

5/1/2010

COVER SHEET

TITLE: Exploring the role of Na^+H^+ exchanger in neonatal brain injury following hypoxia-ischemia

AUTHOR'S NAME: Neil W Kleman

MAJOR: Biochemistry

DEPARTMENT: Biochemistry

MENTOR: Dandan Sun, MD, PhD

DEPARTMENT: Neurological Surgery

MENTOR(2): Pelin Cengiz, MD

DEPARTMENT(2): Pediatrics

YEAR: 2010

(The following statement must be included if you want your paper included in the library's electronic repository.)

The author hereby grants to University of Wisconsin-Madison the permission to reproduce and to distribute publicly paper and electronic copies of this thesis document in whole or in part in any medium now known or hereafter created.

Exploring the role of Na^+/H^+ exchanger in neonatal brain injury following hypoxia-ischemia

We investigated the role of Na^+/H^+ exchanger isoform 1 (NHE-1) in neonatal hypoxia ischemia (HI). HI was induced by unilateral ligation of the left common carotid artery in postnatal day 9 (P9) mice, and subsequent exposure of animals to 8% O_2 for 55 minutes (min). A pre-treatment group received a selective and potent NHE-1 inhibitor HOE 642 (0.5 mg/kg, intraperitoneally) 5 min before HI, then at 24 hour (h) and 48 h after HI. A post-treatment group received HOE 642 (0.5 mg/kg) at 10 min, 24 h, and 48 h after HI. Saline injections were used as vehicle controls. Neuronal degeneration in the ipsilateral hippocampus, striatum, and thalamus was identified with Fluoro-Jade C positive staining and loss of microtubule associated protein 2 (MAP2) expression 72 hr after HI. NHE-1 protein was up-regulated in glial fibrillary acidic protein positive reactive astrocytes. In HOE 642-treated brains, the hippocampal structures were better preserved, and displayed less neurodegeneration and a higher level of MAP2 expression. These findings suggest that NHE-1-mediated disruption of ionic homeostasis contributes to striatal and CA1 pyramidal neuronal injury after neonatal HI.

Neil Kleman / Biochemistry
Author Name/Major

Neil Kleman
Author Signature

5-25-10
Date

Dandan Sun
Mentor Name/Department

D/S
Mentor Signature

5/1/2010

Exploring the role of Na^+H^+ exchanger in neonatal brain injury
following hypoxia-ischemia

Neil Kleman^{2,3}, Pelin Cengiz¹, Kutluay Uluc², Peter Ferrazzano^{1,3}, and Dandan Sun^{2,3}

Depts. of ¹Pediatrics and ²Neurological Surgery, University of Wisconsin School of
Medicine and Public Health, ³Waisman Center, Madison, WI 53705

5/1/2010

ABSTRACT

We investigated the role of Na⁺/H⁺ exchanger isoform 1 (NHE-1) in neonatal hypoxia ischemia (HI). HI was induced by unilateral ligation of the left common carotid artery in postnatal day 9 (P9) mice, and subsequent exposure of animals to 8% O₂ for 55 minutes (min). A pre-treatment group received a selective and potent NHE-1 inhibitor HOE 642 (0.5 mg/kg, intraperitoneally) 5 min before HI, then at 24 hour (h) and 48 h after HI. A post-treatment group received HOE 642 (0.5 mg/kg) at 10 min, 24 h, and 48 h after HI. Saline injections were used as vehicle controls. Neuronal degeneration in the ipsilateral hippocampus, striatum, and thalamus was identified with Fluoro-Jade C positive staining and loss of microtubule associated protein 2 (MAP2) expression 72 hr after HI. NHE-1 protein was up-regulated in glial fibrillary acidic protein positive reactive astrocytes. In HOE 642-treated brains, the hippocampal structures were better preserved, and displayed less neurodegeneration and a higher level of MAP2 expression. These findings suggest that NHE-1-mediated disruption of ionic homeostasis contributes to striatal and CA1 pyramidal neuronal injury after neonatal HI.

5/1/2010

INTRODUCTION

Neonatal hypoxia ischemia (HI) occurs in approximately 1 in 1000 live births (6). The resulting brain injury can lead to developmental disabilities, cerebral palsy or even death (Ferriero, 2004). The mechanisms behind the developing brain injury are not fully understood. Disruption of ionic homeostasis by an overload of intracellular Ca^{+} and Na^{+} has been previously reported (Jiang et al., 2008; Northington et al., 2001; Zhou et al., 2009). Considerable research has focused on the association of metabotropic glutamate receptors with excitotoxic brain injury (Aarts and Tymianski, 2003). Non-glutamate mediated pathways may also contribute to disruption of Na^{+} and H^{+} homeostasis leading to hippocampal injury. A clinical study correlated intracellular alkalosis with severity of injury in term infants following HI (Robertson et al., 2002). Infants with the most alkaline brain pH_i demonstrated more severe brain injury in the first 2 weeks after birth and worse neurodevelopmental outcome at 1 year (Robertson et al., 2002). Excessive activation of the $\text{Na}^{+}/\text{H}^{+}$ exchanger (NHE) may be responsible for this damage.

NHE is a membrane protein that regulates intracellular pH (pH_i) by extrusion of 1 H^{+} in exchange for 1 Na^{+} (Orlowski and Grinstein, 1997). HI causes intracellular acidosis which may trigger over-stimulation and activation of NHE, leading to intracellular Na^{+} overload and secondary brain injury. The NHE isoform 1 (NHE-1) is the most abundant isoform in the rat central nervous system among 9 NHE isoforms (Ma and Haddad, 1997). Inhibition of NHE-1 activity has been shown to reduce damage due to ischemia and reperfusion injury in myocardium and focal cerebral ischemia in adult animal models (Masereel et al., 2003; Avkiran, 2001). Pretreatment of adult gerbils with the amiloride derivative ethylisopropylamiloride (EIPA), a non-selective NHE inhibitor, significantly reduces the extent of CA1 pyramidal neuronal loss following global

5/1/2010

ischemia (Hwang et al., 2008). Administration of the non-selective NHE inhibitor N-methyl-isobutyl-amiloride (MIA) ameliorates neonatal brain injury in a mouse HI model (Kendall et al., 2006). These studies suggest that NHE activation is involved in neuronal injury after HI.

