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The Na⁺/H⁺ Exchanger-1 as a New Molecular Target in Stroke Interventions

Vishal Chanana¹, Dandan Sun² and Peter Ferrazzano^{1,3}

¹Waisman Center, University of Wisconsin, Madison, WI

²Dept. of Neurology, University of Pittsburgh, Pittsburgh, PA

³Dept. of Pediatrics, University of Wisconsin, Madison, WI
USA

1. Introduction

Loss of ion homeostasis plays an important role in the pathogenesis of ischemic cell damage. Ischemia induces accumulation of intracellular Na⁺ ([Na⁺]_i) and Ca²⁺ ([Ca²⁺]_i), and subsequent activation of proteases, phospholipases, and formation of oxygen and nitrogen free radicals. The Na⁺/H⁺ exchanger (NHEs) family is a group of secondary active membrane transport proteins that catalyze the electroneutral exchange of Na⁺ for H⁺ and is important in restoring intracellular pH (pH_i) after ischemia-induced intracellular acidosis. Nine isoforms of NHE (NHE1-9) have been identified in mammalian tissues (Orlowski & Grinstein, 2004). These isoforms differ in their tissue expression, subcellular distribution, kinetic properties, inhibitor sensitivity, and physiological functions. NHE-1 is ubiquitously expressed on the plasma membrane of virtually all mammalian cell types (Sardet et al., 1989). NHE-2-4 are expressed on the plasma membrane, predominantly in the epithelia of the kidney and gastrointestinal tract (Orlowski & Grinstein, 2004). NHE-3 is the only isoform known to recycle between the plasma membrane and the endosomal compartment (D'Souza et al., 1998). NHE-5 expression is concentrated in neurons (Attaphitaya et al., 1999) and may modulate the pH of synaptic vesicles (Szaszi et al., 2002). NHE-6 and NHE-9 are expressed predominantly in endosomal vesicles (Nakamura et al., 2005) and NHE-7 localizes to the trans-Golgi network and associated endosomes (Numata & Orlowski, 2001). NHE-8 has been localized to the plasma membrane of renal proximal tubule epithelial cells, and to endosomal vesicles and the trans-Golgi network (Goyal et al., 2003; Nakamura et al., 2005).

NHE-1 is the most extensively studied isoform, and the most abundant isoform in the CNS (Ma & Haddad, 1997; Orlowski et al., 1992). Research over the past two decades has expanded our understanding of the role of NHE-1 beyond that of simply maintenance of ion homeostasis and cell volume, to an emerging picture of a regulator of many cell functions. NHE-1 plays a role in regulation of cell proliferation, migration (Bussolino et al., 1989), and the microglial respiratory burst (Liu et al., 2010). NHE-1 protein consists of 815 amino acids with a calculated molecular weight of 85 kDa. However, NHE-1 has an apparent size of ~110 kDa due to its N- and O- linked glycosylation in the extracellular loop 1. NHE-1 has

two large functional domains, the highly conserved amphipathic N-terminal domain (~500 amino acids), which is responsible for cation translocation, and a less conserved hydrophilic cytoplasmic C-terminal domain (~315 amino acids), which is crucial for modulating NHE-1 activity (Putney et al., 2002). Activation of NHE-1 has been shown to be a pivotal event in cell damage induced by ischemia and reperfusion in the brain (Horikawa et al., 2001; Hwang et al., 2008; Luo et al., 2005), heart (Liu et al., 1997; Murphy et al., 1991; Wang et al., 2003), liver (Gores et al., 1989), and lungs (Rios et al., 2005). Here we will review recent findings implicating NHE-1 activation as a critical event in the pathogenesis of cellular dysfunction after cerebral ischemia, and the growing evidence supporting the use of NHE inhibitors as neuroprotective agents following cerebral ischemia.

