“FEDERICO II”

UNIVERSITY OF NAPLES

SCHOOL OF MEDICINE

PHD PROGRAM IN NEUROSCIENCE

XXVI CYCLE

PhD THESIS WORK:

ROLE OF SODIUM/CALCIUM EXCHANGER IN NEURONAL DISFUNCTIONS FOLLOWING HYPOXIC-ISCHEMIC INJURY IN NEONATAL MICE

COORDINATOR: PROF. LUCIO ANNUNZIATO

TUTOR: GIUSEPPE PIGNATARO

CANDIDATE: PIERPAOLO CERULLO

ABSTRACT 4

1. PREMISE 6

2. INTRODUCTION 10

2.1 NEONATAL HYPOXIA ISCHEMIA PATHOPHYSIOLOGY 10
2.1.1 MECHANISM OF CELL DEATH: EXCITOTOXICITY 12
2.1.2 OTHER NON-NMDA DEPENDENT PATHWAYS 14
2.1.3 OXIDATIVE STRESS 15
2.1.4 INFLAMMATION 16
2.1.5 NECROSIS AND APOPTOSIS 16
2.2 CLINICAL THERAPIES 17
2.2.1 BARBITURATES 17
2.2.2 ALLOPURINOL 18
2.2.3. ERYTHROPOIETIN 19
2.2.4. ANTICONVULSANTS 21
2.2.4. HYPOTHERMIA 24
2.3 ANIMAL MODELS OF NEONATAL WHITE MATTER INJURY (WMI) 25
2.3.1 RICE AND VANNUCCI MODEL 25
2.3.2 CHRONIC HYPOXIA MODEL 27
2.3.3 INTRAUTERINE HYPOXIA ISCHEMIA MODEL 27
2.3.4 GLIOTOXIC INJURY MODEL 28
2.4 SODIUM/CALCIUM EXCHANGER AS POTENTIAL TARGET IN BRAIN ISCHEMIA 29
2.4.1 SODIUM/CALCIUM EXCHANGER 29
2.4.2 SODIUM/CALCIUM EXCHANGER ROLE IN ISCHEMIC STROKE 31
2.4.3 MECHANISM OF ACTION OF SODIUM/CALCIUM EXCHANGER 33
2.4.4 REGULATION OF SODIUM/CALCIUM EXCHANGER 35
2.4.5 SODIUM/CALCIUM EXCHANGER ACTIVATOR: NEUROUNINA 36

3. AIM OF THE STUDY 39

4. MATERIAL AND METHODS 40

4.1 STUDY DESIGN 40
4.2 PROPIDIUM IODIDE LABELING, TISSUE PREPARATION AND DAMAGE ANALYSIS 42
4.3 IMMUNOHISTOCHEMISTRY 43
4.4 MRNA EXPRESSION ANALYSIS BY REAL-TIME POLYMERASE CHAIN REACTION 44
4.5 PROTEIN EXPRESSION ANALYSIS 45
4.6 INDUCTION OF NEONATAL HI 46
4.7 BEHAVIORAL STUDIES 46
4.7.1 OPEN-FIELD EXPLORATION TEST 47
4.7.2 ELEVATED ZERO MAZE 47
4.7.3 DELAY-DEPENDENT ONE-TRIAL OBJECT RECOGNITION TASK 48
4.7.4 BARNES CIRCULAR MAZE TASK 49

4.7.5 T-MAZE SPONTANEOUS ALTERNATION 50
4.8 STATISTICAL ANALYSIS 50

5. RESULTS 51

5.1 HIE DETERMINES A LOSS OF HYPPOCAMPAL PYRAMIDAL NEURONS THAT PEAKS 72 HOURS AFTER INJURY INDUCTION 51
5.2 ACTIVATION OF NCX WITH NEUROUNINA REDUCES NEURONAL INJURY IN HIPPOCAMPUS 54
5.2.1 QUANTITATIVE ANALYSIS OF NEUROPROTECTION MEDIATED BY NEUROUNINA 55
5.3 NCX1, NCX2 AND NCX3 EVALUATION AFTER NEONATAL HYPOXIA ISCHEMIA 57
5.3.1 NCX1, NCX2 AND NCX3 MRNA EXPRESSION IS REDUCED BY NEONATAL HI 57
5.3.2 NCX1 AND NCX3 PROTEIN EXPRESSION IS REDUCED IN THE HIPPOCAMPUS OF INJURED MICE 58
5.4 NEUROUNINA ADMINISTRATION REVERTS MEMORY DEFICIT IN HI MICE 60 DAYS AFTER INJURY 63
5.4.1 NEUROUNINA PREVENTS ASYMMETRIC BEHAVIOR INDUCED IN MICE BY HI 63
5.4.2 HI MICE TREATED WITH NEUROUNINA IMPROVED PERFORMANCE IN ONE-TRIAL OBJECT RECOGNITION TASK 65
5.4.3 HI MICE TREATED WITH NEUROUNINA SHOWED BETTER PERFORMANCE INTO BARNES CIRCULAR MAZE TASK 67
5.4.4 NEUROUNINA REVERTS THE DISINHIBITED BEHAVIOR OF HI MICE IN OPEN FIELD MAZE 69
5.5.5 NEUROUNINA REVERTS THE DISINHIBITED BEHAVIOR OF HI MICE IN ELEVATED ZERO MAZE 71

6. DISCUSSION 72

7. AKNOWLEDGEMENTS 76

8. REFERENCES 77

ABSTRACT

Background: Hypoxic-ischemic encephalopathy (HIE) accounts for the majority of developmental, motor and cognitive deficits in children, leading to life-long neurological impairments. Since (1) transient brain ischemia followed by reoxygenation alters ionic homeostasis in adult brain and the plasma membrane sodium/calcium exchanger (NCX) plays a fundamental role in the maintenance of ionic homeostasis during brain ischemia, we aim to demonstrate the involvement of NCX in the pathophysiology of HIE.

Methods: Experimental HIE was induced in postnatal day 7 (P7) mice by unilateral elettrocoagulation of the right common carotid artery and subsequent 60 minutes exposure of animals to 8% O2. Expression profiles of NCX from embryos stage to adulthood was done using HI and naïve hippocampus mice. To assess the effect of NCX pharmacological activation, brain infarct volume was evaluated in propidium iodide stained hippocampus sections, obtained at several time points after the administration of the newly synthesized NCX activator, Neurounina. Moreover, the effect of NCX activation on learning and memory was evaluated in P60 mice.

Results: An age-dependent NCX-1 and NCX-3 increased expression was evidenced in immature hippocampus in wild-type untreated animals. By contrast NCX-1 and NCX-3 expression was significantly reduced starting from 7 days until 60 days after the hypoxic-ischemic insult. NCX-2 expression did not show any change in the naïve and HI mice at all considered time intervals. Notably, NCX pharmacological activation by the newly synthesized compound neurounina not only reduced infarct volume but improved also the spatial and object memory 8 weeks after HI induction in P7 mice. These findings suggest that an altered ionic homeostasis mediated by the reduced

expression of NCX contributes to long-term cognitive deficits in neonatal mice exposed to HIE.

1. PREMISE

In 1861, William John Little reported on a series of 68 cases of difficult birth, which he related to later developments of neurological deficits, such as spastic diparesis (a movement disorder that primarily affects the lower limbs). This condition, originally known as Little's disease, became generally known as spasticity or cerebral palsy (CP) (Little, 1861). CP denotes a condition of the brain reflected by movement limitation that is often associated with some degree of cognitive impairment. It is now known that in utero hypoxic-ischemic (HI) events (e.g. placental insufficiency, chronic fetal-to-maternal hemorrhage, stroke, infection and inflammation), perinatal events (e.g. placental abruption, respiratory failure) and neonatal disorders (e.g. chronic lung disease) are associated with acquired brain injuries that lead to CP (Silbereis et al, 2010).

The timing of the vascular event leading to hypoxia-ischemia suggested the classification based on the gestational or postnatal age at diagnosis. The suggested subcategories are (1) fetal hypoxia ischemia, diagnosed before birth by using fetal imaging methods or in stillbirths on the basis of neuropathologic examination, (2) neonatal hypoxia ischemia, diagnosed after birth and on or before the 28th postnatal day (including in preterm infants), and (3) presumed perinatal hypoxia ischemia, diagnosed in infants >28 days of age in whom it is presumed (but not certain) that the ischemic event occurred sometime between the 20th week of fetal life through the 28th postnatal day.

The term hypoxia (anoxia) denotes a partial (or total) oxygen deficiency in one or more tissues of the body including blood (hypoxemia, anoxemia) and it is a direct consequence of asphyxia. The term asphyxia indicates the condition in which lung or placenta gas exchange are altered, leading to a progressive hypoxemia and

hypercapnia with subsequently bradycardia and hypotension. The moderate-severe hypoxia is followed by metabolic acidosis due to accumulation of lactic acid resulting from anaerobic metabolism, instead asphyxia is generally associated with metabolic and breathing acidosis. Ischemia originates from the reduction or blockade of blood flow resulting in hypotension or vessel occlusion. In the fetus or newborn the ischemia is determined by previous hypoxia-acidosis with depressing effect on the cardiovascular system or by vascular occlusion. Normally in this clinical picture hypoxia and cerebral ischemia occur in the same time and this is the reason why it is often defined as hypoxic-ischemic injury (HII).

The hypoxic-ischemic encephalopathy (HIE) is the most important consequence of perinatal asphyxiation and is one of the major cause of neonatal death and neurological disability in children. The estimated incidence is approximately 1-6/1000 in newborns and up to 60% in premature infants (Ferriero, 2004). Half of children with this serious disease presents a clinical neuromotor seriously compromised picture with tetraplegia and spastic paraplegia, the other half shows a variety of neurological problems, such as epilepsy and / or slow in learning and memory capability. The precise reasons why birth injuries, including hypoxic ischemic encephalopathy, happen are not entirely known. There is a great deal of debate in the medical community on the exact cause of this particular condition and on the possibility to prevent and detect it before injury occurs to the newborn. Some research data has found that maternal hypotension, umbilical cord complications, abruption placentae, maternal uterine rupture, and other assorted complications can result in hypoxic ischemic encephalopathy. Sometimes, neonatal brain injury is recognized because it evolves from lethargy to hyper-excitability to stupor during the first three days of life, but it often eludes diagnosis, especially in premature infants with very low birth weight, because obvious signs are lacking or because signs that are present are

attributed to developmental immaturity. Sarnat in 1976 developed the most common scoring scale (Fig.1) (Sarnat & Sarnat, 1976) to distinguish mild, moderate and severe hypoxic ischemic encephalopathy according to the symptoms. Clinically subtle signs and symptoms lead to a delay in the diagnosis of cerebral palsy, learning disabilities, and complex behavioral disorders until later in childhood.

The outcomes of HIE are devastating and permanent, making it a major burden for the patient, the family, and society. In addition, there are currently no available treatments for this condition, and the management of infants with hypoxic/ischemic injury provides mainly primary resuscitation measures. In fact, despite the experimental studies in recent years have contributed to shed light on the complex pathogenesis on which newborn brain damage lays on, strategies for clinical diagnosis and therapeutic intervention are still insufficient.

Therefore, it is critical to identify and develop new therapeutic strategies to alleviate the burden of this disease. However, the lack of available treatment mirrors the paucity in the knowledge of the mechanisms on which this pathology lays on. In fact, the underlying pathophysiology of HIE is not completely known and is difficult to investigate mainly because of the difficulties to recruit comparable HI human newborns and therefore to conduct an appropriate clinical trial. Therefore, neonatal rodents model of HI brain injury have been developed in order to mimic human condition.

	Stage 1	Stage 2	Stage 3
Level of Consciousness	Hyperalert	Lethargic or obtunded	Stuporous
Neuromuscular Control			
Muscle tone	Normal	Mild hypotonia	Flaccid
Posture	Mild distal flexion	Strong distal flexion	Intermittent decerebration
Stretch reflexes	Overactive	Overactive	Decreased or absent
Segmental myoclonus	Present	Present	Absent
Complex Reflexes			
Suck	Weak	Weak or absent	Absent
Moro	Strong; low threshold	Weak; incomplete	Absent
Oculovestibular	Normal	Overactive	Weak or absent
Tonic neck	Slight	Strong	Absent
Autonomic Function	Generalized sympathetic	Generalized parasympathetic	Both systems depressed
Pupils	Mydriasis	Miosis	Variable; poor light reflex
Heart Rate	Tachycardia	Bradycardia	Variable
Bronchial and Salivary Secretions	Sparse	Profuse	Variable
GI Motility	Normal or decreased	Increased; diarrhea	Variable
Seizures	None	Common; focal or multifocal	Uncommon
EEG Findings	Normal (awake)	Early: low-voltage continuous delta and theta Later: periodic pattern Seizures: focal 1-to 1-Hz spike-and-wave	Early: periodic pattern with Isopotential phases Later: totally isopotential
Duration	1-3 days	2-14	Hours to weeks

Fig. 1. Sarnat scoring scale. The clinical characteristics of HIE can be described as mild stage1, moderate stage2, or severe stage 3 (Sarnat & Sarnat, 1976).

2. INTRODUCTION

2.1 NEONATAL HYPOXIA ISCHEMIA PATHOPHYSIOLOGY

Brain hypoxia and ischemia due to systemic hypoxemia, reduced cerebral blood flow (CBF), or both are the primary physiological processes that lead to hypoxic-ischemic encephalopathy (Ferriero, 2004; Grow & Barks, 2002; Perlman, 2004). The initial compensatory adjustment to an asphyxial event is an increase in CBF due to hypoxia and hypercapnia. This is accompanied by a redistribution of cardiac output to essential organs, including the brain, heart, and adrenal glands. A blood pressure (BP) increase due to increased release of epinephrine further enhances this compensatory response.

In adults, CBF is maintained at a constant level despite a wide range in systemic BP. This phenomenon is known as the cerebral autoregulation, which helps maintain cerebral perfusion. The physiological aspects of CBF autoregulation has been well studied in perinatal and adult experimental animals. In human adults, the BP range at which CBF is maintained is 60-100 mm Hg.

Limited data in the human fetus and the newborn infant suggest that CBF is stable over much narrower range of BPs (Papile et al, 1985; Rosenkrantz et al, 1988). Some experts have postulated that, in the healthy term newborn, the BP range at which the CBF autoregulation is maintained may be only between 10-20 mm Hg (compared with the 40 mm Hg range in adults noted above). In addition, the autoregulatory zone may also be set at a lower level, about the midpoint of the normal BP range for the fetus and newborn. However, the precise upper and lower limits of the BP values above and below which the CBF autoregulation is lost remain unknown for the human newborn.

In the fetus and newborn suffering from acute asphyxia, after the early compensatory adjustments fail, the CBF can become pressure-passive, at which time brain perfusion depends on systemic BP. As BP falls, CBF falls below critical levels, and the brain injury secondary to diminished blood supply and a lack of sufficient oxygen occurs. This leads to intracellular energy failure. During the early phases of brain injury, brain temperature drops, and local release of neurotransmitters, such as gamma-aminobutyric acid transaminase (GABA), increase. These changes reduce cerebral oxygen demand, transiently minimizing the impact of asphyxia.

The immature brain may be more vulnerable to oxidative damage than the adult brain due to high concentration of unsaturated fatty acids and the availability of redox-active iron in the developing brain (Lafemina et al, 2006). Moreover, the anatomo-pathological lesions and the consequential long term outcomes are different in the term compared to preterm newborn. In the first group, the cerebral gray matter involvement (cerebral cortex, hippocampus, basal ganglia, cerebellar hemispheres) is predominant, in the second group, white matter injury is more frequent. There are many factors that determine the different topography of perinatal hypoxic-ischemic brain damage: cellular or regional intrinsic vulnerability, vascular factors, nature and timing of the insult, age and maturity of the newborn, contingent factors such as hypoglycemia, sepsis or malnutrition. Since it's very hard to conduct a clinical trial with neonates and also really tough to make a proper diagnosis we can understand why the underlying mechanisms of neonatal HIE remain unclear. Today we know thanks to animal model of HI that neonatal brain injury evolves over days or weeks so different kind of treatment can be attempted as the injury evolves. (Fig. 2) (Ferriero, 2004).

At the cellular level, neuronal injury in hypoxic-ischemic encephalopathy is an evolving process. The magnitude of the final neuronal damage depends on duration

and severity of the initial insult combined to the effects of reperfusion injury, and apoptosis. At the biochemical level, a large cascade of events follow hypoxic-ischemic encephalopathy injury.

In the next paragraphs we will examine the main pathophysiological mechanisms involved in cell death after HIE.