In the current study, we investigated whether inhibition of NHE-1 with the potent inhibitor HOE 642 is neuroprotective after HI in immature brains. We report here that the HOE 642-treated brains displayed better preserved morphology in the hippocampus, accompanied by less neurodegeneration and a higher level of MAP2 expression.

MATERIALS AND METHODS

Materials

Fluoro-Jade C (FJ-C) was from Histo-Chem Inc. (Jefferson, AK). Tissue-Tek O.C.T. compound was from Sakura Finetek (Torrance, CA). Antibodies against microtubule associated protein 2 (MAP2) and glial fibrillary acidic protein (GFAP) were from Sigma (St. Louis, MO) and Dako Inc. (Carpinteria, CA), respectively. Polyclonal NHE-1 antibody was from Abcam Inc. (Cambridge, MA). HOE 642 was a kind gift from Aventis Pharma (Frankfurt, Germany). Goat anti-mouse Alexa Fluor 488-conjugated IgG and goat anti-rabbit Alexa Fluor 546-conjugated IgG were from Invitrogen (Carlsbad, CA). Tissue section rinsing solution CitriSolv was from Fisher Inc. (Hampton, NH) and DPX mounting medium was from Sigma Inc.

Induction of neonatal HI

Postnatal day 9 (P9) C57BL/6J mice were anesthetized with isoflurane (4% for induction, 1% for maintenance), 30% O₂ and 70% N₂. The body temperature of the

5/1/2010

animal was maintained at 37°C with a heating pad. Under a surgical microscope, a midline skin incision (0.5 cm) was made on the neck and the left common carotid artery was exposed and electrically cauterized. The incision was rinsed with 1% lidocaine and sutured with a 6.0 prolene suture. Animals were returned to their dams and observed continuously for 30 minute (min) during a 2 hour (h) recovery period. To induce ipsilateral ischemic injury as described by Vannucci et al (Vannucci and Vannucci, 2005), the animals were placed in a hypoxia chamber (BioSpherix Ltd, Redfield, NY), equilibrated with 8% O₂, 92% N₂ at 37°C, for 55 min. After HI, animals were monitored continuously for 30 min and then checked every 30 min for 2 h and then daily until sacrificed.

Drug administration

To selectively inhibit NHE-1 with its potent inhibitor HOE 642, the animals were randomly divided into four treatment groups: pre-treated, post-treated, and the corresponding two vehicle (saline) controls. The pre-treated group received the initial dose of HOE 642 (0.5 mg/kg) 10 min before HI and subsequently at 24 and 48 h post HI, intraperitoneally (ip). The post-treated group received a dose of 0.5 mg/kg HOE 642 (ip) at 10 min, 24 and 48 h post HI. HOE 642 was administered at multiple time points because the intravenous half-life of HOE 642 is short (40 min in rats, (Scholz et al., 1995)). The two vehicle control groups received an equal volume of saline at the same time points.

Brain tissue preparation

5/1/2010

At 72 h following HI, animals were anesthetized with isoflurane as described above. Animals were transcardially perfused with 4% paraformaldehyde and decapitated. After post-fixation of the brains in 4% paraformaldehyde overnight, brains were stored in a 30% sucrose/PBS solution for 48 h and sectioned (35 or 70 μ m thickness) on a freezing sliding microtome (Leica SM2000R, Leica, IL). The brain sections were either cryoprotected in an antifreeze solution for storage at -20°C or mounted on poly-lysine coated slides.

FJ-C staining and quantification

Mounted brain sections (70 μ m) were dried on a slide warmer at 50°C for 30 min. The following steps were performed in the dark. Sections were treated with 0.06 % KMnO_4 for 15 min. After a brief rinse in ddH₂O, the sections were stained with 0.001 % FJ-C in 1% acetic acid for 25 min on a shaker. Sections were rinsed 3 x 1 min in ddH₂O, and followed by air-drying on the slide warmer at 50°C for 8 min. Slides were then rinsed in CitriSolv and mounted with DPX mounting medium. FJ-C stained images were taken using a Nikon TE300 epifluorescence microscope equipped with a FITC filter. In a blind manner, the total number of FJ-C positive cells in the ipsilateral CA1 region of hippocampus was determined in each slice using Metamorph image analysis software (Molecular Devices, Downingtown, PA). On some slides, a mosaic was obtained using an epifluorescence microscope (Zeiss Axioplan 2, 2.5X objective lens) equipped with a motorized stage and compiled with StereoInvestigator software (MBF Biosciences, Williston, VT) to create whole brain images.

Double immunofluorescence staining

5/1/2010

After rinsing with tris-buffered saline (TBS), brain sections (35 μ m) were incubated with TBS⁺⁺ (0.1% Triton X-100 and 3% goat serum in 0.1 M TBS) for 30 min at 37°C. Sections were double-stained with a monoclonal antibody against MAP2 (1:500), rabbit polyclonal antibody against GFAP (1:500), or polyclonal anti-NHE-1 antibody (1:100) for 1 h at 37°C and then overnight at 4°C. After washing with TBS (3 X 10 min), brain sections were incubated for 1 h at 37°C with goat anti-mouse Alexa Fluor 488-conjugated IgG (for MAP2, 1:200) and goat anti-rabbit Alexa Fluor 546-conjugated IgG (for GFAP, 1:200). Following rinsing with TBS, sections were incubated in To-Pro-3 Iodide (1:1000 in TBS⁺⁺) for 15 min and mounted on slides. Slides were imaged with a Leica DMIRE 2 inverted confocal microscope and Leica microscope software (Leica Inc, IL).

Statistical analysis

Values are expressed as the mean \pm SD. Statistical analysis was performed using Mann-Whitney rank sum test to compare vehicle control and the drug-treated group. ANOVA (the Bonferroni post test) was used in the case of multiple comparisons (SigmaStat, Systat Software, Point Richmond, CA, USA). P-values smaller than or equal to 0.05 were considered statistically significant.

RESULTS

Inhibition of NHE-1 with HOE 642 reduces neuronal injury in hippocampus, striatum, and thalamus

To investigate whether NHE-1 activity plays a role in neuronal injury after HI, the effect of the potent NHE-1 inhibitor HOE 642 was examined. Brain injury was assessed with FJ-C staining, a method that detects degenerative cells (Schmued et al., 2005). As

5/1/2010

shown in **Figure 1A**, hippocampal injury was visible in the ipsilateral hemisphere of the vehicle control animal, as reflected by hippocampal atrophy (**arrowhead in the bright field image**).