2. Na⁺/H⁺ Exchanger isoform-1 (NHE-1) in cerebral ischemia

Ischemia and reperfusion injury is a complex and incompletely understood phenomenon. Ischemia deprives the cell of the energy required for normal cell function and leads to loss of ionic homeostasis within the cell due to opening of ionotropic glutamate receptors (Nishizawa, 2001) and acid sensing non-glutamate-dependent channels (Xiong et al., 2004), as well as activation of ion transport proteins such as NHE-1, Na⁺/K⁺/Cl⁻ cotransporter (NKCC1) (Chen et al., 2005), and the Na⁺/Ca²⁺ exchanger (NCXs) (Hoyt et al., 1998). Reperfusion triggers a cascade of intracellular events including release of reactive oxygen species (ROS) and inflammatory mediators, which exacerbate injury and promote cell death. Ischemia and reperfusion induces intracellular acidosis due to a shift from aerobic to anaerobic glycolysis, and leads to an increase in [Na⁺]_i and [Ca²⁺]_i by mechanisms that include the activation of acid responsive ion transporters (Yao & Haddad, 2004). Recent findings from our group and others highlight the important role of NHE-1 in pH_i regulation after cerebral ischemia and reperfusion.

2.1 NHE-1 mediated intracellular pH regulation

To regulate and maintain constant pH_i, eukaryotic cells express plasma membrane ion transporters such as NHE-1 that protect cells from internal acidification by exchanging extracellular Na⁺ for intracellular H⁺ (Luo et al., 2005). At physiological pH_i, NHE-1 is essentially inactive, despite the large inward Na⁺ gradient established by Na⁺/K⁺-ATPase. However, upon exposure to intracellular acidification, NHE-1 is rapidly activated and uses the electrochemical gradient of Na⁺ to pump H⁺ out of the cell and restore pH_i. Upon restoration of pH_i, NHE-1 activity returns to steady state levels (Pedersen, 2006). Extracellular acidification (low pH_o) or removal of extracellular sodium suppresses this gradient-driven Na⁺/H⁺ exchange (Bobulescu et al., 2005). While NHE-1 serves to maintain homeostasis in the face of normal pH_i fluctuations (which result from changes in metabolic activity), profound acidosis after anoxia can induce a NHE-1 mediated paradoxical alkalinization, a so-called “overshoot” of pH_i restoration. We reported that post-anoxia alkalinization is ablated by pharmacological inhibition of NHE-1 and removal of extracellular sodium (Kintner et al., 2005). Protein kinase inhibitors attenuate this alkalinization, suggesting that activation of NHE-1 involves protein phosphorylation and multiple up-stream regulatory pathways such as extracellular signal-regulated kinases (ERK 1/2), protein kinase A (PKA), and protein kinase C (PKC) (Kintner et al., 2005; Luo et al., 2007; Yao et al., 2001).

2.2 Ionic homeostasis and brain cell function

Secondary active ion transport proteins are important in maintaining steady-state intracellular ion concentrations. NHE-1 plays an important role in regulation of many cellular processes in addition to pH_i and cell volume regulation, such as cell growth, proliferation and differentiation, cell migration and adhesion, cellular immunity, and as cytoskeletal scaffolding for the assembly of intracellular signaling complexes (De Vito, 2006; Luo & Sun, 2007; Luo et al., 2005; Meima et al., 2007; Orłowski & Grinstein, 2004; Pedersen et al., 2006; Xue & Haddad, 2010). Due to their high metabolic rate and rapid changes in metabolic demand, neurons are exposed to frequent fluctuations in pH_i, making efficient acid extrusion mechanisms essential for normal neuronal function. Neurons and astrocytes from mice deficient in the NHE-1 protein (NHE-1^{-/-}) demonstrate decreased basal pH_i and are unable to recover from an acid load (Luo et al., 2005). NHE-1 is the predominant NHE isoform in the CNS (Ma & Haddad, 1997; Orłowski et al., 1992), and as evidence of its importance in normal neurologic function, NHE-1^{-/-} mice exhibit severe neurologic defects and seizures (Bell et al., 1999; Gu et al., 2001).