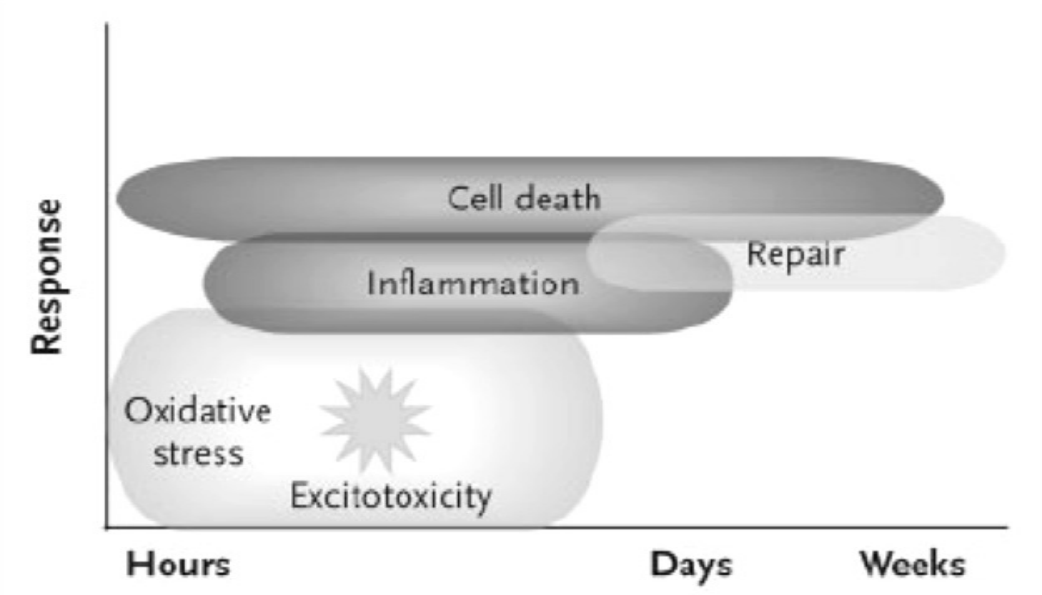


Fig. 2. Mechanisms of Brain Injury in the Term Neonate. Oxidative stress and excitotoxicity, through downstream intracellular signaling, produce both inflammation and repair. Cell death begins immediately and continues during a period of days to weeks. The cell-death phenotype changes from an early necrotic morphology to a pathology resembling apoptosis. This evolution is called the necrosis– apoptosis continuum. (Ferriero, 2004)

2.1.1 MECHANISM OF CELL DEATH: EXCITOTOXICITY

Current research shows the importance of glutamate mediated excitotoxicity following neonatal HI. Glutamate is the main excitatory neurotransmitter in the central nervous system and acts through two broad classes of receptors: ion channel linked ionotropic receptors and metabotropic receptors (mGluR), which are coupled with G-proteins inducing intracellular messenger cascades (Palmada & Centelles, 1998) (Tanabe et al, 1992). Four main subtypes of glutamate-gated channels have been characterized pharmacologically and they have been named according to their

preferred agonist, N-methyl-D-aspartate (NMDA), high affinity kainate (KA), α-amino-3-hydroxy-5- methyl-4-isoxazole propionate (AMPA) and 2-amino-4- Mechanisms Underlying Neonatal Hypoxia Ischemia The Open Drug Discovery Journal, 2010, Volume 2 131 phosphobutyrate (AP4)(Palmada & Centelles, 1998).

A prolonged hypoxia or HI results in depletion of cellular energy stores (ATP), depolarization of neurons and glia and release of excitatory amino acids into the extracellular space, particularly the glutamate. The excessive release of glutamate and/or impaired uptake by glia may result in overactivation of NMDA and AMPA receptors that cause an increase in Ca2+ influx which activates proteases and endonucleases, such as calpains, resulting in the breakage of cytoskeletal and nuclear structure and DNA damage. On the other hand, the influx of Na+ ions via AMPAR causes cell swelling and depolarization. These are the major cell death mechanisms in response to excitotoxicity that eventually results in necrosis or apoptosis.

A large amount of work has been accumulated showing that glutamate extracellular concentrations briskly rise during acute brain injury, thus triggering an influx of Ca^{2+} and Na^{+} ions into neurons through ionotropic glutamate receptor subtypes. This evidence has led to the elaboration of the paradigm of glutamate excitotoxicity that explains ischemic neuronal cell death as a mere consequence of Na^{+} and Ca^{2+} influx through glutamate receptors (Olney & Sharpe, 1969). Although this theory has been guiding basic research in the field of neurodegeneration for almost three decades, more recently it has become the object of serious criticism and reassessment. What has aroused such skepticism among researchers has been the fact that although first, second, and third generation glutamate receptor antagonists have long yielded promising results in animal models of brain ischemia, they have failed to elicit a neuroprotective action in adult stroke and in HI newborns. Therefore, the theory of

excitotoxicity can only explain some of the events occurring in the acute phase of anoxic insult but cannot be seen as a major target for developing new therapeutic avenues for brain ischemia. In the last decade, several seminal experimental works are markedly changing the scenario of research of principal actors of an ischemic event. In fact, it has been shown that some integral plasma-membrane proteins, involved in the control of Ca^{2+}, Na^{+}, K^{+}, H^{+} ions influx or efflux and, therefore, responsible for maintaining the homeostasis of these four cations, might function as crucial players in the brain ischemic process. Indeed, these proteins, by regulating Ca^{2+}, Na^{+}, K^{+}, H^{+} homeostasis, may provide the molecular basis underlying glutamate independent Ca^{2+} overload mechanisms in neuronal ischemic cell death and, most importantly, may represent more suitable molecular targets for therapeutic intervention (Annunziato et al, 2004).

2.1.2 OTHER NON-NMDA DEPENDENT PATHWAYS

Non NMDA-dependent mechanisms can disrupt ionic homeostasis in neonatal brains after HI and contribute to brain damage. This includes Na+/H+ exchanger (NHE) and nonselective Ca2+-activated ATP-sensitive cation channel. Indeed, HI leads to a decrease in the intracellular pH (pHi). To counteract the subsequent acidosis, the Na+/H+ exchanger and H+ pump in the plasma membrane are activated to remove the excess in H+ ions. NHE isoform 1 is the most ubiquitously expressed isoform in the CNS (Luo & Sun, 2007) (Ma & Haddad, 1997). NHE-1 becomes overstimulated following ischemia-induced intracellular acidosis (Avkiran, 2001) and by extracellular regulatory kinase-mediated phosphorylation (Luo & Sun, 2007; Malo et al, 2007). NHE1-mediated ischemic and reperfusion damage largely result from an increase in intracellular Na+, which promotes Ca2+ influx by reverse mode operation of the Na^{+}／Ca^{2+} exchanger NCX (Kintner et al, 2007; Lee et al, 2005). Non NMDA-dependent

mechanisms could also support ionic homeostasis in neonatal brains after HI and reduce brain damage. Na^+ / Ca^{2+} exchanger NCX can facilitate both Ca^{2+} and Na^+ flow in a bidirectional way through the plasma membrane (Blaustein & Lederer, 1999; Philipson & Nicoll, 2000) with a stoichiometry of 3 Na+ ions versus 1 Ca^{2+} ion. Depending on the intracellular levels of Na^+ and Ca^{2+}, NCX can operate in the forward mode by extruding one Ca^{2+} against three entering Na+, using the Na+ gradient across the plasma membrane as a source of energy (Annunziato et al, 2004; Blaustein & Lederer, 1999). Alternatively, in the reverse mode, NCX can function as Na^+ efflux–Ca^{2+} influx. Because of its high exchange capacity, NCX is well-suited for rapid recovery from high intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) and may play an important role in maintaining Ca^{2+} homeostasis and protecting cells from Ca^{2+} overload and eventual death (Annunziato et al, 2004; Blaustein & Lederer, 1999).

2.1.3 OXIDATIVE STRESS

The neonatal brain, with its high concentrations of unsaturated fatty acids, high rate of oxygen consumption, low concentrations of antioxidants, and availability of redox-active iron is particularly vulnerable to oxidative damage (Tan et al, 1996). In the very immature brain, oligodendrocyte progenitor cells and preoligodendrocytes are selectively vulnerable to the depletion of antioxidants or exposure to exogenous free radicals (Sheldon et al, 1998). Mature oligodendrocytes, in contrast, are highly resistant to oxidative stress, owing in part to differences in the levels of expression of antioxidant enzymes and proteins involved in programmed cell death. These characteristics of oligodendrocytes may explain why white matter often is injured selectively in the brain of the premature newborn.

2.1.4 INFLAMMATION

Inflammatory mediators also play an important role in the pathogenesis of HI in immature brains (Hedtjarn et al, 2004). This inflammation could result from maternal infection which is a well known risk factor for the white matter disease in the newborns and leads to neuronal damage and cell loss (Dammann et al, 2002). Inflammation after HI has both beneficial and detrimental effects. Inflammatory cytokines may have a direct toxic effect via increased production of iNOS, cyclooxygenase and free radical release (Perlman, 2007). Expression of interleukin-1-β and tumor necrosis factor α (TNF-α) mRNA has been detected in the area of infarction within 1-4 h after HI (Hagberg et al, 1996). Thus, inflammation may represent a common pathway of brain injury. The beneficial effects of inflammatory cytokines come from their neurotrophic effects. TNF-α knockout mice have increased neuronal cell damage with increased oxidative stress after ischemia, suggesting an important role for TNF- α in neuroprotection. Injury-induced microglial activation was also suppressed in TNF- α knockout (Bruce et al, 1996).

2.1.5 NECROSIS AND APOPTOSIS

Both necrotic and apoptotic cell death occurs following neonatal HI. Necrotic cells show early rupture of the plasma membrane, swelling of intracellular organelles, shrinkage of cell volume and inflammation response activation resulting from the spilled cytosolic contents (Edinger & Thompson, 2004). In contrast, programmed cell death, or apoptosis, is the mechanism for refining cell connections and pathways during brain development. Recent data suggest that apoptosis plays a prominent role in the evolution of hypoxic–ischemic injury in the neonatal brain and may be more

important than necrosis after injury (Hu et al, 2000). During neonatal brain injury, excitotoxicity, oxidative stress, and inflammation all contribute to the cell death by apoptosis or necrosis, depending on the region of the brain affected and the severity of the insult. Signals from cytokine death receptors, for example, result in nitric oxide–mediated necrosis when endogenous inhibitors of apoptosis are abundant (Raoul et al, 2002) and in apoptosis when the inhibitors are deficient. 38 These death-receptor proteins have been documented in the brain and the cerebrospinal fluid of newborns after brain injury, (Felderhoff-Mueser et al, 2003; van Landeghem et al, 2002) suggesting that this pathway may be a potential therapeutic target.

2.2 CLINICAL THERAPIES

It is very difficult to predict during the neonatal period which neonates will suffer the most profound damage after an insult to the central nervous system, since more than 30 percent of neonates presenting with moderate encephalopathy have normal outcomes.

2.2.1 BARBITURATES

The first class of drugs used in HIE infants were barbiturates. This GABAergic agonists show variable success in clinical trials after HI in neonates. Indeed, when thiopental was used in 32 asphyxiated neonates no significant difference was found in the frequency of seizures between control and treated group and it did not appear to have a cerebral sparing effect (Goldberg et al, 1986). Thiopental was begun at a mean age of 2.3 hours and was given as a constant infusion that delivered 30 mg/kg over 2 hours. Treatment was continued at a lower dose for 24 hours. Interestingly,

phenobarbital when given 20 mg/kg intravenously to neonates with HI within 6 hours of life, significantly decreased the incidence of seizures (Singh et al, 2005). Three-year long term follow up of the newborns with HI after 40 mg/kg of phenobarbital therapy revealed better neurological outcome (Hall et al, 1998)'(Shalak & Perlman, 2004). The role of barbiturates in the severely injured population remains unclear (Shalak & Perlman, 2004) .

2.2.2 ALLOPURINOL

Free radicals such as hydroperoxide (H2O2), hydroxyl radical (OH), superoxide radical (O2 −), and peroxynitrite (ONOO–) play a significant role in the development of damage after HI. The primary source of superoxide in reperfused reoxygenated tissues appears to be the enzyme xanthine oxidase, released during ischemia by a calcium triggered proteolytic attack on xanthine dehydrogenase. Allopurinol is a xantine-oxidase inhibitor; in high concentrations allopurinol also scavenges hydroxyl radicals and prevents free radical formation by chelating their catalyst non-protein bound iron. High doses are needed for neuroprotection. Studies in immature rats found that allopurinol treatment 15 min after cerebral hypoxia–ischaemia reduced brain edema and long term brain damage. In this study, the rat pups received either allopurinol (135 mg/kg subcutaneously) or saline. A Cochrane review in 2008 (Chaudhari & McGuire, 2008) suggested that the available data are not sufficient to determine whether allopurinol has clinically important benefits for newborn infants with hypoxic–ischaemic encephalopathy. The review suggested that larger trials are needed which should assess allopurinol as an adjunct to therapeutic hypothermia in infants with moderate and severe encephalopathy and should be designed to exclude clinically important effects on mortality and adverse long-term neurodevelopmental outcomes. Three trials were included in the meta-analysis of

allopurinol for preventing mortality and morbidity in newborn infants with suspected hypoxic–ischemic encephalopathy (Benders et al, 2006; Gunes et al, 2007; Van Bel et al, 1998). Meta-analysis did not reveal a statistically significant difference in the risk of death during infancy or incidence of neonatal seizures. Only one trial assessed neurodevelopment in surviving children and did not find a statistically significant effect. As allopurinol acts through its free radical scavenger property it must be administered early, even during delivery; fortunately allopurinol crosses the placenta. In 2009, the Dutch group demonstrated that maternal allopurinol (500 mg) during fetal hypoxia lowers cord blood levels of S-100B in pregnancies in which fetal hypoxia was suspected at N36 weeks gestation (Torrance et al, 2009); fetal hypoxia was indicated by abnormal/non-reassuring fetal heart rate tracing or fetal scalp pH of b7.20. Following on from this the ALLO-trial has started in the Netherlands (Kaandorp et al, 2010); the proposed trial is a randomized double blind placebo controlled multicenter study in pregnant women at term in whom the fetus is suspected of intra-uterine hypoxia. Allopurinol 500 mg IV or placebo will be administered antenatally to the pregnant woman when fetal hypoxia is suspected.

2.2.3. ERYTHROPOIETIN

Erythropoietin is a glycoprotein hormone that controls erythropoiesis (Patti et al) . Erythropoietin is a cytokine for erythrocyte precursors in the bone marrow and is produced by the peritubular capillary endothelial cells in the kidney. The safety of recombinant human Epo (rEPO) has been demonstrated in clinical studies for the prevention and treatment of anaemia of prematurity (Ohls et al, 2004). Epo is up-regulated in umbilical cord blood from babies who have suffered perinatal asphyxia (Ruth et al, 1990), which may be an endogenous repair mechanism. Several pre-clinical studies have suggested a neuroprotective effect of Epo following hypoxia–

ischaemia and reperfusion (Maiese et al, 2005). Iin neonatal models systemically administered Epo is neuroprotective (Kellert et al, 2007) with early and high dose treatment required to reduce brain injury (5000U/kg), furthermore it promotes neurogenesis (Gonzalez et al, 2007). There is also data suggesting that multiple doses of Epo are required for sustained neuroprotection and reduced tissue loss (Gonzalez et al, 2009). Epo is shown to have neuroprotective effects through different mechanisms such as direct neurotrophic effect, decreased susceptibility to glutamate toxicity, induction of anti-apoptotic factors, decreased inflammation, decreased nitric oxide-mediated injury,

direct antioxidant effects, and protective effects on glia. Interestingly, the antioxidative effect of Epo may be enhanced by the increased erythropoesis utilizating the free-iron which has accumulated following hypoxia–ischaemia (Palmer et al, 1999). The dosage for neuroprotection (1000–30,000 U/kg) is above the range used in anaemia; Epo is a relatively large molecule (30.4 kDa) to cross the blood brain barrier. However following high-dose rEpo administration in non-human primates and sheep, neuroprotective concentrations of Epo was found in the CSF by 2–2.5 h after injection (Juul et al, 2004). High Epo dose was well tolerated in these studies . Pre-clinical studies of the interaction of Epo with hypothermic neuroprotection are required before progression to clinical trials of combined therapy. Interestingly, other neuroprotective agents up-regulate endogenous erythropoietin; for example, xenon up-regulates erythropoietin expression (Ma et al, 2009) and easily crosses the blood brain barrier providing an alternative mechanism to harness erythropoietin protection in the CNS.

Whether higher doses of Epo can improve outcomes without inducing complications is unknown. Concerns over the use of high doses of erythropoietin include possible thrombotic and hematological complications and real safety concerns have arisen in

2 recent adult studies. In a pilot study of out-of-hospital cardiac arrest, patients treated with hypothermia and early high dose Epo (40 000 IU as soon as possible after resuscitation and every 12 h for the first 48 h were compared with case-matched controls who received therapeutic hypothermia alone (Cariou et al, 2008). A higher survival rate with none or minimal cerebral sequelae were seen in the Epo treated patients but adverse effects related to vascular thrombosis occurred (Cariou et al, 2008). In another study of adult stroke patients, a higher death and complication rate was observed in those receiving Epo compared with placebo; complications included intracerebral hemorrhage, brain oedema and thrombotic events (Ehrenreich et al, 2009).