FJ-C positive cells were detected in the ipsilateral hippocampus in CA1, CA2, and CA3 regions as well as in the thalamus (**Figure 1B, arrowhead**). In contrast, in the animals pre-treated with HOE 642, the ipsilateral hippocampus exhibited smaller lesion, reflected by better preserved hippocampal morphology (**Figure 1C, arrow**) and fewer FJ- C positive cells in the pyramidal neuronal layers of CA1, CA2, and CA3 (**Figure 1 D, arrow**). In the post-treatment vehicle control animals, FJ-C positive cells appeared in ipsilateral hippocampus, striatum, thalamus, and some cerebral cortical areas (**Figure 1F, arrowhead**). The animals post-treated with HOE 642 after HI, exhibited neuroprotection in these brain regions, with no FJ-C positive cells visible in the ipsilateral striatum and thalamus, and fewer FJ-C positive cells found in pyramidal neuronal layers of CA2 and CA3 (**Figure 1H**). These data clearly illustrate the neuroprotective effects of inhibiting NHE-1 activity with HOE 642 following HI.

Quantitative analysis of neuroprotection mediated by HOE 642 in CA1 regions

As shown in **Figure 2A (a)**, background, non-specific signal of FJ-C staining was detected in the contralateral hippocampus of P9 brain at 72 h after HI (**arrow**). In contrast, the ipsilateral hippocampus exhibited a higher level of specific FJ-C fluorescence signals in CA1, CA2, CA3, and DG regions [**Figure 2A (b), arrowhead**]. **Figure 2A (c)** illustrates the magnified ipsilateral hippocampal image with a clearly increased number of FJ-C positive cells in the CA1 pyramidal neuronal layers

5/1/2010

(arrowhead). The HOE 642 pre-treated brains showed fewer FJ-C positive cells [Figure 2A (d), arrow].

The CA1 pyramidal neuronal injury was quantified by counting the number of FJ-C positive cells in the vehicle control and in animals treated with HOE 642. Based on the number of FJ-C positive cells, a score was assigned: 0: no FJ-C-positive cells, 1: 1-500, 2: 501-1000, 3: 1001-1500, 4: >1500 FJ-C-positive cells. As shown in Figure 2B, both HOE 642 treatment groups (pre- and post-treated) exhibited statistically significant reduction of FJ-C-positive cells in the ipsilateral CA1 region of hippocampus ($p < 0.05$).

Inhibition of NHE-1 activity by HOE 642 attenuated loss of MAP2 expression following HI

We also investigated hippocampal neuronal injury by examining changes of MAP2 expression. MAP2 is a dendritic protein thought to be involved in microtubule assembly, which is an essential step in neurogenesis. The contralateral hippocampus of the vehicle control mice exhibited abundant MAP2 expression [Figure 3 (a)]. The hippocampal regions, such as stratum pyramidale (SP) and stratum radiatum (SR), show the well organized structure of cell body and dendrites [Figure 3 (c), arrow]. 55 min HI caused an apparent loss of pyramidal neurons and MAP2, accompanied by organization of the SR in the ipsilateral hippocampus [Figure 3 (b)]. These changes were more clearly illustrated in the magnified image [Figure 3 (d)]. In mice pre-treated with HOE 642, the loss of MAP2 expression following HI was attenuated in the ipsilateral hippocampus [Figure 3 (f)]. Structural organization of dendrites was preserved in the stratum radiatum of the ipsilateral hippocampus of the HOE 642 pre-treated brains [Figure 3 (h), arrow].

NHE-1 is expressed exclusively in GFAP positive (GFAP⁺) reactive astrocytes in ipsilateral hippocampus following HI

Reactive astrocytes were detected via GFAP⁺ staining in the ipsilateral hippocampus following HI. In the vehicle control brain after HI, a few GFAP⁺ cells were located in the contralateral stratum oriens (SO), SR, stratum lacunosum and moleculare (SLM) of hippocampus [Figure 3 (c), open arrowhead]. However, the ipsilateral hippocampus showed an increased number of GFAP⁺ cells in the SO, SP, SR and SLM of CA1 region [Figure 3 (b, d)]. Inhibition of NHE-1 activity with HOE 642 did not alter the number of reactive astrocytes, despite its effect on preservation of CA1 neurons and expression of MAP2 in dendrites [Figure 3 (f, h)].

NHE-1 expression of hippocampus after HI

Changes of NHE-1 expression in hippocampus following HI were further characterized by immunofluorescence staining. The contralateral CA1 pyramidal layers in the vehicle control and animals treated with HOE 642 exhibited a low level of NHE-1 expression at 72 h after HI [green, Figure 4A (a, c), arrow]. The immunoreactive signals for NHE-1 were specific because they were absent when the NHE-1 primary antibody was omitted [inset in Figure 4A (a)]. Of interest, many cells in the contralateral SR and SLM were positive for the NHE-1 immunoreactivity [Figure 4A (a, c), open arrowhead] unlike the GFAP⁺ astrocytes. These cells may represent non-reactive astrocytes. In the ipsilateral hippocampus of the vehicle control brain, the CA1 SP layer was disorganized. The striking change in the SR and SLM was an increase in number of cells which had both GFAP and NHE-1 immunoreactivity [Figure 4A (b),

5/1/2010

arrowhead]. These changes are clearly illustrated in the magnified images [**Figure 4A (e, f)**]. The z-section analysis showed that the NHE-1⁺/GFAP⁺ cells exhibited reactive astrocyte morphology with enlarged processes and hypertrophy of the cell body [**Figure 4A (f')**]. Interestingly, NHE-1 was up-regulated in the CA1 pyramidal neurons in the brains of animals treated with HOE 642 [**Figure 4A (d)**]. Treatment of animals after HI with HOE 642 did not attenuate formation of the GFAP⁺ astrocytes in every layer of the ipsilateral hippocampus [**Figure 4A (d, h)**]. Summary data shown in **Figure 4B** indicate that NHE-1 is abundantly expressed in the reactive astrocytes. NHE-1 may play an important role in reactive astrogliosis, which may be contributing to neuronal damage.

DISCUSSION

Expression of NHE-1 in hippocampus

NHE-1 is expressed in virtually all cells and tissues and is, by far, the most abundant plasma membrane NHE isoform in the rat brain (Ma and Haddad, 1997). An *in situ* hybridization analysis showed that NHE-1 mRNA appears at higher levels in the hippocampus, compared to NHE-2, NHE-3 and NHE-4 (Ma and Haddad, 1997). Expression of NHE-1 in rat brains is dependent on the state of development and peaks on day of life 13 (Douglas et al., 2001).