2.3 Role of NHE-1 in cellular dysfunction and cerebral injury during *in vivo* ischemia

Results from *in vivo* experimental studies support the importance of ion transport proteins in ischemia-mediated loss of ion homeostasis. NHE-1 activity in astrocytes (Cengiz et al., 2010), neurons (Manhas et al., 2010), and microglia (Shi et al., 2011) is stimulated following cerebral ischemia. Excessive stimulation of NHE-1 leads to intracellular Na⁺ overload, and in turn causes a rise in intracellular Ca²⁺ due to increased Ca²⁺ influx via reversal of the Na/Ca exchanger. Thus, NHE-1 activity contributes to cerebral ischemic damage in part by disruption of intracellular Na⁺ and Ca²⁺ homeostasis, an event which is characterized by rapid influx of Ca²⁺ and subsequent cell death.

2.3.1 Global ischemia

Global cerebral ischemia entails diminution in cerebral blood flow (CBF) over the entire brain, and is encountered clinically in cardiac arrest. On restoration of CBF, a secondary reperfusion brain injury may occur due to altered ionic homeostasis, increases in ROS, cerebral edema, and inflammatory cascades (Schaller & Graf, 2004). The contribution of NHE-1 activity to global cerebral ischemia has been reported in a number of animal models. In a gerbil model of transient forebrain ischemia, NHE-1 immunoreactivity was markedly increased in CA1 pyramidal neurons as well as in glial cells 4 days following injury, and inhibition of NHE protected CA1 pyramidal neurons and attenuated the activation of astrocytes and microglia (Hwang et al., 2008). Yorkshire-Duroc pigs treated with cariporide (HOE 642), a potent and selective inhibitor of NHE-1, at the onset of a 90 min deep hypothermic circulatory arrest demonstrated improved neurologic recovery (Castellá et al., 2005). Similarly, inhibition of NHE-1 with N-[aminoiminomethyl]-1-methyl-1H-indole-2-carboxamide methanesulfonate (SM-20220) improved neurologic function in a gerbil model of transient global cerebral ischemia (Kuribayashi et al., 2000). Administration of the NHE-1 inhibitor ethylisopropylamiloride (EIPA) prior to bilateral carotid artery occlusion in gerbils resulted in decreased hippocampal neuronal cell death and improved neurologic function (Phillis et al., 1999).

2.3.2 Focal ischemia

Unlike global cerebral ischemia, focal cerebral ischemia entails reduction in regional CBF in a specific vascular territory and is usually encountered clinically as an “ischemic stroke” due to thromboembolic or vaso-occlusive disease. An abundance of *in vivo* studies support the importance of NHE-1 in focal ischemia. The NHE-1 inhibitor SM-20220 reduces infarct size in both transient and permanent focal ischemia models (Kuribayashi et al., 1999). Another NHE inhibitor, Sabiporide, reduces infarct size and edema volume when administered before or after ischemia (Park et al., 2005).

