more pronounced in the ischemic right cortex. The elevation of agmatine after three days of PZC was furthermore of about the same magnitude as after three days of AGM injections alone (Table 1). However, there were two findings amongst the arginine metabolites which might mitigate the issue of PZC's ability to selectively yield a rise in agmatine. One was that AGM-only injections led to higher levels also of l-arginine and NOHA (Table 1), while injections of PZC did not. Also, there was a non-significant trend to reduce citrulline following PZC injections (Table 1). It stands to reason that without agmatinase being inhibited in the AGM-only injected pups, then exogenous agmatine could break down and recycle to produce elevations in arginine and NOHA as well. This is one possible explanation. The present findings with PZC in Table 1 therefore are overall consistent with an inhibition of brain agmatinase.

There were other discrepancies from our earlier findings with AGM in the HI model (Feng et al., 2002, Feng and LeBlanc, 2003). While at the University of Mississippi Medical Center (previous laboratory where AGM-only had been studied in the *in vivo* model), we attempted to study dose response with AGM using the rat pup model of HI (Feng et al., 2002). The dose response curves revealed 50 mg/kg AGM i.p. for three days b.i.d. sufficient to reverse the *in vivo* HI paradigm, but too high doses were problematic. That is, morphological damage to the right cortex (ligated side) was overtaken in the AGM-treated pups. In addition, AGM was capable of suppressing the induction of cortical NO_x produced by HI (Feng et al., 2002). Herein, even though using the same HI schedule (and for that matter the same personnel and much of the same equipment), we could not reproduce the same extent of damage in the cortex. Instead, no cortical damage was noted due to the HI,

itself, at the new site. Likewise, there was no apparent effect from the raised agmatine levels on cortical NO_x which we had anticipated. These discrepancies remain unresolved issues because our previous study had neglected to examine the hippocampal regions of the pups, which became the selective focus of the present study. Such selectivity to the hippocampus may owe to some minor modification we made in the protocol, but which modification remains unclear (see Methods for list of modifications). Our working hypothesis is that difference owes to a cooler surgical room (in Chicago) than used previously (in Mississippi). Whether it was this or some other unrealized modification, there should be cautions when comparing our previous findings with the present results.

Another issue was the absolute concentration of agmatine detected in the present study. We previously reported (Feng et al., 2002) concentrations of agmatine in naïve rat pup cortices averaging 1.06 μ M (138.1 ng/ gm wet weight). By contrast, we now report agmatine in baseline cortices averaged 3.96 μ M (514.5 ng/ gm wet weight: Table 1). Also, we previously reported (Feng et al., 2002) that AGM injection (50 mg/kg i.p.) led to a 28-fold rise in cortical agmatine concentration compared to naïve rat pups at 180 min post-injection. By contrast, we herein found that following 3 days of AGM injections (50 mg/kg i.p. for 3 days b.i.d.) led to a 29.3 % rise in the cortical agmatine concentration in 10-dayold pups (Table 1). While 29.3% is in the proper direction and statistically significant, it led us to reassess the 180 min post-HI in a limited number of pups, and the level of brain AGM was indeed only increased 3-fold by 180 min. These differences between the two studies could again owe to the sparing of the cortex this time by cooler temperatures. Yet, both studies could have benefited from the use of LC mass spectroscopy (LCMS) to provide proof of purity of the agmatine peak.

In summary, this was an exploratory study in which we found support for PZC as a neuroprotective agent against HI both *in vitro* and *in vivo*. The results aligned reasonably with the expectations of an agmatinase inhibitor. However, detailed biochemical studies will be required to definitively prove that PZC's neuroprotection is due to a selective inhibition of agmatinase inhibition. Other future studies are needed to assess PZC's presumed synergy with agmatine and other doses of PZC in other animal species and toxicity models.

Acknowledgments

This research was supported by a grant from the National Institutes of Health, R21 NS054862-1-3. For the analytical chemistry of newly-synthesized PZC, we gratefully acknowledge the Mississippi College Diffraction Facility supported by National Science Foundation grant MRI 0618148, as well as the W. M. Keck Foundation for crystallographic resources. For the brain slice procedure we thank the advice of Dr. Robert Mitchell, and for the slice hypoxia protocol we thank the advice of Dr. Rubin Mestrlil (both of Loyola University Medical Center, Chicago). For immunohistochemical procedures we gratefully acknowledge the advice of Dr. Rita Cowell of the University of Alabama and of Dr. Julie Fudge of the University of Rochester. NY.

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(e)

Figure 1.

(a) Chemical synthesis scheme of piperazine-1-carboxamidine (PZC); (b) ^1H NMR of PZC based on D_2O ppm, δ 3.01 (t, 4H, $-\text{CH}_2-$, $J=5.1\text{Hz}$), and δ 3.52 (t, 4H, $-\text{CH}_2-$, $J=5.1\text{Hz}$); (c) ^{13}C NMR of PZC based on ^{13}C NMR (D_2O , ppm), δ 46.2, 47.9 ($-\text{CH}_2-$), and δ 159.3 (guanidine carbon); (d) Liquid chromatography-tandem mass spectroscopy (LC/MS/MS) analysis based on $[\text{MH}^+] = 129.0$ (protonated PCA), 112 (lost an ammonia), and 87 (protonated piperazine); (e) Deduced x-ray crystal structure of sulphate salt of PZC H^+ cation.