In the current study, we found that NHE-1 was expressed in hippocampal pyramidal neurons and astrocytes throughout the hippocampus. Most importantly, NHE-1 immunostaining was enhanced in both CA1 neurons and reactive astrocytes at 72 h after HI. The z-section analysis using the confocal microscopy confirmed the colocalization of GFAP and NHE-1. These findings are consistent with the recent report showing the elevation of NHE-1 expression in reactive astrocytes in CA1 regions of adult gerbil

5/1/2010

brains following a transient global ischemia (Hwang et al., 2008). Taken together, these studies imply that hippocampal neurons and astrocytes may respond to the intracellular acidosis after HI by up-regulating NHE-1 expression and function. Alternately, we cannot rule out the possibility that some of the NHE-1 immunoreactive signals in the ipsilateral hippocampus may be localized in microglia. Indeed, expression of NHE-1 has been detected in Iba 1-positive cells following global ischemia (Hwang et al., 2008).

Inhibition of NHE-1 with HOE 642 is neuroprotective in neonatal brains after HI

Many reports have shown that NHE inhibition with non-selective inhibitors is neuroprotective against ischemic hippocampal injury. Pretreatment of adult gerbils with the amiloride derivative 5-(N-Ethyl-N-isopropyl)-amiloride (EIPA), a non-selective NHE inhibitor, significantly reduced the extent of CA1 pyramidal neuronal loss at 6 days following global ischemia (Phillis et al., 1999). Pretreatment of P7 mice with the non-specific NHE inhibitor MIA at 30 min before the induction of HI increased the forebrain tissue survival from 44% to 67% (Kendall et al., 2006). In the case when the brain injury was localized to the hippocampus with a mild HI, inhibition of NHE with MIA significantly reduced the injury score and TUNEL-positive cells (Kendall et al., 2006). These reports suggest that NHE plays a role in HI. However, the mechanism of neuroprotection mediated by NHE-1 after HI remains unknown.

In the current study, we examined the effects of selective NHE-1 inhibition with its potent inhibitor HOE 642 on hippocampal injury following HI. HOE 642 is a potent and highly selective NHE-1 inhibitor [IC₅₀ for NHE-1 is 0.01 μ M, NHE-2 1.6 μ M, NHE-3 1000 μ M, (Orlowski and Grinstein, 1997)]. The selectivity of HOE 642 on blocking NHE-1 has been established in parallel studies using HOE 642 in NHE-1^{+/+} neurons,

5/1/2010

NHE-1^{+/+} astrocytes, and NHE-1^{-/-} neurons or astrocytes (Luo et al., 2005; Kintner et al., 2004). HOE 642 selectively inhibits NHE-1 mediated H⁺ extrusion in the NHE-1^{+/+} cells but has no effects in NHE-1^{-/-} neurons or NHE-1^{-/-} astrocytes. Therefore, the neuroprotective benefit with the HOE 642 treatment in this study can be most likely attributed to inhibition of NHE-1.

Our two HOE 642 treatment groups (pre-, and post-HI treatment) clearly demonstrate the efficacy of pharmacological blockade of NHE-1, evident with the decreased neurodegeneration of striatal pyramidal neurons and CA1 neurons and preservation of MAP2 expression and hippocampal organization.

Neuroprotective mechanisms by inhibiting NHE-1

Maintaining pH_i values close to neutrality is a crucial task for a wide variety of cells. It is generally accepted that low pH_i values influence neuronal excitability and inhibit synaptic transmission (Chesler, 2003; Xiong et al., 2000). NHE constitutes the most efficient means of eliminating excessive acid from actively metabolizing cells (Orlowski and Grinstein, 1997). Activation of NHE activity in hippocampal nerve terminals has been detected following intracellular acidification under normoxic conditions (Jang et al., 2006; Trudeau et al., 1999). This elevated NHE activity is accompanied by an increase in [Na⁺]_i and [Ca²⁺]_i as well as increased postsynaptic currents (Jang et al., 2006; Trudeau et al., 1999). The authors attribute these changes to concurrent activation of NHE and the reverse mode of Na⁺/Ca²⁺ exchange in the nerve terminals, which leads to Ca²⁺-mediated excessive release of neurotransmitters.

Robertson et al reported a relationship between brain intracellular alkalosis and the severity of brain injury in term infants with neonatal encephalopathy (Robertson et

5/1/2010

al., 2002). In the current study, we speculate that NHE-1 activity is over-stimulated following HI as a result of protein up-regulation and/or post-translational regulation. HOE 642 may protect CA1 neurons by reducing NHE-1 and $\text{Na}^+/\text{Ca}^{2+}$ exchanger mediated intracellular Na^+ and Ca^{2+} overload, and mitochondrial dysfunction in CA1 neurons. This view is further supported by our recent report that stimulation of NHE-1 in cultured cortical neurons caused dendritic Na^+ accumulation, swelling, and a concurrent loss of Ca^{2+} homeostasis and mitochondrial bioenergetics following oxygen and glucose deprivation (Kintner et al., 2010). It has been suggested that the second wave of injury after 48 h following HI is largely mediated by apoptosis (Northington et al., 2001). In the current study, HOE 642-mediated reduction of FJ-C positive cells at 72 h after HI may reflect a decrease in apoptotic cell death in CA1 pyramidal neurons.

Abundant expression of NHE-1 in reactive astrocytes

Hippocampal astrocytes exhibit an extremely intimate relationship with neurons, which is characterized by a single hippocampal astrocyte contacting several hundred dendrites from multiple neurons and enveloping hundreds of thousands of synapses (Sofroniew and Vinters, 2010). Thus, astrocytes play a direct role in hippocampal neuronal function by maintaining ionic and neurotransmitter homeostasis of the synaptic interstitial fluid. Astrocytes can alter neuronal excitability through changing astrocyte Ca^{2+} or by regulating release of synaptically active “gliotransmitters” (glutamate, ATP, and adenosine) (Halassa et al., 2007;Perea et al., 2009).