Our investigations into the role of NHE-1 in cerebral ischemia have used both genetic and pharmacologic inhibition of NHE-1 in a mouse transient middle cerebral artery occlusion model (MCAO). Mice treated with HOE 642, a potent and selective inhibitor of NHE-1, prior to MCAO demonstrated a 35% reduction in infarct volume compared to vehicle treated controls. NHE-1 heterozygous mice (NHE-1^{+/-}), which demonstrate a ~ 70% reduction in NHE-1 protein expression, exhibited a similar reduction in infarct volumes, establishing the importance of NHE-1 over other NHE isoforms in the CNS (Luo et al., 2005; Wang et al., 2008). With T2-weighted and Diffusion Weighted MRI, we further confirmed that NHE-1^{+/+} mice treated with HOE 642 immediately prior to reperfusion or 60 minutes post-reperfusion, exhibited a significant reduction in infarct volume compared to NHE-1^{+/+} vehicle control mice. NHE-1^{+/-} mice demonstrated a significant reduction in infarct volume on T2 MRI at 72 hours after injury (Ferrazzano et al., 2011). These findings suggest that elevated NHE-1 activity contributes to neuronal injury following ischemia and reperfusion. We subsequently revealed that focal cerebral ischemia triggers a transient stimulation of the extracellular signal-regulated kinase/p90 ribosomal S6 kinase (ERK/p90^{RSK}) pathway that contributes to ischemic damage in part via phosphorylation of NHE-1 protein (Manhas et al., 2010). The NHE-1-mediated [Na⁺]_i overload causes reverse function of the Na⁺/Ca²⁺ exchanger, elevating [Ca²⁺]_i and enhancing the p38 mitogen-activated protein kinase (MAPK) and/or nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Liu et al., 2010). NHE-1 activity also plays a detrimental role in mitochondrial Ca²⁺ overload and mitochondrial dysfunction after ischemia as evidenced by attenuation of ischemia-induced cytochrome C release from mitochondria after NHE-1 inhibition (Wang et al., 2008). Interestingly, when NHE-1 activity is blocked either pharmacologically or by genetic knockdown, microglia activation and proinflammatory cytokine formation is significantly reduced in ischemic brains after MCAO (Shi et al., 2011). Taken together, these results strongly support that NHE-1 is activated after cerebral ischemia and worsens ischemic brain injury.

2.3.3 Hypoxia/Ischemia

Hypoxia-ischemia (HI) is a common cause of brain injury in neonates (Ferriero, 2004). We recently investigated the role of NHE-1 using a mouse model of neonatal hypoxia-ischemia as described by Vannucci (Vannucci & Vannucci, 2005). In these studies, post-natal day 9 mice (P9) underwent unilateral carotid artery ligation and subsequent exposure to 55 minutes of 8% O₂. Following carotid ligation, mice were treated with HOE 642 either immediately before or 10 minutes following exposure to hypoxia (Cengiz et al., 2010). Following HI, vehicle-treated control brains exhibited astrogliosis in the ipsilateral hippocampus, and reactive astrocytes expressed an abundant level of NHE-1. Inhibition of NHE-1 before or after HI resulted in decreased neurodegeneration in striatum, thalamus

and hippocampus and improved performance on tests of motor learning and memory (Cengiz et al., 2010). These findings suggest that NHE-1 mediated disruption of ionic homeostasis can contribute to CA1 pyramidal neuronal injury after neonatal HI. Moreover, T2 weighted and Diffusion Tensor (DTI) MRI revealed that NHE-1 inhibition with HOE 642 after HI resulted in improved white matter injury in the corpus callosum, which correlated with improvements in memory and learning (Cengiz et al., 2011).

2.4 Role of NHE-1 in cellular dysfunction during *in vitro* ischemia

Extensive *in vitro* studies have established that ischemia stimulates NHE-1 by reduction in pH_i or via signaling pathways such as ERK-p90^{rsk}, PKA or PKC (Dunbar & Caplan, 2001; Herrera et al., 1994; Kintner et al., 2007a; Li et al., 2004). The role of NHE-1 in cerebral ischemia has been mainly examined in two types of *in vitro* ischemic models, oxygen glucose deprivation/reoxygenation (OGD/REOX) or the hypoxic, acidic, ion-shifted Ringers's solution (HAIR). Superfused brain slices also represent a useful preparation to study acid-base disturbance that occurs in the mammalian brain during *in vitro* ischemic conditions.