2.2.4. ANTICONVULSANTS

Data from both animal and human studies suggest that seizures amplify neonatal hypoxic–ischemic brain damage (Glass et al, 2009; Wirrell et al, 2001). In a recent study of newborns with HIE with MRS used to assess tissue metabolic integrity, the severity of seizures was independently associated with brain injury (Calabresi et al, 2003; Miller et al, 2002). These results provide some support for the hypothesis that effective prevention of neonatal seizures could attenuate brain injury. Anticonvulsant drugs (AEDs) are being studied as possible neuroprotective agents on the basis of the similar cascade of cellular events triggered by seizures and acute hypoxia–ischaemia. Two drugs of particular interest in perinatal asphyxia are topiramate and levetiracetam. opiramate is an AED drug used safely in children (Kim et al, 2009). Topiramate has actions on many cell pathways: it acts through modulation of alphaamino- 3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate and gamma-aminobutyric acid (GABA)A-activated ion channels and voltage-activated Na+ and Ca++ channels, which may reduce excessive glutamate release after hypoxia-

ischaemia (Herrero et al, 2002; Taverna et al, 1999; Zona et al, 1997). Topiramate suppresses the pre-synaptic voltage-sensitive sodium channel of excitatory synapses and enhances GABA-mediated chloride flux and GABA-evoked chloride currents in murine brain neurons and increases seizure threshold(White et al, 1997). In a neonatal rat stroke model, neither topiramate nor hypothermia alone conferred protection, whereas combined treatment with topiramate and hypothermia improved functional performance and reduced severity of brain injury compared with delayed hypothermia alone (O'Brien et al, 2006). Other studies have shown that topiramate on its own is neuroprotective in neonatal rat pups although the post-treatment window appears to be relatively narrow (2 h) (Noh et al, 2006). In another p7 rodent model post-insult topiramate was protective against hypoxic– ischemic white matter injury and decreased subsequent neuromotor deficits (Follett et al, 2004). In this study topiramate attenuated AMPA-kainate receptor-mediated cell death and calcium influx, as well as kainateevoked currents in developing oligodendrocytes, similar to the AMPA– kainate receptor antagonist 6-nitro-7-sulfamoylbenzo-(f)quinoxaline- 2,3-dione (NBQX). Notably, protective doses of NBQX and topiramate did not affect normal maturation and proliferation of oligodendrocytes (Follett et al, 2004). Topiramate was shown to protect against white matter injury in a newborn piglet model when given as infusion 1 h after hypoxia– ischaemia followed by maintenance doses for 3 days(Schubert et al, 2005). Interestingly, topiramate administration did not affect seizure frequency. Two dose regimens were used: a low dose (loading dose of 20 mg/kg and a maintenance dose of 10 mg/kg/day) or a higher dose (loading dose of 50 mg/kg followed by 20 mg/kg/day). Topiramate reduced cell loss in a dose dependent way, however there was concern that increased apoptosis was seen in the white matter of high dose treated animals. Topiramate is an attractive agent to consider for clinical trials, however pre-clinical studies are needed to assess

the effect of topiramate on neurodegeneration. One study has suggested that although Topiramate and Levetiracetam alone did not induce cell death in 8 day old rat pups, when both drugs were given in combination with phenytoin, then cell death was increased (Kim et al, 2007a). In addition further study on the effect of hypothermia on the metabolism of Topiramte is needed as some studies have shown a reduction in both absorption and elimination of topiramate with therapeutic hypothermia (Filippi et al, 2009). Levetiracetam was approved by the US FDA in 1999; in the last 10 years it has become one of the first-line AEDs and has been evaluated in small uncontrolled studies and case series as an off-label treatment for status epilepticus, for nonepileptic neurological conditions and for some psychiatric conditions. Levetiracetam has not been approved for any indication other than chronic epilepsy. In a recent small pilot study of the treatment of neonatal seizures with levetiracetam, there were no adverse effects and all six patients treated with oral levetiracetam became seizure free within 6 days (Furwentsches et al, 2010). In addition to its efficacy in seizure treatment, some studies suggest that levetiracetam is neuroprotective (Mazarati et al, 2004). Levetiracetam was administered by intraperitoneal bolus injections of 5.5, 11, 22 and 44 mg/kg 30 min before occlusion followed by a continuous 24 h infusion of 1.25, 2.6, 5.1 and 10.2 mg/kg per h, respectively. Levetiracetam administration reduced the infarct volume by 33% at he highest dose tested (Hanon & Klitgaard, 2001). Although there is a lack of clinical experience with levetiracetam, this agent is worth investigating further as a neuroprotective agent as in contrast to other conventional AEDs such as phenobarbitone and phenytoin, it does not induce cell death in the developing brain even at high dose (Kim et al, 2007b).

2.2.4. HYPOTHERMIA

Hypothermia is the only standard treatment at present approved for HIE and several clinical trials for establishing a better protocol for the use of hypothermia in HI are in progress. Therapeutic hypothermia reduces the brain energy use rate and inhibits many of the processes involved in primary and secondary energy failure (Laptook, 2009b).

The hypothermia neuroprotective mechanisms are not completely understood. Possible mechanisms include (1) reduced metabolic rate and energy depletion; (2) decreased excitatory transmitter release; (3) reduced alterations in ion flux; (4) reduced apoptosis due to hypoxic-ischemic encephalopathy; and (5) reduced vascular permeability, edema, and disruptions of blood-brain barrier functions.

The clinical efficacy of therapeutic hypothermia in neonates with moderate-to-severe hypoxic-ischemic encephalopathy has been evaluated in 7 randomized controlled trials (Azzopardi et al, 2009; Eicher et al, 2005a; Eicher et al, 2005b; Gluckman et al, 2005; Jacobs et al, 2011; Shankaran et al, 2005; Simbruner et al, 2010; Zhou et al, 2010). Inclusion criteria varied slightly. Criteria from the larger trials (NICHD, CoolCap, and TOBY) are summarized as follows:

- Near-term infants born at 36 weeks' gestation or more with birth weight of 1800-2000 g or more, younger than 6 hours at admission
- Evidence of acute event around the time of birth - Apgar score of 5 or less at 10 minutes after birth (In the study by Shankaran et al, this needed to be in conjunction with either evidence of acute perinatal event or need for assisted ventilation for at least 10 min.(Shankaran et al, 2005)), severe acidosis, defined as pH level of less than 7 or base deficit of 16 mmol/L or less (cord blood or any

blood gas obtained within 1 h of birth), continued need for resuscitation at 10 minutes after birth

- Evidence of moderate to severe encephalopathy at birth - Clinically determined (at least 2 of the following: lethargy, stupor, or coma; abnormal tone or posture; abnormal reflexes [suck, grasp, Moro, gag, stretch reflexes]; decreased or absent spontaneous activity; autonomic dysfunction [including bradycardia, abnormal pupils, apneas]; and clinical evidence of seizures), moderately or severely abnormal amplitude-integrated electroencephalography (aEEG) background or seizures (CoolCap and TOBY)

Therapeutic hypothermia when applied within 6 hours of birth and maintained for 48-72 hours is a promising therapy for mild-to-moderate cases of hypoxic-ischemic encephalopathy (Laptook, 2009a; Shankaran, 2009). Although many components of its implementation remain to be optimized, hypothermia therapy is increasingly offered to infants with moderate-to-severe hypoxic-ischemic encephalopathy. Some even argue that not discussing hypothermia therapy as an option with parents is unethical (Perlman & Shah, 2009; Wilkinson, 2009).

2.3 ANIMAL MODELS OF NEONATAL WHITE MATTER INJURY (WMI)

Due to the difficulties to recruit and conduct clinical trial with newborns several animal models have been developed in order to study the mechanism underlying neonatal brain injury (Fig. 3) .

2.3.1 RICE AND VANNUCCI MODEL

The model of neonatal HI was firstly developed by Vannucci and Rice in neonatal rats (Rice et al, 1981). The Vannucci/Rice method involves a unilateral carotid artery

ligation of postnatal day (P7) rats followed by exposure to 8%oxygen/balanced nitrogen at 37°C. The unilateral carotid artery ligation alone does not induce ischemic damage due to the intact circle of Willis. Exposure of neonatal rats to 8%oxygen after unilateral common carotid ligation results in hypoxemia and hypocapnia produced by hyperventilation (Vannucci & Vannucci, 2005). Hypocapnia compensates for the metabolic acidosis which is due to lactic acid accumulation secondary to anaerobic glycolysis. Therefore, this model causes damage to ipsilateral hippocampus, striatum, thalamus and cortex (Welsh et al, 1982).The selection of the age of the neonatal rodent and duration of hypoxia vary among researchers. In rats, the accepted age has remained at P7, which correlates histologically to a 32 to 34 week human neonate (Vannucci & Vannucci, 2005). In mice, the most frequently used age is P9, although P7 to P10 are commonly used (Barks et al, 2008; Doverhag et al, 2008; Stone et al, 2008). Seven to 10 day old mouse brains are similar to a third-trimester neonatal brains, in terms of cellular proliferation, cortical organization, synapse number, neurotransmitter synthetic enzymes and electrophysiology (Liu et al, 1999).The time course to induce HI related brain injury varies among different animal species and ages. Neonatal rats exposed to hypoxia for over three hours can induce brain injury before significant mortality occurs (Vannucci & Vannucci, 2005) . Severe forebrain injury was observed following 150 minutes (min) of HI in P7 rats (Jiang et al, 2008; Northington et al, 2001). Similarly, time course of HI varies from 15 min (Jiang et al, 2008) to 2.5 hours (h) in P7 mice (Nakanishi et al, 2009). Thirty min of hypoxia can result in moderate injury and 1 h in severe injury in P7 mice (Kendall et al, 2006).Necrotic neurodegeneration is seen in the ipsilateral cortex and striatum at 3 h post HI (Northington et al, 2001). Apoptotic cell death is detected in the thalamus at 24 h post HI (Northington et al, 2001). At 48 h post HI, cells show both necrotic and apoptotic appearances in the ipsilateral cortex and striatum in rats

(Northington et al, 2001). In mice, damage is only detected in the ipsilateral hippocampus at 48 h post HI(Kendall et al, 2006).

2.3.2 CHRONIC HYPOXIA MODEL

Infants with chronic lung disease as a complication of premature birth suffer multiple hypoxic episodes and are at higher risk of developing CP. Neonatal rodent models involving either continuous or intermittent hypoxic rearing have been proposed as models of this type of brain injury and display some pathological features of developmental WMI (Back et al, 2006; Chahboune et al, 2009; Ment et al, 1998; Scafidi et al, 2009).

2.3.3 INTRAUTERINE HYPOXIA ISCHEMIA MODEL

Intrauterine models of transient global ischemia have been described in the rat and rabbit by housing of pregnant dams in hypoxic conditions or by abruption of placental blood flow by inflation of a balloon catheter inserted into the uterine artery, respectively (Cai et al, 1998; Cai et al, 1995; Hersey et al, 1995). In both rat and rabbit, this model recapitulates many histopathological aspects of developmental WMI (Derrick et al, 2007). In rabbits, in addition to lesions throughout the cerebral gray and white matter, some animals develop spontaneous intraventricular hemorrhage with ventriculomegaly and periventricular white matter loss. Detailed neurobehavioral assessments have demonstrated that the surviving rabbit kits display a wide range of hypertonic motor deficits that resemble CP. This is currently the only model that permits such detailed clinico-pathological correlations with neurobehavioral outcome and neuro-radiological assessment (Drobyshevsky et al, 2005). This model has also been used extensively to study the cellular mechanisms of WMI. For example, it was recently shown that the susceptibility to WMI during

development closely coincided with the timing of appearance of preOLs (Buser et al, 2010).

2.3.4 GLIOTOXIC INJURY MODEL

Understanding the repair response after demyelination and oligodendrocyte death might be relevant to understanding the failure of myelination during developmental insults. In this respect, gliotoxic models, which are traditionally used to study demyelinating diseases such as multiple sclerosis (MS), might prove useful for understanding developmental WMI. The two main toxin-based models of demyelination in adult white matter are (1) systemic administration of cuprizone, which induces widespread CNS demyelination, or (2) focal injection of lysolecithin or ethidium bromide, which typically induce focal lesions in spinal cord white matter or caudal cerebellar peduncle, respectively (Blakemore & Franklin, 2008). Although such models lack an inflammatory basis, which might be an important consideration in neonatal WMI, they might allow targeted investigation of the responses of oligodendrocyte progenitors to WMI and define mechanisms involved in regeneration and repair. In addition, they might be useful for assessing mechanisms of myelin regeneration that are conserved in both neonatal and adult white matter disorders, such as MS.

Model	Species	Features WMI	Advantages	Disadvantages
Rice-Vannucci (HIE)	Mouse, rat	Oligodendrocytes: •cell death •alterations Axonal damage Neural cell death Microglial activation	Widely used Use of transgenic mice	•Not adapted to large animals •Variability between animals
Chronic hypoxia	Mouse, Rat	Oligodendrocyte: •minimal cell death •alterations Moderate neural cell death Ventriculomegaly	Generates chronical global hypoxia that is common in premature human infants	•Lacks gliosis •Inflammatory response poorly characterized •Relatively mild WMI
In utero ischemia	Rabbit	Lesions to multiple forebrain structures (cortex, thalamus and basal ganglia) and white matter tracts Intraventricular hemorrhage	Rabbits exhibit hypertonic motor deficits similar to those observed in CP	•Not amenable to genetic •experiments •Requires specialized •infrastructure and •expertise
Gliotoxic injury	Mouse, rat	Demyelinization and oligodendrocyte cell death in focal lesion	Can be used in adult transgenic mice	•Not adapted to neonatal stage •Lacks inflammatory component

Fig. 3. Overview of common animal models for studying developmental white matter injury

2.4 SODIUM/CALCIUM EXCHANGER AS POTENTIAL TARGET IN BRAIN ISCHEMIA

2.4.1 SODIUM/CALCIUM EXCHANGER

The Na^+/Ca^{2+} exchanger (NCX) is one of the major membrane proteins involved in Ca^{2+} extrusion at the plasma membrane. The regulation of Ca^{2+} and Na^+ homeostasis is a crucial physiological phenomenon in neurons. In fact, Ca^{2+} ions play a key role as a second messenger in the cytosol and in the nucleus (Choi, 1988), while the Na^+ ion regulates the cellular osmolarity, inducing action potentials (Lipton, 1999), and it is involved in the signal translation (Yu & Choi, 1997). The control of this regulation is

delegated to ionic channels selective for Ca^{2+} and Na^{+}, to Na^{+} pumps, Ca^{2+} ATP-dependent and to NCX (Blaustein & Lederer, 1999). The NCX family, which exchanges three Na^{+} ions for one Ca^{2+} ion or four Na+ ions for one Ca2+ ion depending on $[Na^{+}]_i$ and $[Ca2^{+}]_i$ (Fujioka et al, 2000; Kang & Hilgemann, 2004; Reeves & Hale, 1984) consists of three dominant genes coding for the three different isoforms of the exchanger: NCX1 (Nicoll et al, 1990), NCX2 (Li et al, 1994), and NCX3 (Nicoll et al, 1996) proteins. These three genes appear to be dispersed, since NCX1, NCX2, and NCX3 have been mapped in mouse chromosomes 17, 7, and 12, respectively (Nicoll et al, 1996). At the post-transcriptional level, at least 12 NCX1 and 3 NCX3 proteins are generated through alternative splicing of the primary nuclear transcripts. These variants arise from a region of the large intracellular f-loop, are encoded by six small exons defined A to F, and are used in different combinations in a tissue-specific manner. To maintain an open reading frame, all splice variants must include either exon A or B, which are mutually exclusive (Quednau et al, 1997). NCX1 is composed of 938 amino acids in the canine heart and has a molecular mass of 120 kDa and contains nine transmembrane segments (TMS). NCX1 amino terminus (N-terminal) is located in the extracellular space, whereas the carboxyl terminus (C-terminal) is located intracellularly. The nine transmembrane segments can be divided into an N-terminal hydrophobic domain, composed of the first five TMS (1–5), and into a C-terminal hydrophobic domain, composed of the last four TMS (6–9). These two hydrophobic domains are important for the binding and the transport of ions. The first (1–5) TMS are separated from the last four (6–9) TMS through a large hydrophilic intracellular loop of 550 amino acids, named the f-loop (Nicoll et al, 1999). Although the f-loop is not implicated in Na^{+} and Ca2+ translocation, it is responsible for the regulation of NCX activity.