Hippocampal astrocytes respond to HI by developing reactive astrogliosis, which is reflected by up-regulation of GFAP expression, astrocyte hypertrophy, and proliferation (Xiong et al., 2009;Hwang et al., 2008;Ouyang et al., 2007). In the current

5/1/2010

study, we detected a sharp elevation in GFAP expression in reactive astrocytes of the CA1 regions following HI, which was accompanied by hypertrophy. Interestingly, NHE-1 and GFAP were colocalized in the SR, SO regions of the ipsilateral hippocampus at 72 h following HI. Surprisingly, we did not find the decrease in NHE-1/GFAP double-stained cells or attenuation of the HI-induced GFAP up-regulation in the HOE 642-treated brains, despite the reduced CA1 neuronal injury. This leads us to speculate that NHE-1 activity in reactive astrocytes may be detrimental to neuronal survival. HOE 642 may offer neuroprotection in part via inhibition of NHE-1 activity in reactive astrocytes, indirectly protecting hippocampal neurons by reducing the detrimental effects of reactive astrocytes. This interpretation is supported by a recent report that NHE activity in hippocampal astrocytes was indeed stimulated after hypoxia (Bevensee and Boron, 2008). Currently, it is not clear how blocking NHE-1 activity in reactive astrocytes can reduce neuronal injury in hippocampus following HI. We hypothesize that this may result from reducing NHE-1 mediated Na^+ influx and astrocyte swelling, which can increase the driving force for Na^+ dependent glutamate transporter, and promote glutamate uptake from the synaptic interstitial fluid. The decrease in NHE-1- and $\text{Na}^+/\text{Ca}^{2+}$ exchange may also prevent Ca^{2+} elevation in reactive astrocytes and reduce gliotransmitter release and pro-inflammatory cytokine release.

In the current study, we investigated a role for NHE-1 in hippocampal injury after HI by pharmacologically blocking NHE-1 activity with its potent inhibitor HOE 642. The vehicle control HI brains exhibited neuronal degeneration in the ipsilateral striatum, thalamus and CA1 pyramidal neurons at 72 h. HI also triggered moderate astrogliosis in the ipsilateral hippocampus. Interestingly, the GFAP⁺ reactive astrocytes expressed an

5/1/2010

abundant level of NHE-1. Pharmacological inhibition of NHE-1 activity reduced neurodegeneration in striatum, thalamus and hippocampus. These findings suggest that NHE-1 mediated disruption of ionic homeostasis can contribute to CA1 pyramidal neuronal injury after neonatal HI. The HOE 642 post-treatment results indicate the potential of targeting NHE-1 in the development of more effective therapies for HI in neonates.

Acknowledgement

We would like to thank technical assistance from Douglas Kintner and Margaret Griesemer.

Author Disclosure Statement

This work was supported in part by University of Wisconsin Department of Pediatrics Research & Development Grant (Cengiz P), 1UL1RR025011 from the Clinical and Translational Science Award program of NCCR and NIH (Ferrazzano P), AHA SURF 09UFEL2260340 (Kleman N), NIH grants RO1NS38118 and RO1NS48216 and AHA EIA 0540154 (Sun D), NIH grants RO1NS42803 and RO1NS060120 (Messing A) and NIH P30 HD03352 (Waisman Center).

LIST OF ABBREVIATIONS

Abbreviations used are: CA (1,2,3), *Cornu Ammonis*; DG: dentate gyrus; GFAP: glial fibrillary acidic protein; HI: hypoxia-ischemia; MAP2: microtubule associated protein 2; NHE: Na⁺/H⁺ exchanger; NHE-1: Na⁺/H⁺ exchanger isoform 1; SO: stratum oriens; SP:

5/1/2010

stratum pyramidale; SR: stratum radiatum; SLM: stratum lacunosum moleculare.

REFERENCES

1. Aarts MM, Tymianski M (2003) Novel treatment of excitotoxicity: targeted disruption of intracellular signalling from glutamate receptors. *Biochem Pharmacol* 2003 Sep 15 ;66 (6):877 -86 66: 877-886.
2. Avkiran M (2001) Protection of the ischaemic myocardium by Na^+/H^+ exchange inhibitors: potential mechanisms of action. *Basic Res Cardiol* 96: 306-311.
3. Bevensee MO, Boron WF (2008) Effects of acute hypoxia on intracellular-pH regulation in astrocytes cultured from rat hippocampus. *Brain Res* 1193: 143-152.
4. Chesler M (2003) Regulation and modulation of pH in the brain. *Physiol Rev* 83: 1183-1221.
5. Douglas RM, Schmitt BM, Xia Y, Bevensee MO, Biemesderfer D, Boron WF, Haddad GG (2001) Sodium-hydrogen exchangers and sodium-bicarbonate co-transporters: ontogeny of protein expression in the rat brain. *Neuroscience* 102: 217-228.
6. Ferriero DM (2004) Neonatal brain injury. *N Engl J Med* 351: 1985-1995.
7. Halassa MM, Fellin T, Haydon PG (2007) The tripartite synapse: roles for gliotransmission in health and disease. *Trends Mol Med* 13: 54-63.
8. Hwang IK, Yoo KY, An SJ, Li H, Lee CH, Choi JH, Lee JY, Lee BH, Kim YM, Kwon YG, Won MH (2008) Late expression of Na^+/H^+ exchanger 1 (NHE1) and neuroprotective effects of NHE inhibitor in the gerbil hippocampal CA1 region induced by transient ischemia. *Exp Neurol* 212: 314-323.
9. Jang IS, Brodwick MS, Wang ZM, Jeong HJ, Choi BJ, Akaike N (2006) The $\text{Na}(+)/\text{H}(+)$ exchanger is a major pH regulator in GABAergic presynaptic nerve terminals synapsing onto rat CA3 pyramidal neurons. *J Neurochem* 99: 1224-1236.
10. Jiang X, Mu D, Biran V, Faustino J, Chang S, Rincon CM, Sheldon RA, Ferriero DM (2008) Activated Src kinases interact with the N-methyl-D-aspartate receptor after neonatal brain ischemia. *Ann Neurol* 63: 632-641.
11. Kendall GS, Robertson NJ, Iwata O, Peebles D, Raivich G (2006) N-methyl-isobutyl-amiloride ameliorates brain injury when commenced before hypoxia ischemia in neonatal mice. *Pediatr Res* 59: 227-231.
12. Kintner DB, Chen X, Currie J, Chanana V, Ferrazzano P, Chiu SY, Baba A, Matsuda T, Cohen MS, Orlowski J, Taunton J, Sun D (2010) Excessive Na^+/H^+ exchange in disruption of dendritic Na^+ and Ca^{2+} homeostasis and mitochondrial dysfunction following *in vitro* ischemia. *J Biol Chem*.