2.4.1 Cell cultures

NHE-1 activity is stimulated during *in vitro* ischemia and subsequent reoxygenation and contributes substantially to neuronal and glial cell injury. Acutely isolated CA1 neurons exhibit a tri-phasic response to 5 minutes of anoxia. During anoxia, an initial acidification progresses to alkalinization that is followed by further alkalinization on exposure to reoxygenation. This alkalinization is attenuated by reduction of external pH, removal of extracellular sodium, or inhibition of NHE-1 (Sheldon & Church, 2002; Yao et al., 2001). Additionally, inhibition of PKA can block post-anoxia alkalinization, suggesting cAMP-dependent signaling pathways for NHE-1 activation (Sheldon & Church, 2002).

We have demonstrated that NHE-1 is essential in pH_i regulation using an internal acid load in cultured cortical neurons (Luo et al., 2005). Additionally, we found that activation of NHE-1 after OGD/REOX results in a significant increase in neuronal [Na⁺]_i. This rise in [Na⁺]_i following OGD is significantly attenuated in HOE 642-treated or NHE-1^{-/-} neurons, and cell death is reduced (Luo et al., 2005). In a separate study, we demonstrated that NHE-1-mediated Na⁺ entry leads to reverse activation of the Na⁺/Ca²⁺ exchanger (NCX_{rev}) and rise in [Ca²⁺]_i, which contribute to the selective dendritic vulnerability to *in vitro* ischemia (Kintner et al., 2010). Taken together, our studies suggest that NHE-1 activity in neurons is significantly stimulated in response to the metabolic acidification associated with an ischemic insult. This ischemia-induced increase in NHE-1 activity causes intracellular Na⁺ and Ca²⁺ overload, and eventually leads to cell death.

In another series of studies, we examined the role of NHE-1 in ischemic astrocyte damage using OGD/REOX in cultured cortical astrocytes and found that NHE-1 is the primary pH regulatory mechanism after ischemia. Astrocyte NHE-1 activity is increased by ~ 1.8 fold during REOX (Kintner et al., 2004), and depends on ERK1/2 signaling pathways (Kintner et al., 2005). OGD/REOX results in a drop in pH_i by 0.29 pH units (Kintner et al., 2004), and inhibition of NHE-1 results in a further decrease of pH_i. Additionally, we observed that OGD/REOX triggers a ~5-fold increase in [Na⁺]_i and 26% increase in astrocyte cell volume. This increase in [Na⁺]_i and cell swelling are significantly reduced either with HOE 642 treatment or in NHE-1^{-/-} astrocytes (Kintner et al., 2004). Using the HAIR model in

astrocytes, we found a similar increase in $[Na^+]_i$ which could be abolished by the NHE-1 inhibitor HOE 642 (Kintner et al., 2007b). It has been reported that the expected rise in $[Ca^{2+}]_i$ after HAIR exposure is inhibited by NHE-1 inhibition with HOE 694 (Bondarenko et al., 2005). Taken together, these results indicate that NHE-1 activity raises $[Na^+]_i$ which fosters reversal of the Na^+/Ca^+ exchanger leading to increased intracellular Ca^{2+} and astrocyte cell death.

More recently, new evidence supports a role of NHE-1 in microglial pH_i regulation. Microglia activation by lipopolysaccharide (LPS), phorbol myristate acetate (PMA), or OGD/REOX triggers a concurrent stimulation of NHE-1 and NADPH oxidase (Liu et al., 2010). The elevation in NHE-1-mediated H^+ extrusion prevents intracellular acidosis, allowing for sustained NADPH oxidase function (Liu et al., 2010). Moreover, the coupling of NHE-1 activation with NCX_{rev} activates $[Na^+]_i$ and $[Ca^{2+}]_i$ dependent signaling, which promotes the microglial respiratory burst and production of proinflammatory cytokines (Liu et al., 2010).