2.4.2 SODIUM/CALCIUM EXCHANGER ROLE IN ISCHEMIC STROKE

In an *in vivo* model of cerebral ischemia, reproduced in our laboratories, based on permanent middle cerebral artery occlusion (pMCAO) it has been observed a downregulation of about 90% of the levels of expression of NCX1 and NCX3 in ischemic core and in the peri-ischemic regions. In other brain districts, belonging to the ischemic penumbra, after cerebral ischemia, there was an increase of the levels of RNA messenger of NCX3 and NCX1. In contrast, in the same regions, the pMCAO causes a decrease in mRNA expression of NCX2. The up-regulation of NCX3 in peri-infarct tissue has been interpreted as a compensatory mechanism to offset the reduced activity of NCX2, that in the course of ischemia is down-regulated and to keep at a proper homeostasis ions Na^+ and Ca^{2+}. In essence, the expression of NCX1 and NCX3 after permanent middle cerebral artery occlusion (pMCAO) in rats is regulated in a differential manner, depending on the region involved in the insult (Boscia et al, 2006; Pignataro et al, 2004). Furthermore, antisense-induced downregulation of NCX1 and NCX3 or genetic ablation of NCX3 worsens the experimentally-induced ischemic damage in mice and rats (Molinaro et al, 2008). Evidence for NCX3 neuroprotective role relies in the remarkable broadening ofthe infarct volume occurring when NCX3 protein is knocked down with aselective antisense oligonucleotide, thereby worsening the neurologic deficits (Pignataro et al, 2004). Accordingly, it has been recently showed in ischemic NCX3–/– mice thatNCX3 exerts a neuroprotective effect (Molinaro et al, 2008). For instance, in homozygous ncx3−/− mice subjected to MCAO, an increased brain damage occurs (Molinaro et al, 2008). In addition, the silencing of NCX1 and NCX3 expression by RNA interference increases cerebellar granule neurons vulnerability to Ca^{2+} overload and excitotoxicity (Bano et al, 2005; Secondo et al, 2007). Moreover, the vulnerability to chemical hypoxia of BHK cells overexpressing NCX1 or NCX3 considerably

increases when either NCX1 or NCX3 is silenced (Bano et al, 2005; Secondo et al, 2007). Finally, ischemic rats treated with NCX1 or NCX3 antisense display a remarkable enlargement of the infarct volume (Pignataro et al, 2004).In a recent paper published by Pignataro et al, it has been shown that among the three NCX brain isoforms, NCX1 represent a new molecular effector involved in a neuroprotective mechanism named “ischemic preconditioning” (Pignataro et al, 2013; Pignataro et al, 2011). In effect, the brain possesses internal defense mechanisms that can be triggered by several stimuli. Among these mechanisms, preconditioning has recently attracted a great deal of interest. Preconditioning is a phenomenon whereby a subliminal injurious stimulus applied before a longer harmful ischemia (Dirnagl et al, 2003; Gidday, 2006; Kirino, 2002) is able to exert a remarkable neuroprotection, thus establishing a state of tolerance to anoxic conditions. Pignataro has demonstrated that among the three NCX brain isoforms, NCX1 and NCX3 represent two molecular effectors involved in the neuroprotective mechanisms of ischemic preconditioning. So, results of Pignataro work support the importance of NCX1 and NCX3 in the pathogenesis of ischemic lesion and, most important, offer a new possible interpretation of the neuroprotective mechanism elicited by ischemic preconditioning. Furthermore the overexpression of NCX1 and NCX3 observed during preconditioning may be related to their ability to counteract the dysregulation of intracellular Na+, ($[Na^+]_I$) and Ca^{2+}, ($[Ca^{2+}]_I$) homeostasis occurring in the brain under anoxic conditions corresponding to an harmful ischemia. In a rat model of cerebral transient ischemia the reduction in NCX1 expression induced by the ischemic insult alone (Boscia et al, 2009; Pignataro et al, 2004) was completely prevented when the animals were exposed to preconditioning alone. Indeed, since NCX1 and NCX3 silencing partially prevented ischemic neuroprotection mediated by preconditioning these results have shown that it would be reasonable to tune up a

pharmacological strategy able to modulate NCX1. This approach has been derived from the evidence that is possible to hypothesize that the increased expression of certain proteins induced by a neuroprotective strategy like preconditioning could render the brain tissue ready to withstand subsequent, more severe brain conditions. Interestingly, the activation of these mechanisms has appeared to be long lasting, as relatively to sodium/calcium exchanger protein the upregulation of NCX1 and NCX3 was still present even after 72 h after preconditioning induction, thus suggesting that both NCX1 and NCX3 might be considered as two possible effectors of delayed preconditioning. More important, the increased expression of NCX1 and NCX3 observed at early time points does not necessarily implicated that the neuroprotection might have occured at the same time points. The results mentioned in this study have suggested that, in order to reduce the extension of the infarct volume after a harmful ischemic insult an enhancement of NCX1 and NCX3 expression and/or activity might be desirable.

2.4.3 MECHANISM OF ACTION OF SODIUM/CALCIUM EXCHANGER

The NCX family, which exchanges three Na^+ ions for one Ca^{2+} ion or four Na+ ions for one Ca2+ ion depending on $[Na^+]_i$ and $[Ca2^+]_i$ (Fujioka et al, 2000; Kang & Hilgemann, 2004; Reeves & Hale, 1984) consists of three dominant genes coding for the three different isoforms of the exchanger: NCX1 (Nicoll et al, 1990), NCX2 (Li et al, 1994), and NCX3 (Nicoll et al, 1996) proteins. These three genes appear to be dispersed, since NCX1, NCX2, and NCX3 have been mapped in mouse chromosomes 17, 7, and 12, respectively (Nicoll et al, 1996). At the post-transcriptional level, at least 12 NCX1 and 3 NCX3 proteins are generated through alternative splicing of the primary nuclear transcripts. These variants arise from a region of the large intracellular f-loop, are encoded by six small exons defined A to F,

and are used in different combinations in a tissue-specific manner. To maintain an open reading frame, all splice variants must include either exon A or B, which are mutually exclusive (Quednau et al, 1997). NCX1 is composed of 938 amino acids in the canine heart and has a molecular mass of 120 kDa and contains nine transmembrane segments (TMS). NCX1 amino terminus (N-terminal) is located in the extracellular space, whereas the carboxyl terminus (C-terminal) is located intracellularly. The nine transmembrane segments can be divided into an N-terminal hydrophobic domain, composed of the first five TMS (1–5), and into a C-terminal hydrophobic domain, composed of the last four TMS (6–9). These two hydrophobic domains are important for the binding and the transport of ions. The first (1–5) TMS are separated from the last four (6–9) TMS through a large hydrophilic intracellular loop of 550 amino acids, named the f-loop (Nicoll et al, 1999). Although the f-loop is not implicated in Na^+ and Ca2+ translocation, it is responsible for the regulation of NCX activity.

NCX can facilitate both Ca^{2+} and Na^+ flow in a bidirectional way through the plasma membrane (Blaustein & Lederer, 1999; Philipson & Nicoll, 2000) with a stoichiometry of 3 Na+ ions versus 1 Ca^{2+} ion. Depending on the intracellular levels of Na^+ and Ca^{2+}, NCX can operate in the forward mode by extruding one Ca^{2+} against three entering Na+, using the Na+ gradient across the plasma membrane as a source of energy (Annunziato et al, 2004; Blaustein & Lederer, 1999). Alternatively, in the reverse mode, NCX can function as Na^+ efflux–Ca^{2+} influx. Because of its high exchange capacity, NCX is well-suited for rapid recovery from high intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) and may play an important role in maintaining Ca^{2+} homeostasis and protecting cells from Ca^{2+} overload and eventual death (Annunziato et al, 2004; Blaustein & Lederer, 1999).

2.4.4 REGULATION OF SODIUM/CALCIUM EXCHANGER

During brain ischemia there is a dramatic impairment of mechanisms regulating the homeostasis of sodium and calcium ions between the internal neurons and their extracellular melieu. One of the crucial actors of these biochemical deleterious events leading to neuronal cell pain is represented by the sodium/calcium exchanger (conventionally referred to as NCX). The activity of this membrane antiporter protein has been focused for several years of experimental work of our research group. The exchanger acts by modulating in excitable cells the intracellular concentrations of Na^+ and Ca^{2+} ions, thus providing for the maintenance of cell ions homeostasis. NCX mediates ions fluxes of Ca^{2+} and Na^+ ions across the plasma membrane in a bidirectional way (Annunziato et al, 2004; Blaustein & Lederer, 1999; Philipson & Nicoll, 2000). There are several factors that regulate the activity of the exchanger sodium calcium, among which: (i) the concentration of the two cations transported, Na^+ and $Ca^{2+;}$ (ii) the intracellular pH; (III) compounds related to metabolism, ATP, PIP2, PKA and PKC, and (iv) reactive oxygen species (ROS) and reactive nitrogen species (RNS). The concentration of calcium regulates NCX through CBD (Ca^{2+}-binding domain) in the same way the Na^+ ion plays a regulatory function. In particular when the sodium concentration increases, it binds to the site of transport of the heat exchanger, and after this influx of sodium, is an inactivation of the same. This process of inactivation, is very similar to the phenomenon that occurs in the voltage-gated ion channels, and is called sodium-dependent inactivation. The exchanger can also be regulated by the intracellular pH. A strong acidity inhibits the activity of NCX leaving it under a constant steady state, in fact, reductions in pH value below 0.4, can induce an inhibition of NCX than 90%. The ATP, which acts as a donor of phosphate groups, can increase the activity of the exchanger in different ways. First, activating the G protein-coupled receptors for endogenous and exogenous ligands. As a

second, ATP can stimulate the activity of NCX through the pathway involving PKC or PKA; each isoform of NCX is presumed to have several phosphorylation sites. Finally, another mechanism by which NCX can be activated requires the production of lipid PIP2. In fact, this lipid binds the XIP region of the loop "f" eliminating the inactivation of NCX, thus stimulating its function. Interestingly, the depletion of ATP within the cell act differently on the three isoforms of the exchanger by inactivating both NCX1 and NCX2 but not by influencing the activity of NCX3 (Secondo et al, 2007) . The sodium-calcium exchanger is sensitive to reactive oxygen species, in fact by altered redox cell can result increases the activity of NCX (Annunziato et al, 2004). Among the main factors that regulate the activity of NCX can be considered, quite rightly, miRNAs that have already amply demonstrated to fine-tune their target genes at the post-transcriptional level.

2.4.5 SODIUM/CALCIUM EXCHANGER ACTIVATOR: NEUROUNINA

In recent years there has been great interest in the identification of new compounds capable of increasing NCX activity to limit the extension of ischemic brain damage (Annunziato et al, 2009). Only NCX inhibitors were available including 3-amino-6-chloro-5-[(4-chloro-benzyl)amino]-N-[[(2,4-dimethylbenzyl) amino]iminomethyl]-pyrazinecarboxamide (CB-DMB) (Secondo et al, 2009), KB-R7943 (Watano et al, 1999), SEA0400 (Matsuda et al, 2001), SM-15811 (Hasegawa et al, 2003) , SN- 6 (Iwamoto et al, 2004; Iwamoto & Kita, 2006) , and YM-244769 [132]. These inhibitors had a number of interesting features. In particular, KB-R7943 preferentially inhibits NCX3 more than NCX1 and NCX2 (Iwamoto et al, 2001), whereas SEA0400 and SN-6 preferentially block NCX1 rather than NCX2 and NCX3 (Iwamoto et al, 2004; Iwamoto & Kita, 2006). However, despite their potency, these compounds possess some nonspecific actions against other ion channels and receptors (Pintado et al,

2000; Reuter et al, 2002). In one of our recent work we developed a potent NCX activator, named neurunina. Starting from the structure of one of the most potent NCX inhibitors, SM-15811, we synthesized a new compound, 7-nitro-5-phenyl-1-(pyrrolidin-1-ylmethyl)-1H-benzo[e][1,4]diazepin-2(3H)-one. Neurounina was investigated in order to verify the effect on NCX1, NCX2, and NCX3 activity in the forward and reverse modes of operation by means of Ca^{2+} radiotracer, Fura-2 microfluorimetry, and patch-clamp techniques, and thereafter, with the help of chimera strategy, deletion, and site-directed mutagenesis, it were identified the molecular determinants of this compound on NCX structure. Neurounina, whose structure is illustrated in the inset of figure 4, increases NCX1 and NCX2 activity in a concentration-dependent manner but it is not active on NCX3. More important, since NCX activity is involved in stroke pathophysiology, it was examined the putative protective effects of neurounina on in vitro and in vivo models of cerebral ischemia. This compound provided with an high lipophilicity index and exerted a neuroprotective action in an animal model of stroke (tMCAO) (Molinaro et al, 2013) (Fig 5).

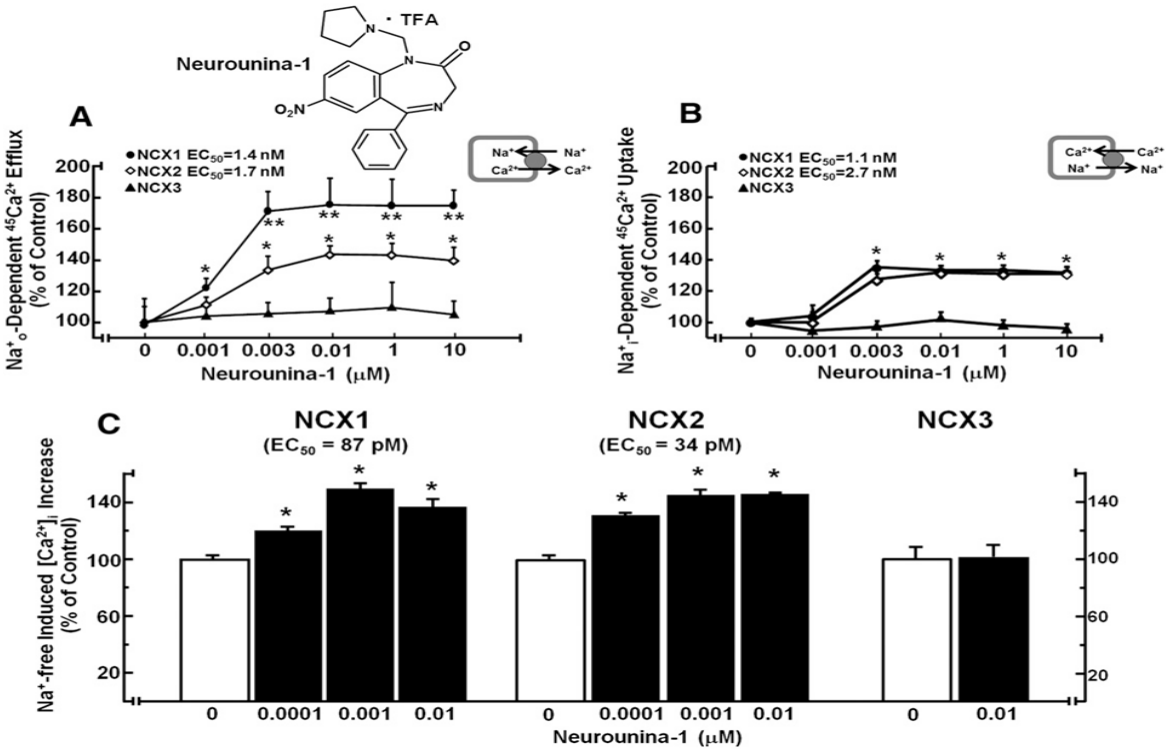


Fig. 4. Effect of neurounina-1 on NCX1, NCX2, and NCX3 activity measured by Na^+-dependent $^{45}Ca^{2+}$ influx/efflux and Fura-2-monitored Ca^{2+} techniques. Inset shows the chemical structure of neurounina-1. (**A** and **B**) concentration-response curves of the effects of neurounina-1 on Na_o dependent $^{45}Ca^{2+}$ efflux and Na_o-dependent $^{45}Ca^{2+}$ uptake, respectively, in BHK cells expressing NCX1, NCX2, or NCX3. (**C**) effect of neurounina-1 (0.0001–0.01 μM) on NCX1, NCX2, and NCX3 activity measured by single-cell Fura-2 AM microfluorimetry (n = 60 cells for each group). (Molinaro et al, 2013).