5/1/2010

13. Kintner DB, Su G, Lenart B, Ballard AJ, Meyer JW, Ng LL, Shull GE, Sun D (2004) Increased tolerance to oxygen and glucose deprivation in astrocytes from Na⁺/H⁺ exchanger isoform 1 null mice. *Am J Physiol Cell Physiol* 287: C12-C21.
14. Luo J, Chen H, Kintner DB, Shull GE, Sun D (2005) Decreased neuronal death in Na⁺/H⁺ exchanger isoform 1-null mice after in vitro and in vivo ischemia. *J Neurosci* 25: 11256-11268.
15. Ma E, Haddad GG (1997) Expression and localization of Na⁺/H⁺ exchangers in rat central nervous system. *Neuroscience* 79: 591-603.
16. Masereel B, Pochet L, Laeckmann D (2003) An overview of inhibitors of Na(+)/H(+) exchanger. *Eur J Med Chem* 38: 547-554.
17. Northington FJ, Ferriero DM, Graham EM, Traystman RJ, Martin LJ (2001) Early Neurodegeneration after Hypoxia-Ischemia in Neonatal Rat Is Necrosis while Delayed Neuronal Death Is Apoptosis. *Neurobiol Dis* 8: 207-219.
18. Orłowski J, Grinstein S (1997) Na⁺/H⁺ exchangers of mammalian cells. *J Biol Chem* 272: 22373-22376.
19. Ouyang YB, Voloboueva LA, Xu LJ, Giffard RG (2007) Selective dysfunction of hippocampal CA1 astrocytes contributes to delayed neuronal damage after transient forebrain ischemia. *J Neurosci* 27: 4253-4260.
20. Perea G, Navarrete M, Araque A (2009) Tripartite synapses: astrocytes process and control synaptic information. *Trends Neurosci* 32: 421-431.
21. Phillis JW, Estevez AY, Guyot LL, O'Regan MH (1999) 5-(N-Ethyl-N-isopropyl)-amiloride, an Na⁺-H⁺ exchange inhibitor, protects gerbil hippocampal neurons from ischemic injury. *Brain Res* 839: 199-202.
22. Robertson NJ, Cowan FM, Cox IJ, Edwards AD (2002) Brain alkaline intracellular pH after neonatal encephalopathy. *Ann Neurol* 52: 732-742.
23. Schmued LC, Stowers CC, Scallet AC, Xu L (2005) Fluoro-Jade C results in ultra high resolution and contrast labeling of degenerating neurons. *Brain Res* 1035: 24-31.
24. Scholz W, Albus U, Counillon L, Gogelein H, Lang HJ, Linz W, Weichert A, Scholkens BA (1995) Protective effects of HOE-642, a selective sodium-hydrogen exchange subtype 1 inhibitor, on cardiac ischaemia and reperfusion. *Cardiovasc Res* 29: 260-268.
25. Sofroniew MV, Vinters HV (2010) Astrocytes: biology and pathology. *Acta Neuropathol* 119: 7-35.
26. Trudeau LE, Parpura V, Haydon PG (1999) Activation of neurotransmitter release in hippocampal nerve terminals during recovery from intracellular acidification. *J Neurophysiol* 81: 2627-2635.

5/1/2010

27. Vannucci RC, Vannucci SJ (2005) Perinatal hypoxic-ischemic brain damage: evolution of an animal model. *Dev Neurosci* 27: 81-86.
28. Xiong M, Yang Y, Chen GQ, Zhou WH (2009) Post-ischemic hypothermia for 24h in P7 rats rescues hippocampal neuron: association with decreased astrocyte activation and inflammatory cytokine expression. *Brain Res Bull* 79: 351-357.
29. Xiong ZQ, Saggau P, Stringer JL (2000) Activity-dependent intracellular acidification correlates with the duration of seizure activity. *J Neurosci* 20: 1290-1296.
30. Zhou M, Xu W, Liao G, Bi X, Baudry M (2009) Neuroprotection against neonatal hypoxia/ischemia-induced cerebral cell death by prevention of calpain-mediated mGluR1alpha truncation. *Exp Neurol* 218: 75-82.

FIGURE LEGENDS

Figure 1. Inhibition of NHE-1 with HOE 642 reduces neuronal damage in hippocampus and striatum.

FJ-C staining was performed to assess neuronal damage in the ipsilateral hippocampus and striatum at 72 h following HI. **A, C, E, G:** brightfield images. **B, D, F, H:** FJ-C staining. **A, B, E, F:** vehicle (saline) controls. **C, D:** Pre-treatment with the initial dose of HOE 642 (0.5 mg/kg, ip) administered at 5 min before HI induction and three subsequent doses (0.5 mg/kg, ip) administered at 24 h and 48 h after HI. **G, H:** Post-treatment with HOE 642 (0.5 mg/kg, ip) administered at 10 min, 24 h and 48 h after HI. Multiple doses were used to maintain an effective drug concentration (half-life of HOE 642 is 40 min in rats, (Scholz et al., 1995). **Arrowhead:** severe degeneration; **Arrow:** reduced cell degeneration. Scale bar: 1 mm.

Figure 2. Quantification of HOE 642 mediated protection in CA1 hippocampal neurons.

5/1/2010

A. Brain sections were harvested at 72 h after 55-min HI and stained with FJ-C. **a, b:** FJ staining images of the contralateral and ipsilateral hippocampus in the vehicle control mouse. **c, d:** Magnified images of CA1 region of the ipsilateral hippocampus in vehicle control or HOE 642 pre-treated mouse. **Arrowhead:** severe degeneration; **Arrow:** reduced degeneration. Specific hippocampal regions were indicated, SO: stratum oriens; SP: stratum pyramidale; SR: stratum radiatum; SLM: stratum lacunosum. Scale bar: 1 mm (a, b) and 100 μ m (c, d). **B.** Data are expressed as mean neurological damage score \pm S.D. A score was given as: 0: no FJ-C positive cells, 1: 1-500, 2: 501-1000, 3: 1001-1500, 4: >1500 FJ-C positive cells.

Figure 3. Changes of MAP2 in the ipsilateral hippocampus following HI.