2.4.2 Brain slice

Few studies have used brain slice preparations to examine acid-base homeostatic disturbances during ischemia. In hippocampal slices, hypoxia induces a significant drop in both pH_i and pH_o , and a brief alkaline peak is also occasionally observed (Fujiwara et al., 1992; Melzian et al., 1996; Roberts & Chih, 1997). In slice preparations from various brain regions, hypoxia causes acidosis with an approximately 0.8-1.2 pH_i unit drop (Ballanyi et al., 1996; Knopfel et al., 1998; Pirttila & Kauppinen, 1994). Cytosolic calcium changes are observed during ischemia in cortical brain slices that can be only partially inhibited by combined blockade of ion channels (Bickler and Hansen, 1994). Only one report shows a direct involvement of NHE mediated pH_i regulation in slice preparations. In brainstem slices from neonatal rats exposed to 10 minutes of anoxia, intracellular pH drops by 0.1-0.3 pH units in neurons. Inhibition of NHE with amiloride increases this anoxia-induced intracellular acidification (Chambers-Kersh et al., 2000).

2.5 NHE-1 inhibitors and potential therapies

Despite decades of research, the effective treatment and prevention of cerebral ischemic injury remains challenging. Inhibition of NHE-1 with either pharmacological agents or genetic ablation has been demonstrated to significantly reduce brain damage after ischemic insult, in both *in vitro* and *in vivo* models. These encouraging findings suggest the potential use of NHE inhibitors as neuroprotective therapies after cerebral ischemia.

2.5.1 Pharmacological approach

Two major classes of pharmacological agents are currently used to inhibit NHE-1 activity (Putney et al., 2002). The first class of drugs includes amiloride and its 5' alkyl-substituted derivatives (Counillon et al., 1993; Yu et al., 1993), such as ethylisopropylamiloride (EIPA), dimethylamiloride (DMA), 5-*N* (methylpropyl)amiloride (MPA), 5-(*N*-methyl-*N*-isobutyl)-amiloride (MIBA), and 5-(*N*, *N*-hexamethylene) amiloride (HMA). These agents are more effective inhibitors of NHE-1 than amiloride but have relatively weak selectivity toward NHE-1. The simultaneous replacement of the pyrazine ring by a phenyl and of the 6-chloro by sulfomethyl leads to another class of inhibitors that includes the benzoylguanidines and derivatives such as HOE 694 (Counillon et al., 1993) and HOE 642 (cariporide) (Scholz et al.,

1995). Both classes are more specific for NHE-1 than NHE-3, with the amiloride compounds demonstrating ~10²-fold increased specificity and the HOE compounds ~10³- to 10⁵-fold more NHE-1 specificity. The HOE compounds are viewed as the most promising agents for treatment of ischemia-reperfusion injury due to their selectivity for NHE-1, and excellent solubility, resorption, and bioavailability profiles (Scholz et al., 1999; Baumgarth et al., 1997; Xue & Haddad, 2010). HOE compounds are competitive inhibitors of Na⁺ binding at the extracellular cation-binding site (Baumgarth et al., 1997; Counillon et al., 1993; Kinsella & Aronson, 1981; Mahnensmith & Aronson, 1985), while the amiloride derivatives also act non-competitively (Warnock et al., 1988). More recently, several new molecules have been designed as potential NHE blockers based on the bicyclic template, including SM-20220, SM-20550, BMS-284640, T-162559, and TY-12533, which have also shown promising results in *in vivo* studies of cerebral ischemia (Kitayama et al., 2001). The IC₅₀ for the human NHE-1 are as follows: Amiloride = 10.7 μM, Cariporide = 0.08 μM, T-165229 = 13 nM. Importantly, the NHE inhibitors HOE 642 and SM-20220 not only reduce cell death and edema, but also improve neurological function in *in vivo* ischemia models, and have demonstrated benefits when administered after ischemia (Kintner et al., 2007b; Kuribayashi et al., 2000).