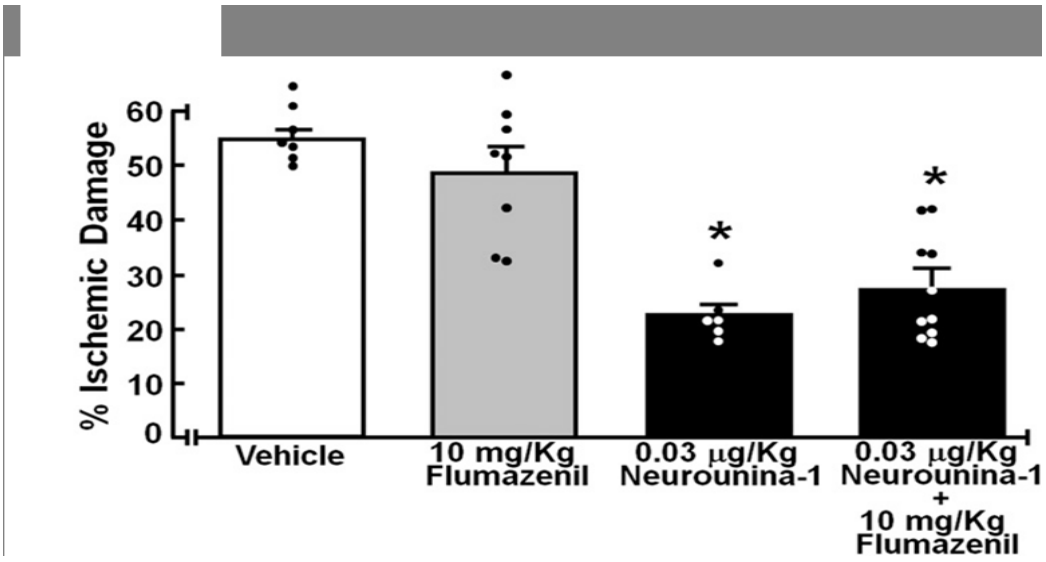


Fig. 5. After stroke onset. Each column represents the mean ± S.E.M. of the percentage of the infarct volume compared with the ipsilateral hemisphere. Each dot represents the single value measured. *P< 0.05 vs. control group. **P < 0.05 vs. 0.01 mg/kg treated group.(Molinaro et al, 2013)

3. AIM OF THE STUDY

Hypoxic-ischemic encephalopathy (HIE) accounts for the majority of developmental, motor and cognitive deficits in children, leading to life-long neurological impairments. Since transient brain ischemia followed by reoxygenation alters ionic homeostasis in adult brain and the plasma membrane sodium/calcium exchanger (NCX) plays a fundamental role in the maintenance of ionic homeostasis during brain ischemia, we aim to demonstrate the involvement of NCX in the pathophysiology of HIE. In particular the purpose of the present study was (1) to evaluate the protein expression profile of NCX1, NCX2 and NCX3 in the brain of mice subjected to neonatal hypoxia ischemia till adulthood; (2) to evaluate the effect of NCX activator on the development of HIE hippocampal lesion; and (3) to verify whether long term behavioral dysfunctions caused by HIE could be prevented by activating NCX (Fig. 6).

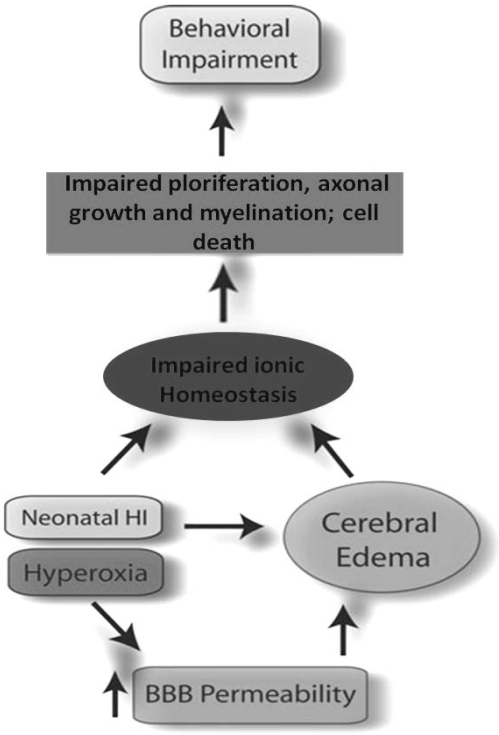


Fig. 6. Neonatal HI leads to behavioral impairment caused in part by development of dysregulation of ionic homeostasis HI-induced.

4. MATERIAL AND METHODS

4.1 STUDY DESIGN

In order to accomplish the proposed objectives, the study has been divided in three phases:

Phase 1. NCX1, NCX2 and NCX3 expression was evaluated in hyppocampus of mice subjected to neonatal Hypoxic ischemic injury (Fig. 7). The experimental groups were randomized as following:

Group 1. Naive C57BL mice were sacrified at different time intervals in order to verify the physiological hyppocampal expression of NCX1 and NCX3 (E16, p0, p5, p7, p10, p14, p21, p28 and p60) and the relative mRNA (p10 and p14)

Group 2. p7 C57BL mice were subjected to only hypoxia and were sacrified 3 and 7 days (p10 and p14) after injury in order to verify NCX1 and NCX3 mRNA expression

Group 3. p7 C57BL mice were subjected to Hypooxia-Ischemia and were sacrified at different time intervals after injury (p10, p14, p21, p28 and p60), to verify NCX1, NCX2 and NCX3 protein expression and the relative mRNA (p10 and p14) in order to compare with physiological levels.

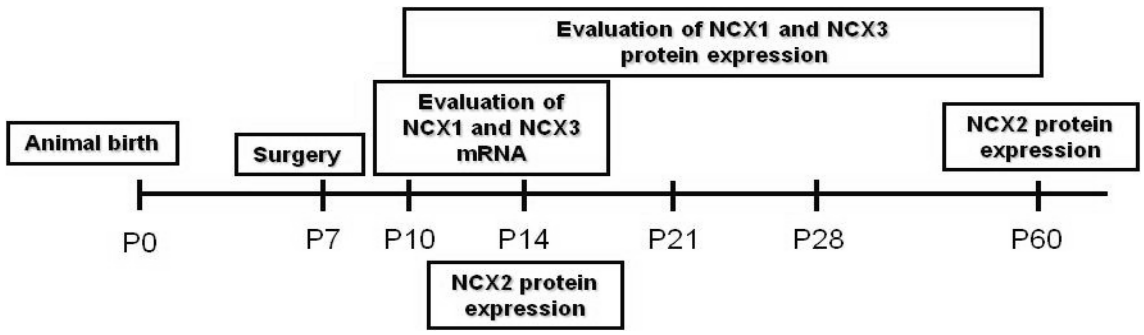


Fig. 7. Phase 1 experimental program

Phase 2. NCX activator, Neuronina, was administered after injury in order to verify neuroprotective activity in acute phase of HI injury (Fig. 8). The experimental groups were randomized as following:

Group 1. p7 C57BL mice were subjected to Hypooxia-Ischemia. 2 hours after the surgical procedure of Hypoxia/ischemia induction, animals were treated with 100 µL i.p. of saline solution

Group 2. p7 C57BL mice were subjected to Hypooxia-Ischemia. 2 hours after the surgical procedure of Hypoxia/ischemia induction, animals were treated with 100 µL of Neurounina saline solution at dose of and 30 mg/kg i.p.

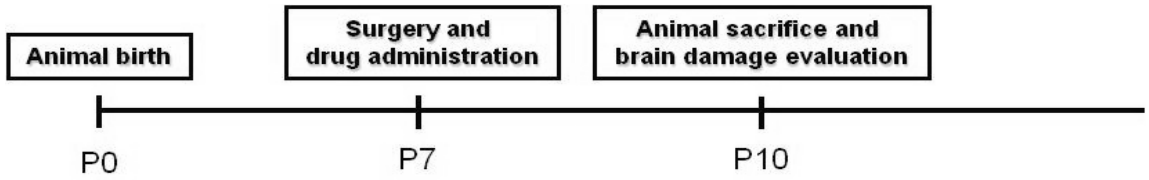


Fig. 8. Phase 2 experimental program

Phase 3. Neurounina compuond was administered in adult mice 60 days after injury, 3-5 hours before behavioral test evaluation in order to verify whether the increased NCX activity was able to ameliorate behavioral deficits induced by neonatal hypoxia ischemia (Fig. 9). The experimental groups were randomized as following:

Group 1. p7 C57BL mice were subjected to Hypooxia-Ischemia. Around 53 days after injury, p60 animals were treated with 100 µL of Neurounina saline solution at dose of and 30 mg/kg i.p. 3-5 hours before behvioral tests.

Group 2. p7 C57BL mice were subjected to Hypooxia-Ischemia. Around 53 days after injury, p60 animals were treated with 100 µL of saline solution with i.p. injection 3-5 hours before behvioral tests.

Group 3. p60 C57BL naive mice were treated with 100 µL of Neurounina saline solution at dose of and 30 mg/kg i.p. 3-5 hours before behvioral tests.

Group 4. p60 C57BL naive mice were treated with 100 µL of saline solution at with i.p. injection 3-5 hours before behvioral tests.

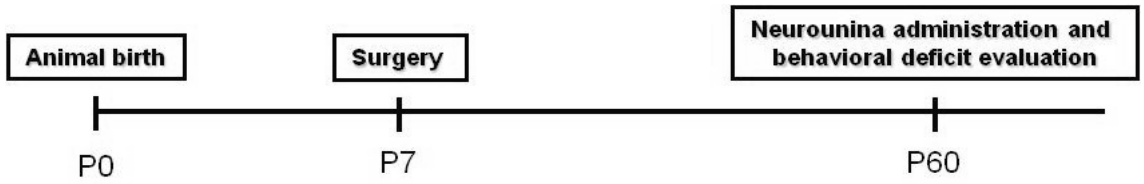


Fig. 9. Phase 3 experimental program

4.2 PROPIDIUM IODIDE LABELING, TISSUE PREPARATION AND DAMAGE ANALYSIS

Five microliters of propidium iodide (PI, 1 mg/mL in distilled water, Sigma) were injected into the right lateral brain ventricle of newborn mice. Twenty minutes after injection, pups were deeply anesthetized and perfusion-fixed with 4% paraformaldehyde in 0.1 mol/L PBS. Brains were rapidly removed on ice and processed for antigen retrieval by immersing overnight in 10 mmol/L sodium citrate buffer (pH 6.0, 4 °C) and boiling in the same buffer for 3 min. After boiling, brains were cryoprotected with 30% sucrose/PBS (72 h, 4 °C). Preliminary experiments have shown that the boiling procedure does not affect the distribution of the dye

(Carloni et al, 2007; Unal Cevik & Dalkara, 2003). The brains were cut on a cryostat into coronal sections (thickness 20μm) and then processed for immuno-fluoscence analyses. In initial experiments, propidium iodide dye has been injected into the right lateral ventricle of both control (neither hypoxic nor ischemic; n=3) and sham-operated hypoxic animals (subjected only to the hypoxic procedure; n=2) and no dye detection was found. Sections were analyzed under a microscope (Nikon E400). The infarct area PI-stained was calculated comparing it with whole ipsilateral hippocampus to determine the percentage of area damaged. The tissue loss instead, was calculated comparing the area of ipsilateral and controlateral emisphere.

4.3 IMMUNOHISTOCHEMISTRY

Immunostaining and confocal immunofluorescence procedures were performed as previously described (Boscia et al., 2009). PI-labeled cryosections were incubated with 1.5% normal blocking serum for 1 h at room temperature and then incubated overnight at 4 °C with the following antibodies: neuron-specific nuclear protein (anti-NeuN, mouse monoclonal antibody; 1:500; Chemicon); glial fibrillary acidic protein (GFAP, rabbit polyclonal antibody; 1:300; DAKO). In other experiment, the cryosections were incubated with 1.5% normal blocking serum for 1 h at room temperature and then incubated overnight at 4 °C with the following antibodies: mouse monoclonal anti-NCX1 (1:500) rabbit polyclonal anti-NCX3 (1:4000), neuron-specific nuclear protein (anti-NeuN, mouse monoclonal antibody; 1:500; Chemicon). Sections processed for NeuN were incubated with the corresponding biotinylated secondary antibody, and the peroxidase reaction was developed using 3,3-diaminobenzidine/4-HCl as a chromogen. Sections processed for immunofluorescence analysis were incubated with the corresponding fluorescent-

labeled secondary antibodies (Alexa 488/Alexa 594-conjugated anti-mouse/anti-rabbit IgGs). Images were observed using a Zeiss LSM510 META/laser scanning confocal microscope. Single images were taken with an optical thickness of 0.7 m and a resolution of 1024 × 1024. In double-labeled sections, the pattern of immunoreactivity for both antigens was identical to that seen in single-stained material. Controls of the methods in the double immunofluorescence experiments included replacement of the primary antisera with normal serum (1:200). To control for a possible cross-reactivity between IgGs in double immunolabeling experiments, some sections were processed through the same immunocytochemical sequence except that primary antisera were replaced with normal serum, or only one primary antibody was applied, but the full complement of secondary antibodies was maintained. In addition, the secondary antibodies utilized were highly pre-adsorbed to the IgGs of numerous species. Tissue labeling without primary antibodies was also tested to exclude autofluorescence. No specific staining was observed under these control conditions, thus confirming the specificity of the immunosignals.

4.4 MRNA EXPRESSION ANALYSIS BY REAL-TIME POLYMERASE CHAIN REACTION

Mice ipsilesional hemispheres belonging to mice underwent only to hypoxia and to hypoxic-ischemic insult both 3 and 7 days after surgery were frozen on dry ice. Brain samples were ground into powered dry ice, then Trizol Reagent solution (Invitrogen, Milan, Italy) was added. Total RNA was extracted and purified in accordance with the manufacturer's protocol. For reverse transcription, 2.0 mg of each extracted RNA was digested with DNase and reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, Segrate, Milan, Italy). Amplification was performed using Power Sybr

Green PCR Master Mix (Applied Biosystem, Milan, Italy) according to the manufacturer's protocol. All data were normalized to hypoxanthine phosphoribosyltransferase 1 mRNA levels and expressed as percentage of the mRNA levels detected in sham-operated animals.

Sequences of the primers used were the following:

NCX1-forward, 5 -CCGTGACTGCCGTTGTGTT-3 ; NCX1-reverse, 5 -GCCTATAGACGCATCTG-CATACTG-3 ; NCX3-forward, 5 -CCTCTGTGCCA-GATACATTTGC-3 ; NCX3-reverse, 5 -CC-AAACCAATACCCAGGAAGAC-3 ; hypoxanthine-guanine phosphoribosyl transferase (HGPRT)-forward, 5 -TCCATTCCTATGAC-TGTAGATTTTATCAG-3 ; HGPRT-reverse, 5 -AACTTTTATGTCCCCCGTTGACT-3.

4.5 PROTEIN EXPRESSION ANALYSIS

Whole-cell protein extracts from dissected areas were obtained and processed as previously described (Secondo et al, 2007). Nitrocellulose membranes were incubated with anti-NCX1 antibody (rabbit poly-clonal; Swant, Bellinzona, Switzerland; 1:1000 dilution), anti-NCX2 antibody (rabbit polyclonal; AlphaDiagnostics International, San Antonio, TX; 1:1000 dilution), anti-NCX3 antibody (rabbit polyclonal; 1:2000; kindly provided by K. D. Philipson and D. Nicoll, University of California, Los Angeles, CA) and anti- β-tubulin (Sigma-Aldrich rabbit polyclonal 1:1000 dilution). These nitrocellulose membranes were first washed with 0.1% Tween 20 and then incubated with the corresponding secondary antibodies for 1 h (GE Healthcare, Little Chalfont, UK). Immunoreactive bands were detected with the ECL (GE Healthcare). The optical density of the bands (normalized with-β tubulin) (Sigma, St. Louis, MO) was determined by Chemi-Doc Imaging System (Bio-Rad, Segrate, Italy)

4.6 INDUCTION OF NEONATAL HI

Postnatal day 7 (P7) C57BL6J mice were anesthetized with isofluorane (4% for induction, 1% for maintenance), 30% O_2, and 70% N_2O. The body temperature of the animal was maintained at 37C during the whole procedure with a heating pad. Under a surgical stereo microscope, a midline skin incision (0.5 cm) was made on the neck, and the right common carotid artery was exposed, isolated and electrically cauterized. The incision was rinsed with 1% lidocaine and sutured with a 6.0 polypropylene (Prolene) suture. Animals were returned to their dams and observed continuously for 30 min during a recovery period of 1 hour. To induce ipsilateral ischemic injury as described by Vannucci et al., the animals were placed into an hypoxic chamber, perfused with an equilibrated gaseous mixture made from 8% O_2 and 92% N_2 at 37 °C; the chamber was placed in a water bath heated to 37 °C for 60 minutes. After HI, animals were monitored continuously for 30 min and then checked every 30 min for 2 h and then daily until they were sacrificed.

4.7 BEHAVIORAL STUDIES

At two months of life mice were analyzed in behavioral performance tests. Experiments were performed under diurnal lighting conditions between 10:00 A.M. and 5:00 P.M. Neurounina-1 saline solution was administered (dose 30 mg/kg i.p) 3-5 hours before the behavioral test. Exploratory behavior was acquired with a high definition digital camera while on-line videotracking of animals was computed using a custom software written in Matlab environment.

4.7.1 OPEN-FIELD EXPLORATION TEST

The open field apparatus consisting of a transparent Plexiglas square arena (50x50 cm, 40 cm high) was placed in a homogenously lit experimental room with several large-scale environmental visual cues. Briefly, mice were placed in the arena and were allowed to explore it for 5 min. Total traveled distance was measured using an automated tracking device. Moreover, the time spent by animals in three concentric areas was automatically measured as an index of anxious behavior (Kassed & Herkenham, 2004); (Kazlauckas et al, 2005). Since the probability to explore an area is proportional to the size of the area, the time spent in each area was also divided by the percentage size of the total area to obtain unbiased estimates. At the end of the test, the number of fecal boluses was counted to evaluate anxious behavior. The same apparatus was also used for the object recognition task.