Changes of MAP2 and GFAP in the ipsilateral hippocampus were examined at 72 h after HI. The vehicle control and HOE 642 pre-treatment were described in Figure 1 legend. Specific hippocampal regions (SO, SP, SR, SLM) were indicated similarly to Figure 2 legend. **a, b, e, f:** Double immunostaining for MAP2 (green) and GFAP (red) in the CA1 regions. ToPro-3 Iodide (blue) was used as a nuclear staining. **c, d, g, h:** 40 x magnification of the CA1 region indicated in the white boxes. **Arrowhead:** severe degeneration; **Arrow:** reduced degeneration; **Open arrowhead:** GFAP⁺ reactive astrocytes. Scale bar: 300 μ m (a, b, e, f) and 75 μ m (c, d, g, h). Images were representative from n = 8 in each group.

Figure 4. Changes of NHE-1 expression in hippocampus following HI.

A. Expression of NHE-1 was determined in P1-P30 mouse brains. 150 μ g total protein was loaded per lane and the immunoblot was probed for NHE-1 and β -tubulin on the same blot. **B.** Immunofluorescence staining in the CA1 region of brains at 72 h after HI.

5/1/2010

NHE-1 (red), GFAP (green) and To-Pro-3 (blue). Specific hippocampal regions (SP, SR, SLM) were indicated similarly to Figure 2 legend. **a, b**: vehicle control brain. **c, d**: HOE 642 post-treated brain. **e-h**: magnified regions indicated the box in a-d. **f'**: z-section image in f. **C**. Summary data. Data are expressed as mean \pm S.D. n = 4-5.

Arrowhead: NHE-1/GFAP double-positive cells. Scale bar: 75 μ m (a-d, e-h) and 10 μ m (in f'); **Arrow**: CA1 neuron; **open arrowhead**: non-reactive astrocytes lacking GFAP expression.