2.5.2 Transgenic approach

While pharmacological studies indicate that NHE-1 plays a central role in cerebral ischemia-reperfusion injury, the use of pharmacologic inhibitors to study ion transport function raises questions regarding dosing, absorption, species specific T_{1/2}, and non-specific effects. For this reason, confirmation by an alternative method using NHE-1 knockdown mice is warranted. NHE-1^{-/-} mice exhibit neurologic abnormalities, seizures, ataxia, and growth retardation, and do not survive into adulthood (Bell et al., 1999; Gu et al., 2001). Therefore, NHE-1^{-/-} mice are useful for cultures of NHE-1 null neurons, astrocytes and microglia, but cannot be used for *in vivo* studies. NHE-1^{+/-} mice express <50% of NHE-1 protein levels, and are useful for *in vivo* studies of the role of NHE function after cerebral ischemia. A marked decrease of infarct volume, microglial activation and proinflammatory cytokine formation is found in NHE-1^{+/-} mice after MCAO (Luo et al., 2005). NHE-1^{+/-} and NHE-1^{-/-} cortical neurons and astrocytes demonstrate decreased cell death after OGD/REOX (Luo et al., 2005).

The fact that NHE-1 inhibitors applied during or after cerebral ischemia protect the brain against ischemic damage is now well established in animal studies. Despite the uniformity of results from animal models, a number of challenges remain before NHE-1 inhibitors can be translated into clinical use. Questions regarding safety, optimal dose, and timing of administration remain to be addressed, and large animal studies demonstrating improved functional outcomes are still lacking.

3. Conclusion

NHE-1 plays a pivotal role in maintaining tissue ionic homeostasis under normal physiological conditions. However, excessive stimulation of NHE-1 appears to be a major contributor to cellular damage in ischemic conditions. The proposed mechanism for injury induced by NHE-1 activation includes accumulation of [Na⁺]_i, subsequent [Ca²⁺]_i overload via reverse activation of the Na⁺/Ca²⁺ exchanger, and eventual cell death. Additionally, activation of MAPKs, and release of excitatory amino acids and ROS also contribute to cell

damage and death after ischemia. NHE-1 inhibitors have been demonstrated to be neuroprotective in both *in vitro* and *in vivo* ischemia models, making NHE-1 an attractive therapeutic target for cerebral ischemia. Thus, mechanisms of NHE-1 activation in ischemia continue to present an interesting focus for future research in this field.

4. Acknowledgment

This work was supported by NIH grants R01NS48216 and R01NS38118 (D. Sun), 1UL1RR025011 from the Clinical and Translational Science Award (CTSA) program of the National Center for Research Resources (NCRR) (P. Ferrazzano), and P30HD03352 (Waisman Center).

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Advances in the Preclinical Study of Ischemic Stroke

Edited by Dr. Maurizio Balestrino

ISBN 978-953-51-0290-8

Hard cover, 530 pages

Publisher InTech

Published online 16, March, 2012

Published in print edition March, 2012

This book reports innovations in the preclinical study of stroke, including - novel tools and findings in animal models of stroke, - novel biochemical mechanisms through which ischemic damage may be both generated and limited, - novel pathways to neuroprotection. Although hypothermia has been so far the sole "neuroprotection" treatment that has survived the translation from preclinical to clinical studies, progress in both preclinical studies and in the design of clinical trials will hopefully provide more and better treatments for ischemic stroke. This book aims at providing the preclinical scientist with innovative knowledge and tools to investigate novel mechanisms of, and treatments for, ischemic brain damage.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Vishal Chanana, Dandan Sun and Peter Ferrazzano (2012). The Na⁺/H⁺ Exchanger-1 as a New Molecular Target in Stroke Interventions, *Advances in the Preclinical Study of Ischemic Stroke*, Dr. Maurizio Balestrino (Ed.), ISBN: 978-953-51-0290-8, InTech, Available from: <http://www.intechopen.com/books/advances-in-the-preclinical-study-of-ischemic-stroke/the-na-h-exchanger-1-as-a-new-molecular-target-in-stroke-interventions>

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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