4.7.2 ELEVATED ZERO MAZE

The elevated zero maze is a modification of the elevated plus maze used for assessing anxiety-related behaviors. Use of the circular maze removes any ambiguity in data interpretation as there is no center zone (Lister, 1990; Rodgers et al, 1997; Shepherd et al, 1994). The elevated circular platform (40 cm off the ground, 50 cm in diameter) had two enclosed arenas opposite each other (5 cm wide with 15 cm high walls) and two open arenas (5 cm wide). At the start of the test, each mouse was lowered by its tail into the open arena of the maze and allowed to explore the maze for 300 s. Activity of the mouse was monitored via an overhead camera connected to a computer in a separate room using video acquisition and ANY-maze analysis software (Stoelting, Wood Dale, IL). Data analyzed included percentage of time spent in the open versus closed arenas and the total distance traveled in the maze.

4.7.3 DELAY-DEPENDENT ONE-TRIAL OBJECT RECOGNITION TASK

Two different objects that differed in terms of height, color, shape, and surface texture were used. Both of them had sufficient weight to ensure that the mice could not displace them, had no ethological significance for the mice, and had never been paired with a reinforcer. Indeed, pilot studies have ensured that mice of the C57BL/6J genetic background strain cannot discriminate between objects and have no preference for one object over another (Ennaceur & Delacour, 1988)¨(Dere et al, 2007). Procedure. Mice were subjected to two types of trials: a sample trial and a test trial. During the sample trial, which took place 1 d after exposure to the open field so as to allow the animals to habituate to the test apparatus, all mice were presented with the first pair of identical objects. Briefly, the animal was placed on one side of the open field, whereas, on the opposite side, two copies of the same objects were placed 10 cm from the two corners. After 10 min, the animal was removed from the open field and were returned to their home cage (Ennaceur & Delacour, 1988); (Dere et al, 2007). During the trial phase, instead, H/I and WT mice were tested for item recognition (10 min) 5 min and 24h after sample trial phase. The animal was again released into the open field, now containing two objects: the familiar one used during the sample trial and the novel object, which was randomly positioned (Ennaceur & Delacour, 1988); (Dere et al, 2007).The time spent by each mouse in exploring the two objects was measured using an automated tracking device that record the mouse position coordinate and the two objects coordinates to match them. Exploration of an object was assumed when the mouse approached an object, when touched it with either its vibrissae, snout, or forepaws. The percentage of time spent exploring the novel object, compared with the total time spent exploring both objects, was recorded and considered as a measure of object recognition: recognition index =

t novel / (t novel + t familiar) X 100. Recognition index values >50 suggest a preference for the novel object, values close to 50 suggest no recognition, and values well below 50 suggest a preference for the familiar object (Dere et al, 2007; Ennaceur & Delacour, 1988; Kassed & Herkenham, 2004; Kazlauckas et al, 2005).

4.7.4 BARNES CIRCULAR MAZE TASK

The behavioral apparatus used in this experiment consisted of a white circular platform (1.22 m diameter) elevated 40 cm above the floor, with 36 equally spaced holes (each 5 cm diameter) around the periphery (5 cm from the perimeter). Only one hole led to a dark escape box (5cm X5cm X11cm) that was fixed in relation to the distal environmental cues and contained some dust-free sawdust bedding which was changed between trials. The platform surface was randomly rotated from trial to trial to prevent the use of local olfactory, visual, or tactile cues and was brightly illuminated from above. The illumination served as motivation to escape in the dark box (Bach et al, 1995); (Seeger et al, 2004). During procedure mice were gently picked up from the tail and placed in the middle of the platform. The direction faced by the animals at the start position was random and changed from trial to trial. After 5 min, if the mice did not find the goal they were gently directed toward the correct hole, and allowed to descend into the escape box where they were left for 1min (Bach et al, 1995); (Seeger et al, 2004).On the following 5 d, test trials (one trial per day) were performed under identical conditions. Each trial ended when the mouse entered the goal tunnel or after a maximum time of 5 min. The amount of time it took the mice to enter the escape hole (escape latency) was recorded (Bach et al, 1995); (Seeger et al, 2004).

4.7.5 T-MAZE SPONTANEOUS ALTERNATION

This procedure was carried out in an enclosed “T” shaped maze (Med Associated, St. Albans, VT) in which long arm of the T (47 cm × 10 cm) serves as a start arm and the short arms of the T (35 cm × 10 cm) serve as the goal arms. In this task the mouse was placed in the start arm and after 5 seconds the door was opened and the mouse was allowed to choose and explore one of the goal arms. When the mouse had fully entered the choice arm (tail tip all the way in) a guillotine door was closed and the mouse was confined to the choice arm for 30 s. The mouse was then removed, the guillotine door lifted and the next trial initiated. This was repeated for a total of 9 trials. If the mouse did not make a choice within 2 min the trial was ended and advanced to the next. At the conclusion of each trial the maze was cleaned of urine and feces.

4.8 STATISTICAL ANALYSIS

The data were evaluated as means ± SEM. Statistically significant differences among means were determined by ANOVA followed by Student-Newman-Keuls test. The threshold for statistical significance data was set at $P < 0.05$. The non-parametric data related to some behavioral test, were analyzed using the non-parametric Kruskal-Wallis test, followed by the Nemenyi test for the non-parametric multiple comparison Statistical significance was accepted at the 95% confidence level ($p<0.05$).

5. RESULTS

5.1 HIE DETERMINES A LOSS OF HYPPOCAMPAL PYRAMIDAL NEURONS THAT PEAKS 72 HOURS AFTER INJURY INDUCTION

Hippocampal injury was detectable in the ipsilateral hemisphere of HI mice, as reflected by hippocampal atrophy. PI–positive cells were detected in the ipsilateral hippocampus in CA1, CA2, and CA3 regions as confirmed by immunohistochemical analysis (Fig. 10), the PI-stained area was compared with whole ipsilateral hippocampus to determine the percentage of damage 24h, 48h, 72h and 7d after injury. The biggest damage area was obtained 72h after insult (Fig. 11). 53 days after injury (p60 mice), there were not PI stained cells but the number of neurons was significantly reduced reaching a value close to 40% (Fig. 12).

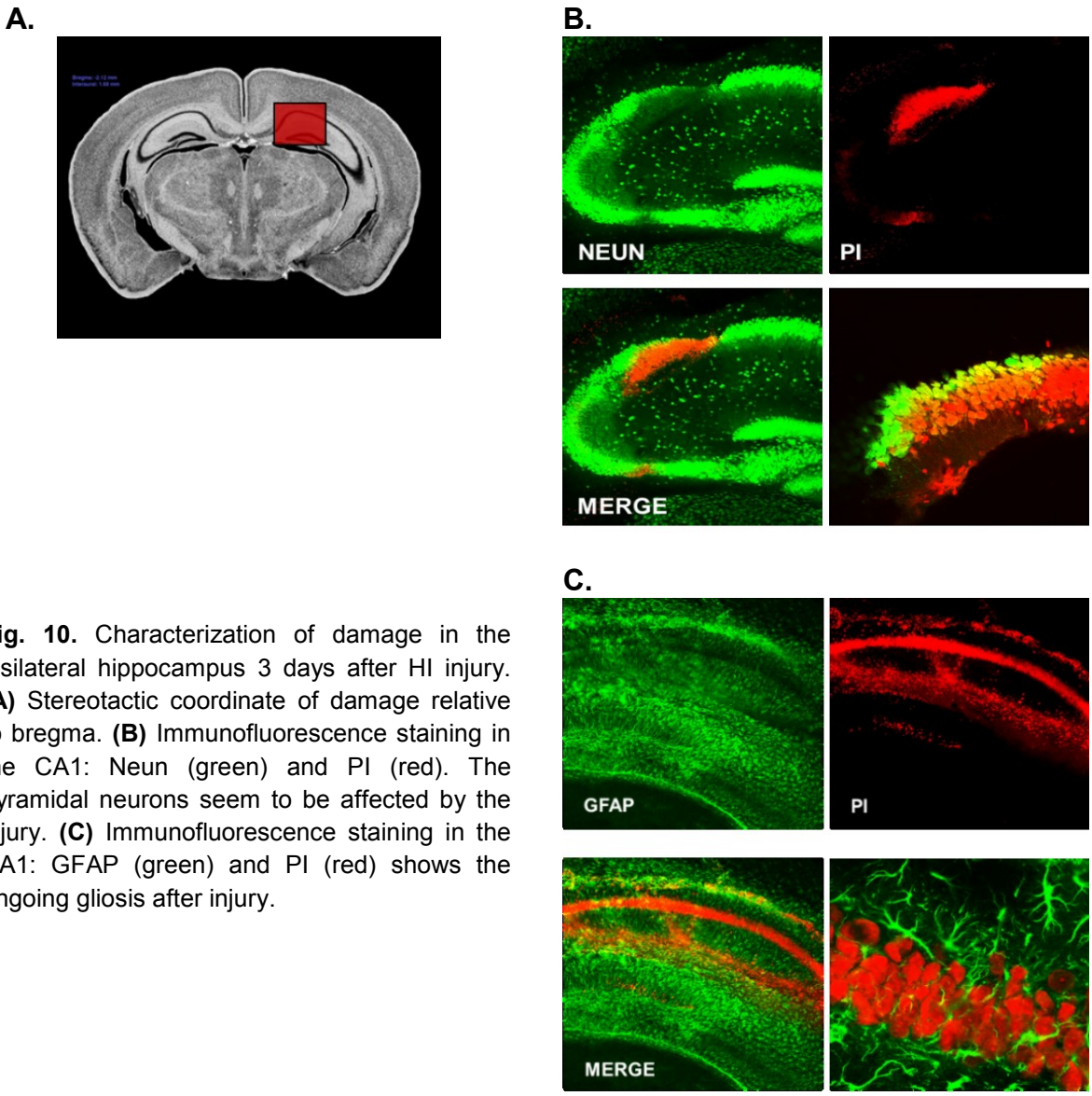


Fig. 10. Characterization of damage in the ipsilateral hippocampus 3 days after HI injury. **(A)** Stereotactic coordinate of damage relative to bregma. **(B)** Immunofluorescence staining in the CA1: Neun (green) and PI (red). The pyramidal neurons seem to be affected by the injury. **(C)** Immunofluorescence staining in the CA1: GFAP (green) and PI (red) shows the ongoing gliosis after injury.

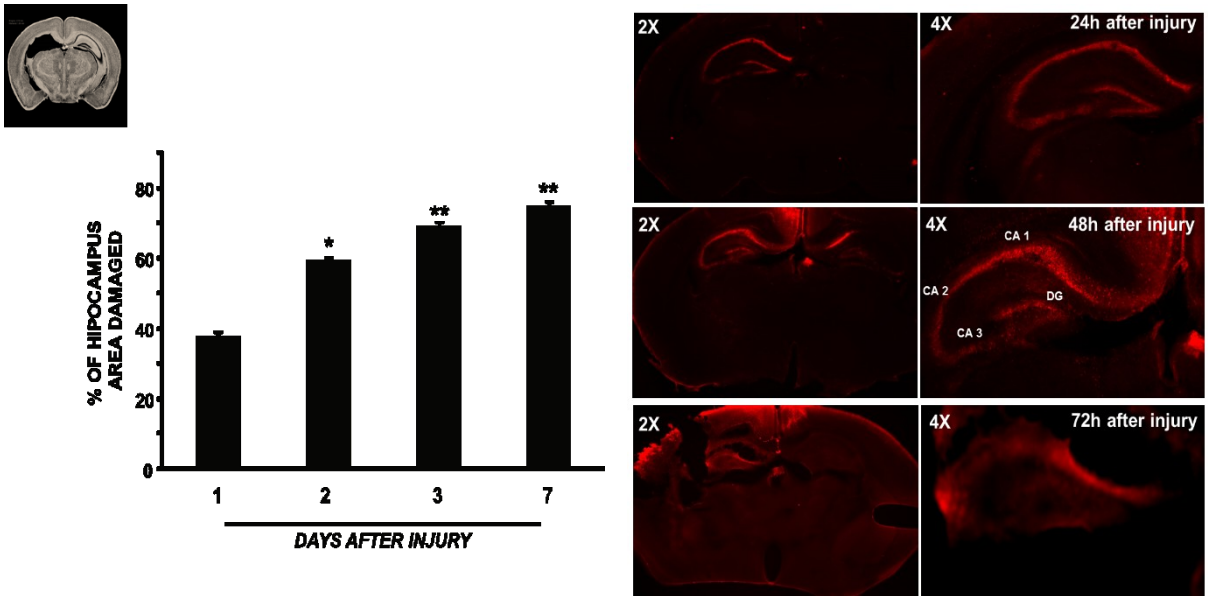


Fig. 11. PI-stained area was compared with whole ipsilateral hippocampus to determine the percentage of damage 24h, 48h, 72h and 7d after injury. There is a peak of damage around 72h after injury.

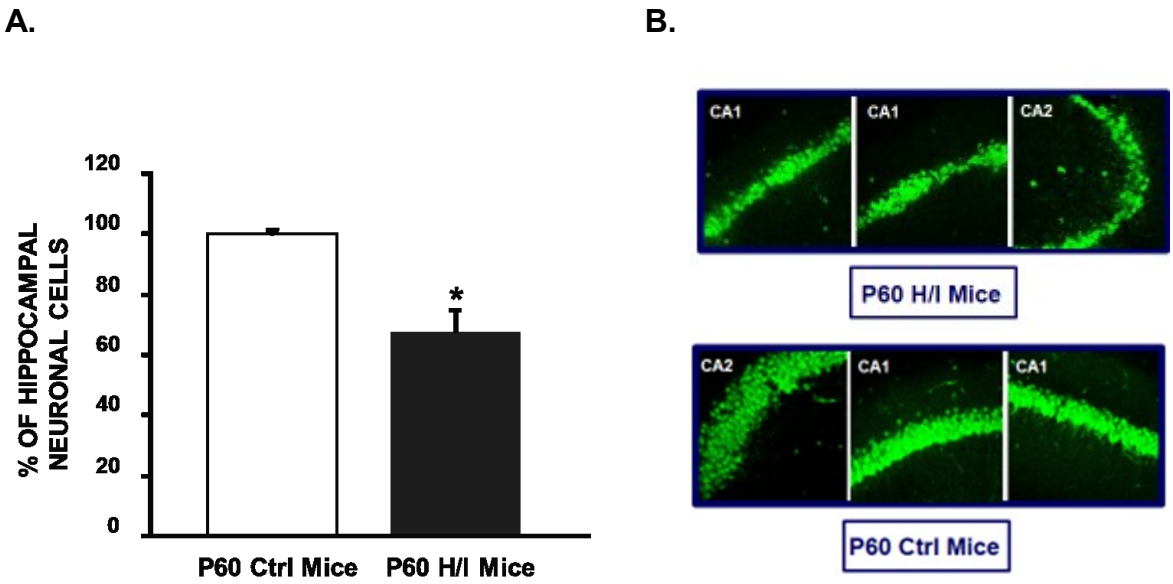


Fig. 12. In p60 mice (53 days after injury) there were not PI stained cells but the number of neurons in CA1 CA2 and CA3 was reduced about 40%. **(A)** Counting of hippocampal neuronal cells. **(B)** Immunofluorescence staining of hippocampus of control and HI mice (Neun green).

5.2 ACTIVATION OF NCX WITH NEUROUNINA REDUCES NEURONAL INJURY IN HIPPOCAMPUS

To investigate whether NCX activity plays a role in neuronal injury after HI, the effect of the potent NCX activator Neurounina on H/I related brain injury was examined. Brain damage was assessed with Propidium Iodide technique, a method that detects degenerative cells (Carloni et al, 2007; Unal Cevik & Dalkara, 2003) . As shown in figure 13. , hippocampal injury was visible in the ipsilateral hemisphere of HI vehicle treated animals, as reflected by hippocampal atrophy. 72h after injury, PI–positive cells were detected in the ipsilateral hippocampus in CA1, CA2, and CA3 regions. In contrast, in the animals Neurounina treated after injury, the ipsilateral hippocampus exhibited smaller lesions, reflected by better preserved hippocampal morphology (Fig. 13) and fewer PI–positive cells in the pyramidal neuronal layers of CA1, CA2, and CA3. In the vehicle treatment animals, PI–positive cells appeared in ipsilateral hippocampus and sometime in striatum, thalamus, and some cerebral cortical areas. The animals treated with neurounina after HI induction exhibited neuroprotection in these brain regions, with no PI–positive cells visible in the ipsilateral striatum and thalamus, and fewer PI–positive cells were found in pyramidal neuronal layers of CA1, CA2 and CA3. Moreover comparing controlateral and ipsilateral emisphere it was evaluated the significant reduction of tissue loss in the Neurounina treated animals when compared to vehicle treated animals. These data clearly highlighted the neuroprotective effects of increasing NCX activity with Neurounina after hypoxia-ischemia in acute phase.