5/1/2010

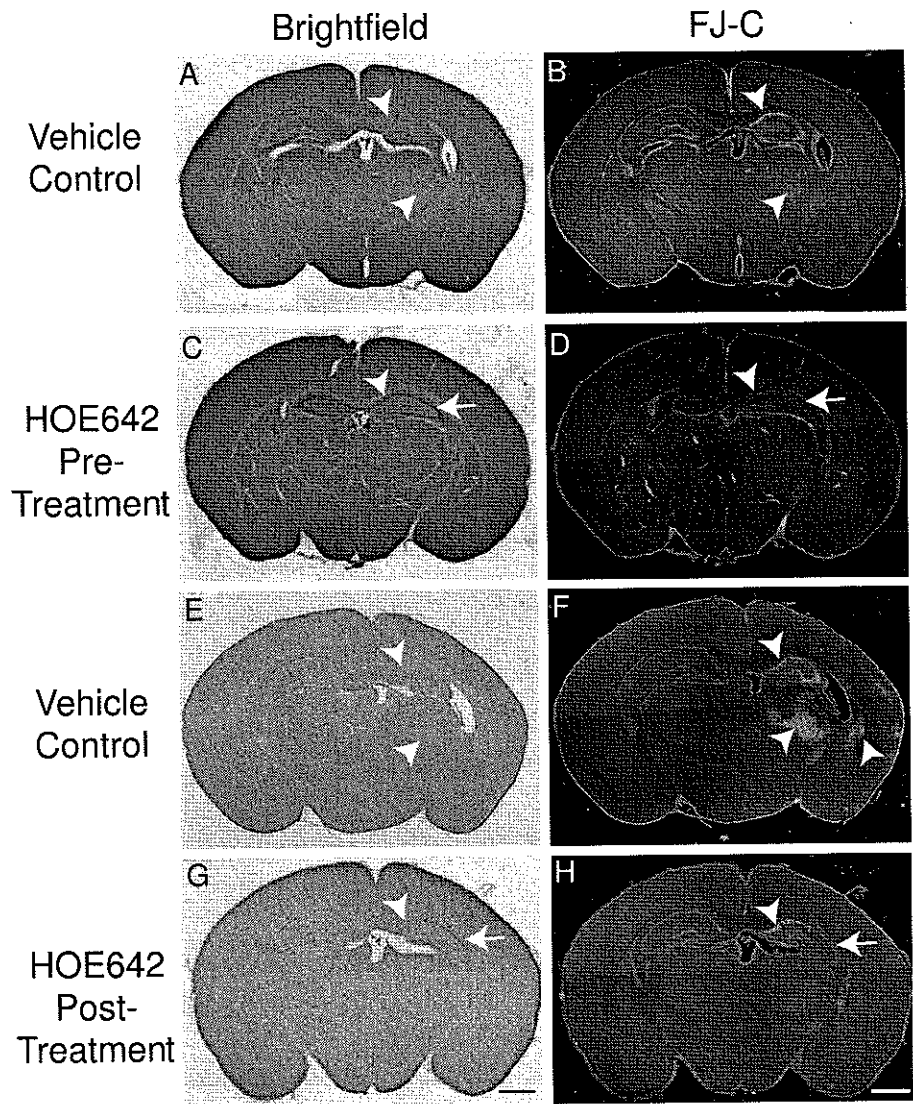


Figure 1

5/1/2010

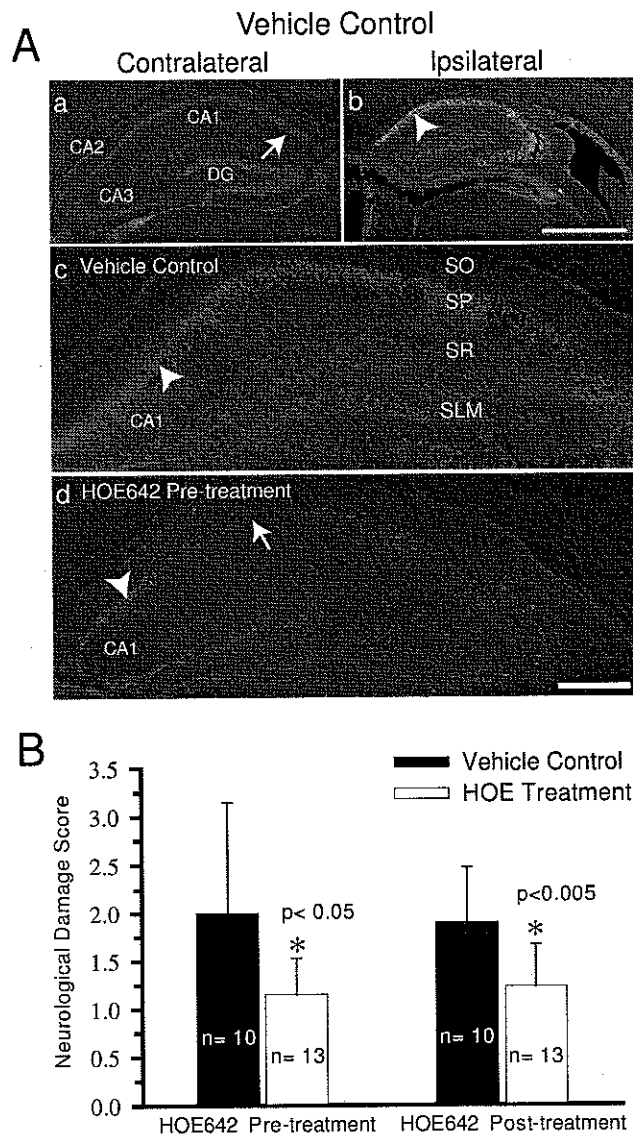


Figure 2

5/1/2010

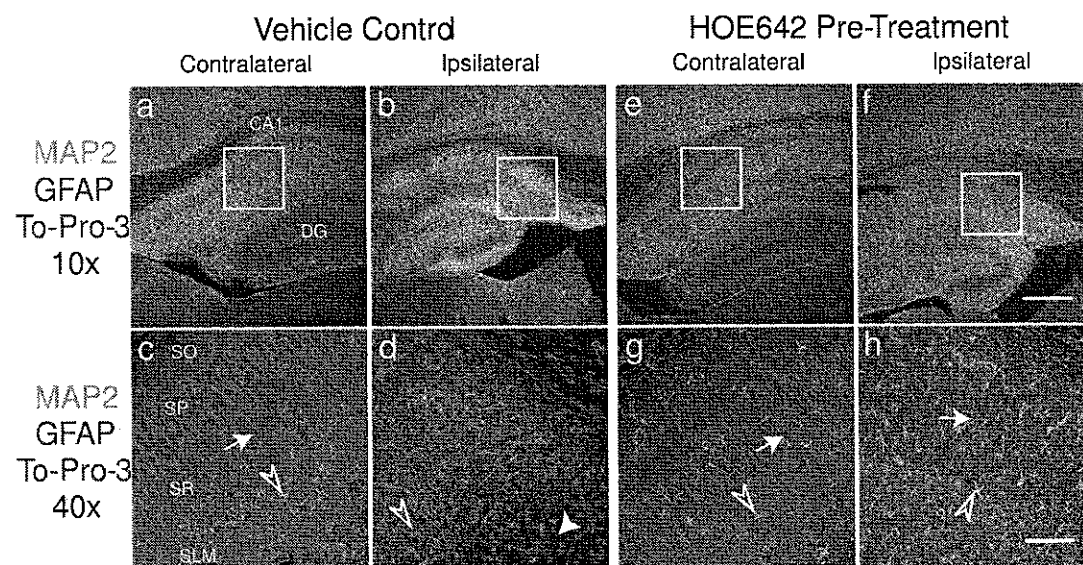


Figure 3

5/1/2010

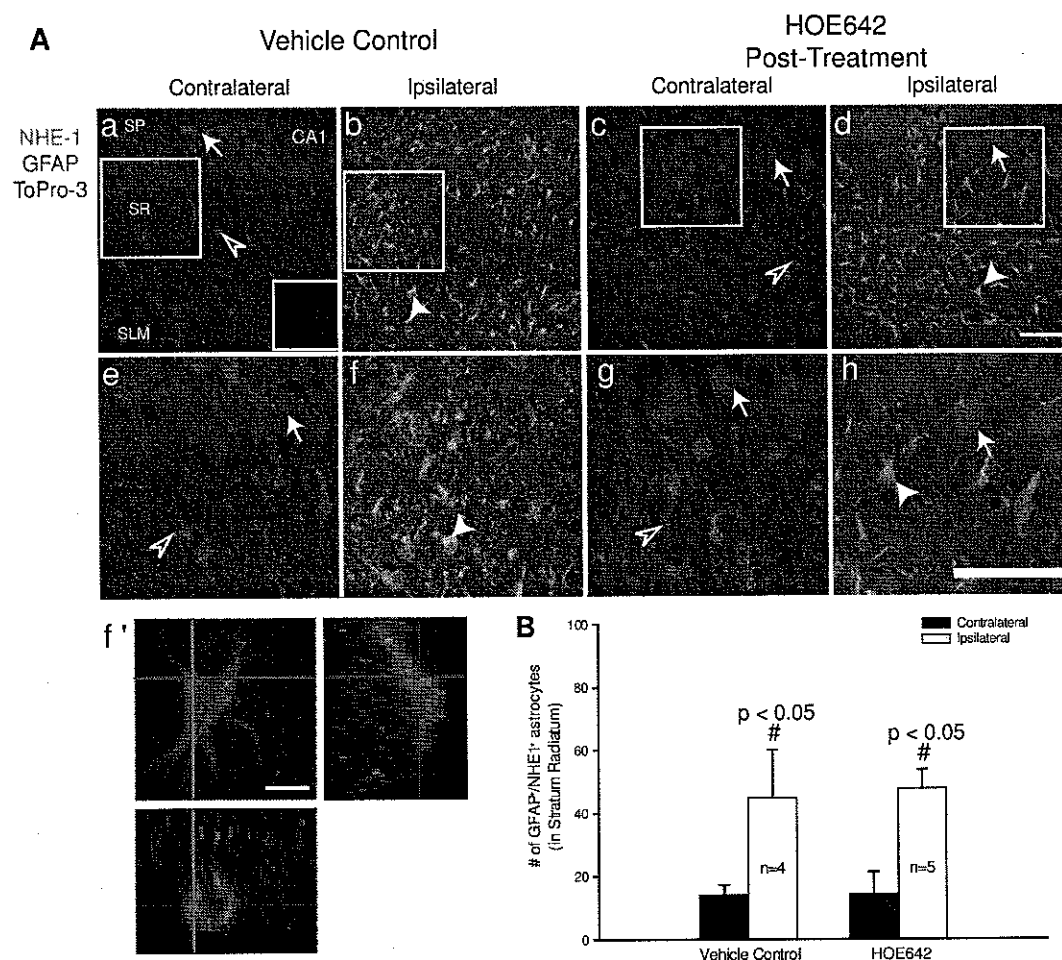


Figure 4