Figure 2.

Piperazine-1-Carboxamidine Or Agmatine are Neuroprotective Added Directly to Hippocampal-Entorhinal Cortex Slice Cultures After Hypoxia With Low glucose.

A. Propidium iodide stain shows damaged regions unless agmatine (AGM) or piperazine-1-carboxamidine (PZC) were added to the media after hypoxia-low glucose. Abbreviations of the template: AGM, Agmatine; CA1, CA2, CA2, hippocampal regions of Ammon's horn; DG, dentate gyrus; ENT, entorhinal cortex; PZC, piperazine carboxamidine; SUB, subicular complex.

B. Densitometric images were quantified from three separate experiments and for visual purposes adjusted to percent maximal necrosis in each experiment. For the statistical analysis, the 6 columns of raw data were subjected to two-way ANOVA using the InStat program, yielding $p = 0.01$. The post-hoc saline (SAL) comparison between hypoxia-low glucose and normoxia was statistically significant: $p = 0.02$. Under hypoxia-low glucose the following treatments were also statistically significant : SAL vs. +AGM, $p = 0.02$; SAL vs. +PZC, $p = 0.02$. None of the other post-hoc (Bonferroni or Tukey-Kramer) comparisons approached statistical significance.

Fig. 3.
Mid Brain Hippocampal Neuroprotection by Piperazine-1-Carboxamidine Following Hypoxic Ischemia In Vivo

A. Anti-Neun monoclonal antibody staining intensity in cryostat-prepared mid-hippocampus sections from 10-day-old rat pups under three treatments. Results show right-side selective damage of neurons after hypoxic-ischemia period (HI) mostly in hippocampal regions (CA1, CA2, CA3) but not the dentate gyrus (DG) in saline treated pups (SAL TX). Such damage was not seen in pups treated for three days with piperazine-1-carboxamidine (PZC TX, 50 mg/kg i.p., b.i.d.) or the sham-operated controls (SHAM). The sham animals (SHAM) had not undergone hypoxic ischemia.

B. Densitometric areas were quantified from at least five separate experiments and for visual purposes adjusted to the right:left brain intensity of each region for each experiment. The idealized ratio (1.0) is shown as a dotted line. For statistical analysis, the 3 columns of raw data (not ratios) in region-specific manner were subjected to repeated measures ANOVA using the InStat program, yielding overall $p = 0.01$ except for the dentate gyrus. The post-hoc saline comparisons to PZC were statistically significant (at least at $p = 0.02$) in the CA1, CA2 and CA3 regions. None of the other post-hoc (Bonferroni or Tukey-Kramer) comparisons approached statistical significance.

Table 1

Effect Of Piperazine-1-Carboxamidine Or Arginine Injections On Brain Cortical Concentrations Of Arginine And Its Metabolites Three Days After Rat Pup Hypoxic-Ischemia

Conc. (nM)→ Treatment↓	Arginine		Citrulline		NOHA		Agmatine		Ornithine		Putrescine	
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
Experimental controls treated with saline 3 days post HI (n = 41)	4389 ± 395 ^a	4046 ± 304 ^c	6853 ± 469	6192 ± 314	762 ± 83 ^b	767 ± 110	3966 ± 275 ^d	3950 ± 225 ^e	4432 ± 417	4042 ± 240	3321 ± 308	2690 ± 175
PZC injections 3 days post HI (n = 14)	4912 ± 587	5403 ± 768	5470 ± 508	5632 ± 326	802 ± 164 ^f	460 ± 72 ^f	4898 ± 472 ^d	5277 ± 430 ^e	4355 ± 589	5521 ± 1054	4367 ± 922	4847 ± 1076
Agmatine injections 3 days post HI (n = 22)	6007 ± 439 ^a	5850 ± 557 ^c	6339 ± 566	6621 ± 490	1199 ± 152 ^{b,g}	866 ± 68 ^g	5005 ± 453 ^d	5229 ± 758 ^e	5292 ± 305	5089 ± 406	4412 ± 914	4586 ± 1155

After inducing the hypoxic-ischemia model (HI), rat pups were injected with either saline, piperazine-1-carboxamidine (PZC, 50 mg/kg b.i.d.), or agmatine (50 mg/kg b.i.d.) from just after the hypoxia chamber until mid-day-3. Three hours after the last morning injection they were sacrificed. Pups derived from at least 5 litters per group. Shown are arginine and metabolite concentrations in the Left (L) or Right (R) cortical hemispheres expressed as group means ± SEMs. Statistically noteworthy comparisons between treatments (One-way ANOVA, post-hoc) were:

Other statistically noteworthy comparisons between brain sides (paired t test) were:

No other comparisons were statistically different.

^a p = 0.037;

^b p = 0.023;

^c p = 0.01;

^d p = 0.069 (each way);

^e p = 0.047 (each way).

^f p = 0.067

^g p = 0.035.