5.2.1 QUANTITATIVE ANALYSIS OF NEUROPROTECTION MEDIATED BY NEUROUNINA

As shown in figure 13, background, a nonspecific signal of PI staining was detected in the contralateral hippocampus of brain at 72 h after HI. In contrast, the ipsilateral hippocampus exhibited a higher level of specific PI fluorescence signals in CA1, CA2, CA3, and DG regions. The animals neurounina treated showed at the brain level fewer PI–positive cells (Fig. 13) and a better tissue preservation compared with control animals. The hippocampal injury was quantified by measuring the percentage of area damaged in the vehicle control and in the animals treated with neurounina. Based on the percentage of PI-positive (Fig. 14), neurounina treatment group exhibited statistically significant reductions of PI–positive area in the ipsilateral CA1, CA2 and CA3 regions of the hippocampus ($p<0.05$). Moreover the percentage of tissue loss in the group of HI mice treated with neurounina, calculated comparing the ipsilateral hemisphere area with contralateral hemisphere area, was significantly decreased if compared to H/I miche treated with vehicle (Fig. 14B).

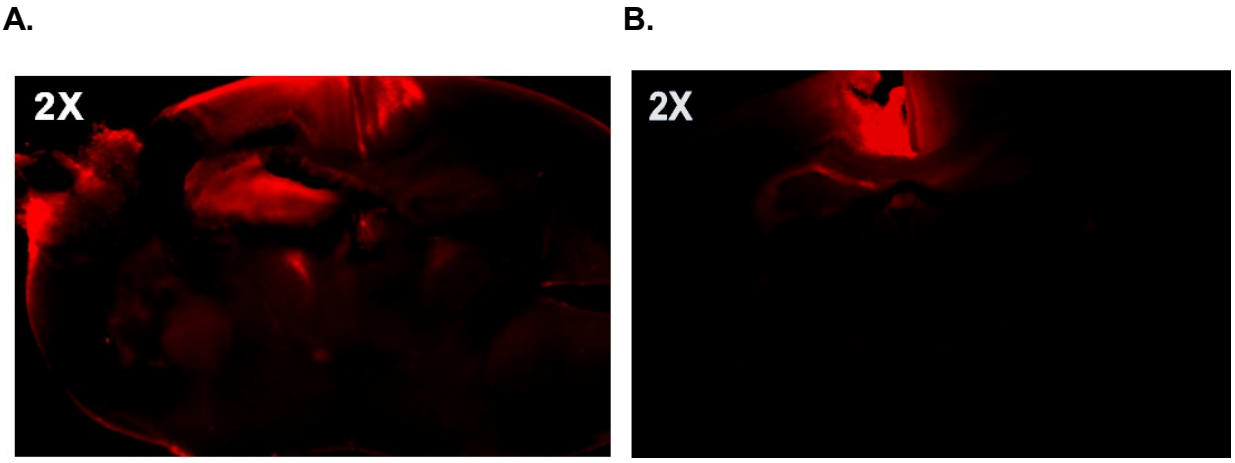


Fig. 13. Neurounina animals treated after injury, exhibited smaller lesions, reflected by better preserved hippocampal morphology and fewer PI–positive cells in the pyramidal neuronal layers of CA1, CA2, and CA3. **(A)** Vehicle treated animal **(B)** Neurounina treated animal

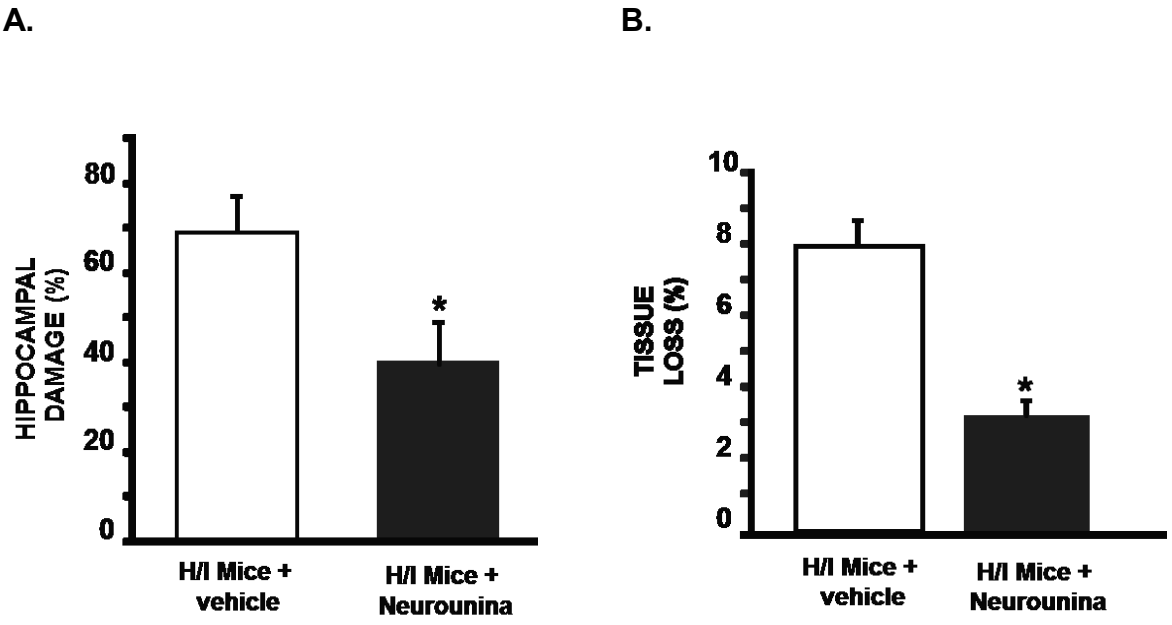


Fig. 14. Quantitative analysis of neuroprotection mediated by Neurounina. **(A)** PI-stained area was compared with whole ipsilateral hippocampus to determine the percentage of damage ($P<0.05$). **(B)** the percentage of tissue loss was calculated comparing the ipsilateral hemisphere area with contralateral hemisphere area in both experimental groups ($P<0.05$).

5.3 NCX1, NCX2 AND NCX3 EVALUATION AFTER NEONATAL HYPOXIA ISCHEMIA

5.3.1 NCX1, NCX2 AND NCX3 MRNA EXPRESSION IS REDUCED BY NEONATAL HI

To assess NCX1 and NCX3 mRNA after hypoxia ischemia, a real-time polymerase chain reaction was performed 3 and 7 days after injury in the ipsilateral hemisphere in the three experimental groups: Naive, only hypoxia and Hypoxia-ischemia mice. Three days after hypoxia-ischemia NCX1 and NCX3 mRNA expression was significantly reduced than control mice but with no difference compared to only hypoxia mice (Fig 15A). Seven days after injury NCX1 and NCX3 mRNA levels were reduced to almost 50% compared to only hypoxia mice that recovered the physiological levels (Fig. 15B).

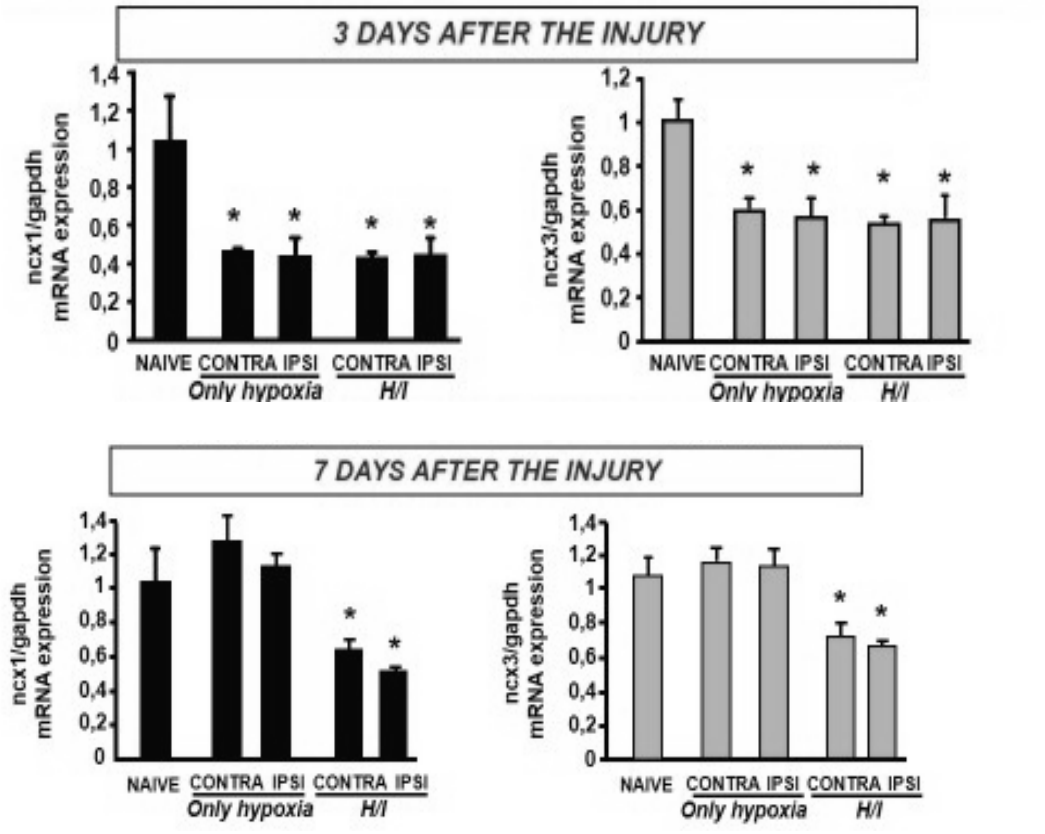
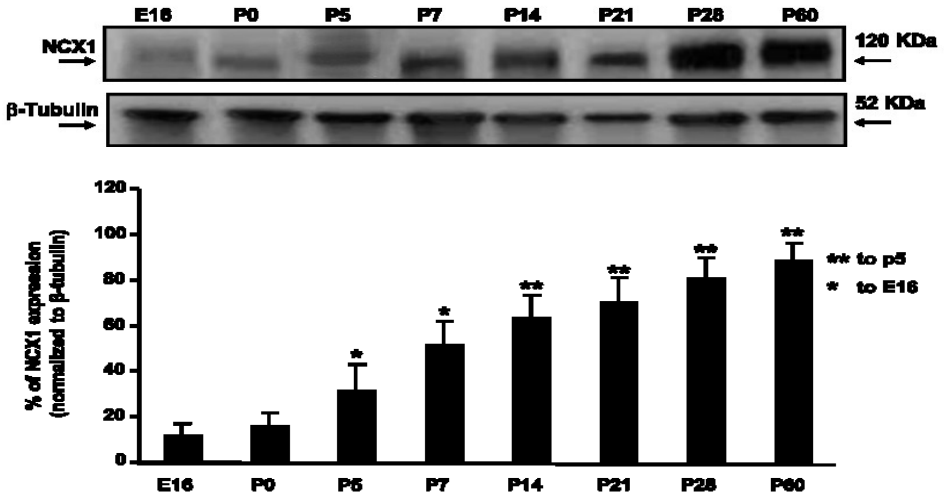


Fig. 15. NCX1 and NCX3 mRNA in the ipsilateral hemisphere after hypoxia or hypoxia-ischemia. **(A)** 3 days after injury NCX1 and NCX3 mRNAs were significantly reduced only hypoxia and Hypoxia-ischemia groups compared to naive mice. **(B)** 7 days after injury NCX1 and NCX3 mRNA levels were reduced to almost 50% compared to only hypoxia mice that recovered the physiological levels.

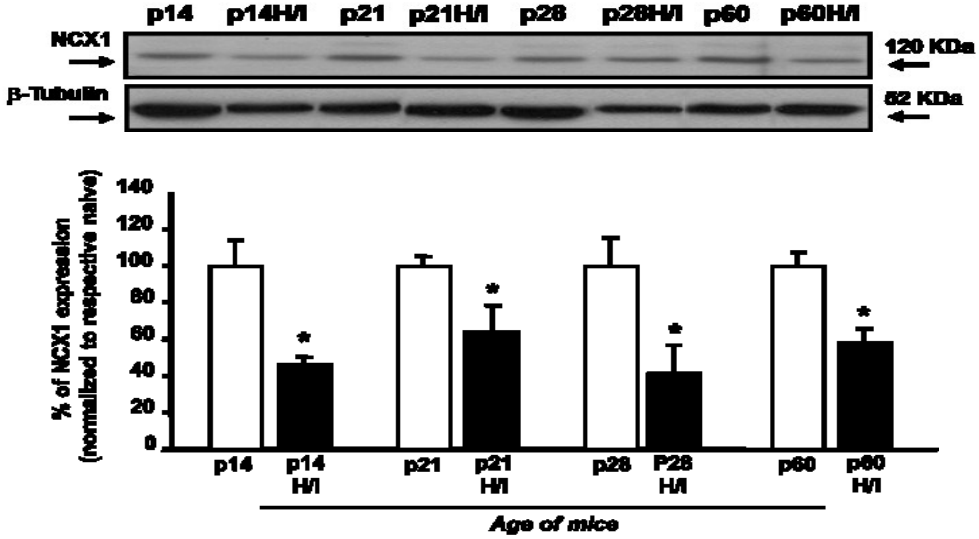
5.3.2 NCX1 AND NCX3 PROTEIN EXPRESSION IS REDUCED IN THE HIPPOCAMPUS OF INJURED MICE

The expression of NCX-1 and NCX-3 proteins was evaluated by Western Blot analysis. A developmental-dependent elevation of NCX-1 and NCX-3 expression was noted in immature hippocampus. A low level of the two proteins was detected in E16-p0 hippocampus and increased in the P5-P21 hippocampus. P21-P60 hippocampus exhibited a sustained level of NCX-1 and NCX-3 expression. The β-tubulin III protein loading controls were similar among these samples (Fig. 16 A). To assess the role played by the three different NCX proteins in the H/I mice, NCX-1 and NCX-3 expression was evaluated in the ipsilateral hippocampus of mice subjected to Neonatal Hypoxia-Ischemia at different time points: 1, 3, 7, 14, 21 and 60 days after injury and compared with the expression of the two proteins in the same brain region of naïve mice (Fig. 16 B-C-; 17 B-C). NCX1 and NCX3 expression was significantly reduced from 7 days until 60 days after the Hypoxic-ischemic insult (Fig. 16 B-C-; 17 B-C). These data were confirmed with immunohistochemical analysis in 7 days after injury mice, compared with control mice (Fig. 18-19). By contrast, NCX2 expression did not show any change in the two experimental groups (naïve and H/I mice) 7 and 60 days after injury (Fig. 20).

A.



B.



C.

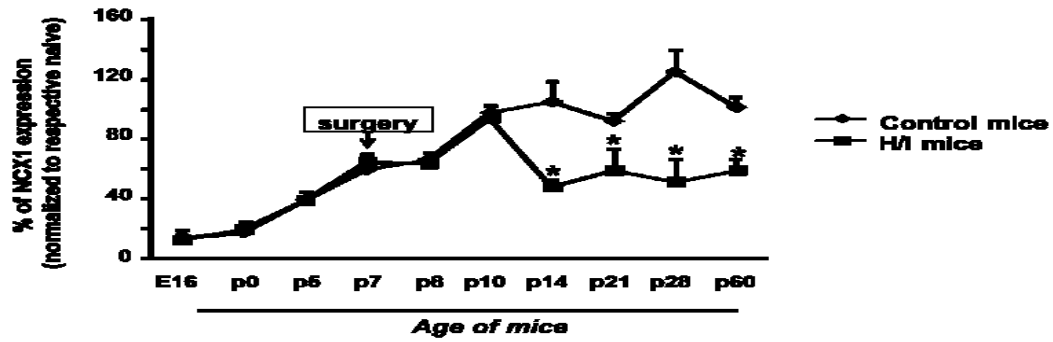


Fig. 16.(A) NCX1 protein increases in an age dependent manner in brain hippocampus of c57 naive mice. **(B-C)** NCX1 protein expression in hippocampus of mice subjected to hypoxic ischemic injury was reduced and did not recover the physiological expression level at all time point considered.

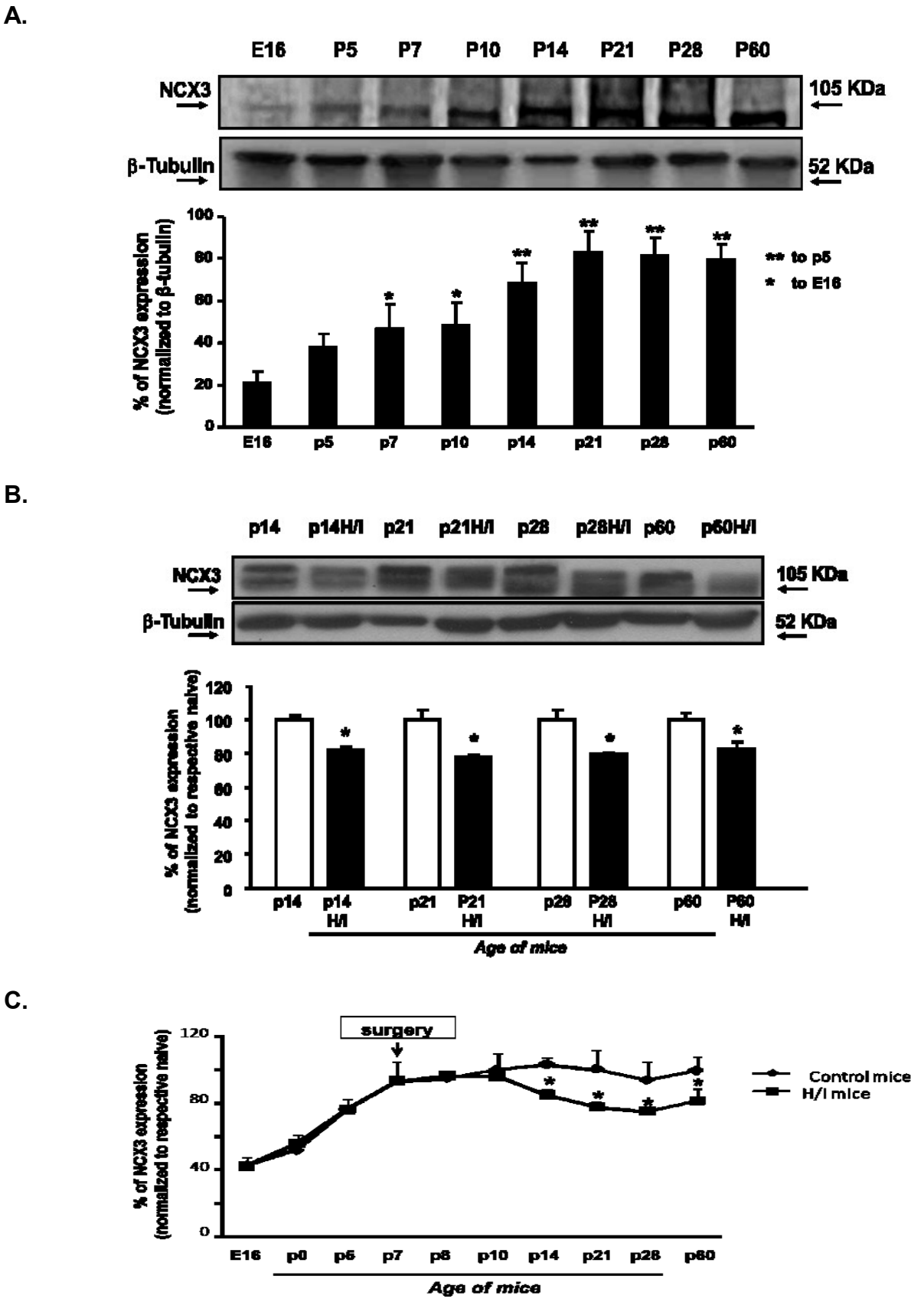


Fig. 17. (A) NCX3 protein increases in an age dependent manner in brain hippocampus of c57 naive mice. **(B-C)** NCX3 protein expression in hippocampus of mice subjected to hypoxic ischemic injury was reduced and did not recover the physiological expression level at all time point considered.

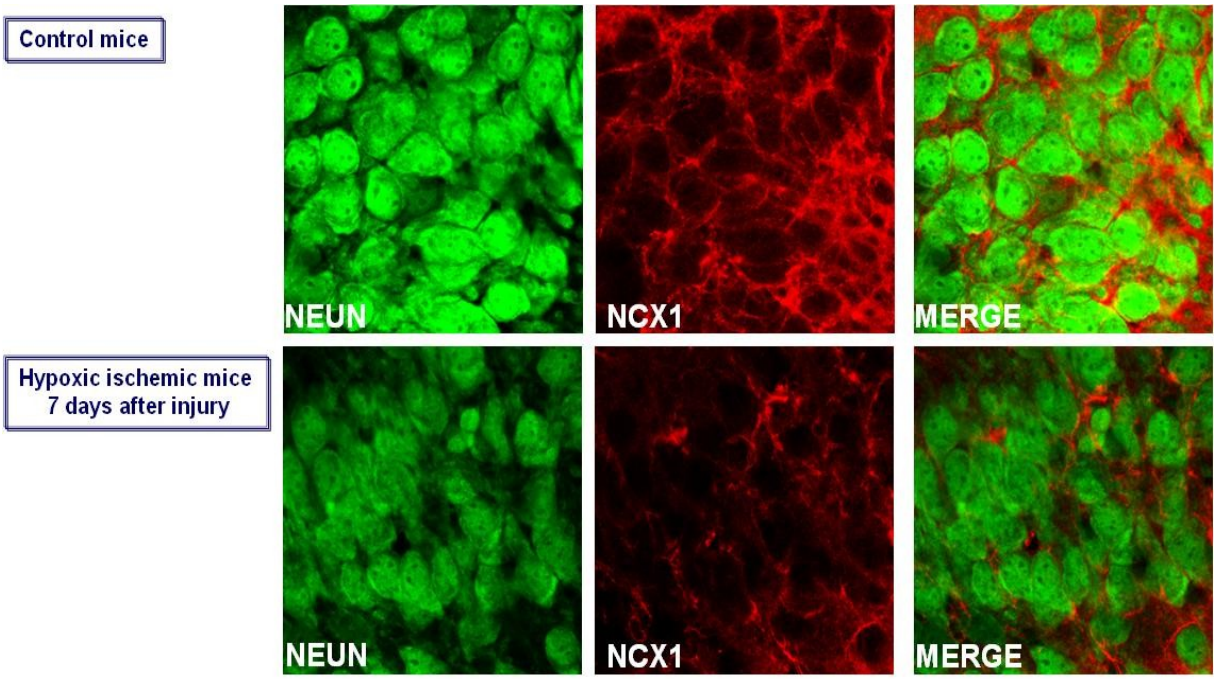


Fig. 18. Immunofluorescence staining 7 days after injury. NCX1 expression in hippocampal pyramidal neurons is reduced in hypoxic ischemic mice. Neun (green) NCX1 (red)

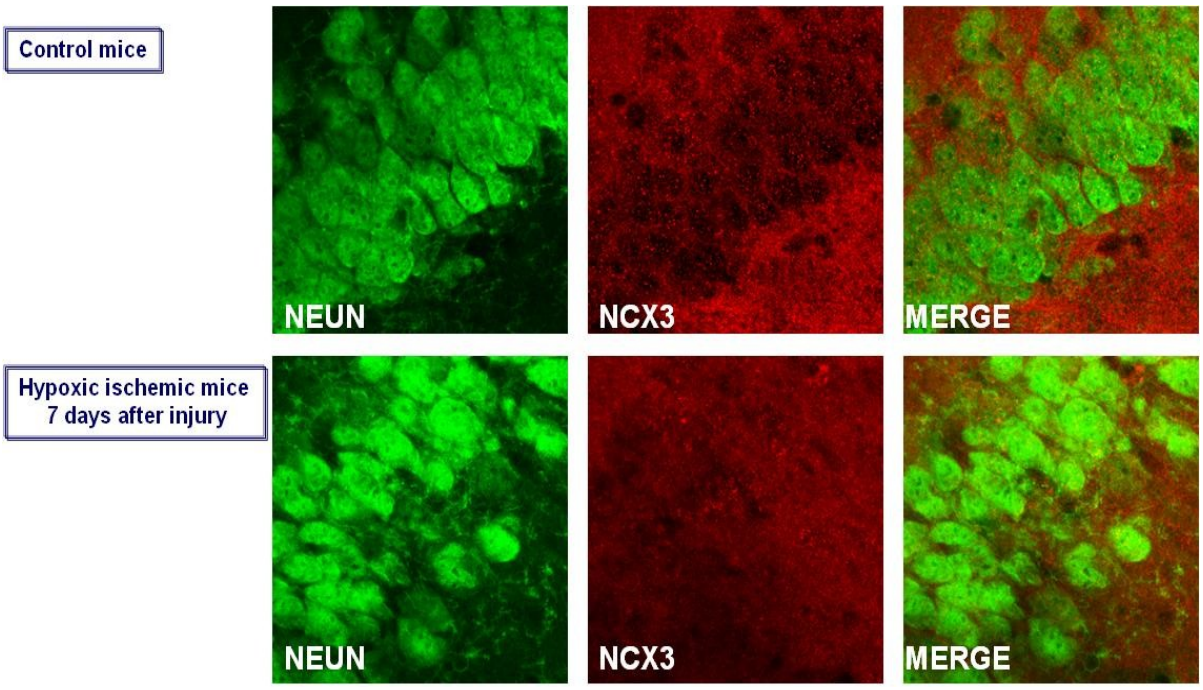


Fig. 19. Immunofluorescence staining 7 days after injury. NCX3 expression in hippocampal pyramidal neurons is reduced in hypoxic ischemic mice. Neun (green) NCX3 (red)

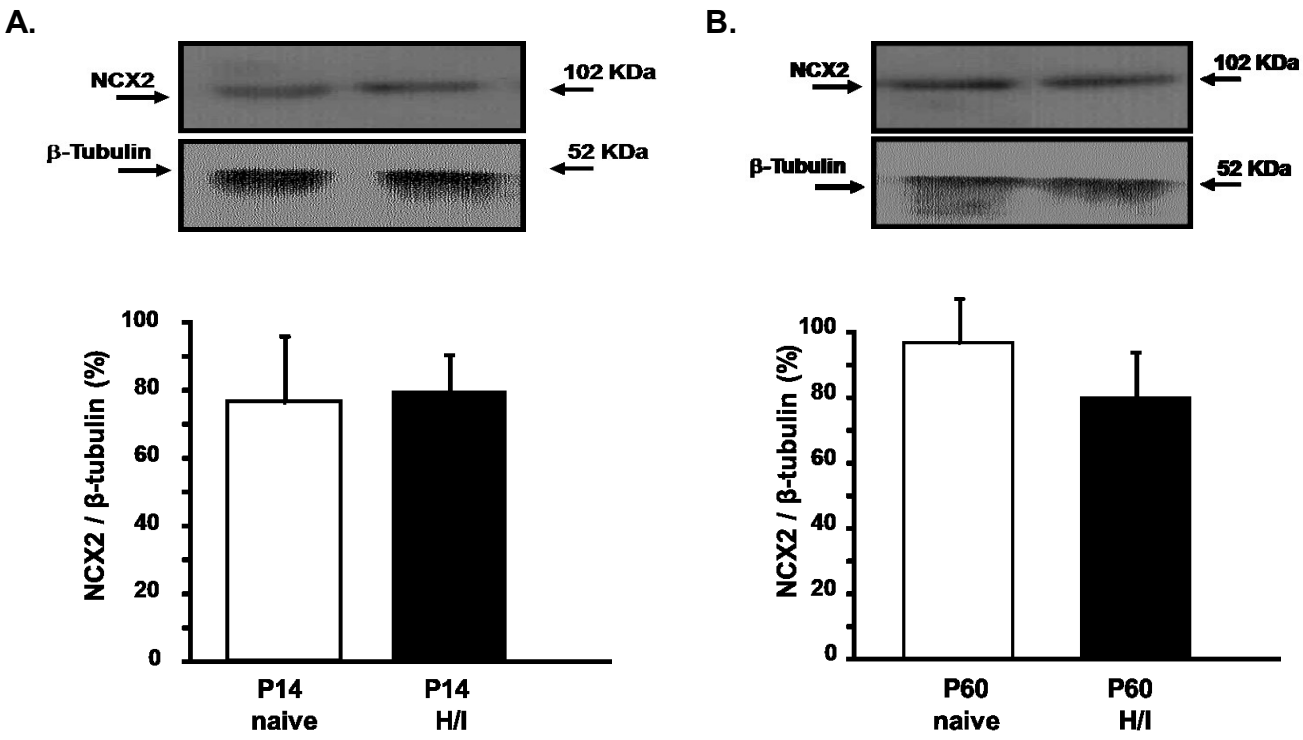


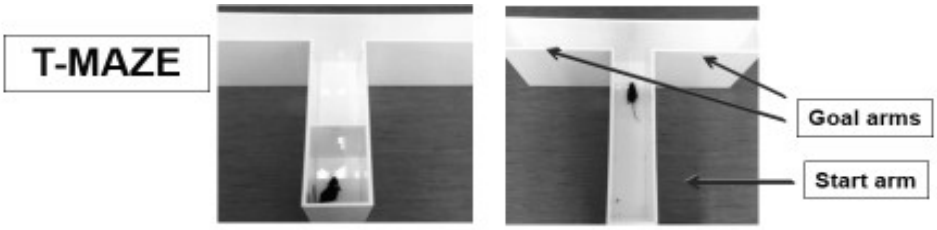
Fig. 20. NCX2 protein expression in hippocampus of naive injured mice, **(A)** 7 days after insult (p14) and **(B)** 53 days after insult (p60).

5.4 NEUROUNINA ADMINISTRATION REVERTS MEMORY DEFICIT IN HI MICE 60 DAYS AFTER INJURY

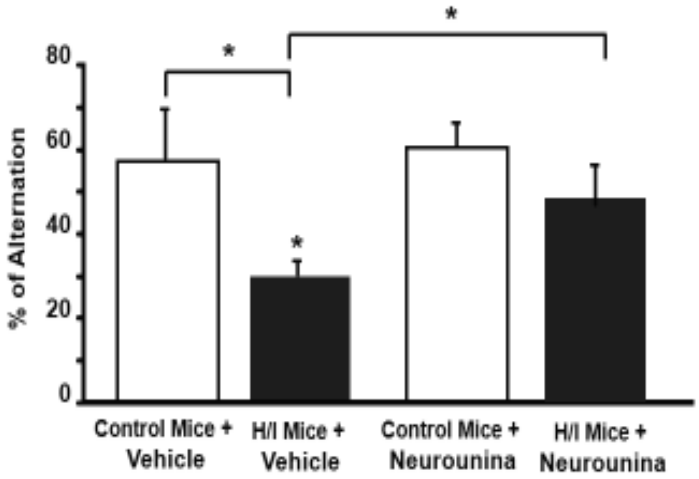
5.4.1 NEUROUNINA PREVENTS ASYMMETRIC BEHAVIOR INDUCED IN MICE BY HI

Spontaneous alternation is expressed as a percentage and refers to ratio of arm choices differing from the previous choice to the total number of arm entries. Alternation is used like proof of short term memory deficit. HI mice alternated between the arms of the maze less than control mice significantly ($P< 0.05$), supporting the hypothesis of a deficit of working memory (Fig. 21B). HI mice treated 3-5h before the test with neurounina showed improved outcomes rather than HI mice vehicle treated ($P> 0.05$) and in the same time they did not show any significant difference with control groups. This result could be altered by an asymmetric development of brain so we looked at the different arms choices of the mice and we discovered that HI mice, injured in right emisphere, preferentially choose the right arm, this particular result it could be referred to brain asimmetry but it is not evident in the mice treated with Neurunina (Fig 21C).

A.



B.



C.

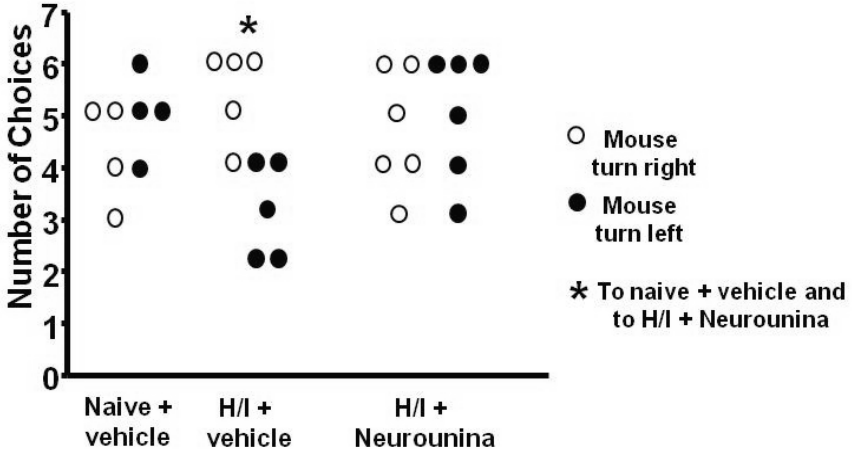


Fig. 21. (A) Apparatus overview. **(B)** HI mice alternated between the arms of the maze less than control mice significantly (P< 0.05), supporting the hypothesis of a deficit of working memory (Fig.). HI mice Neurounina treated show improved outcomes rather than HI mice vehicle treated (P> 0.05) and in the same time they did not show any significant difference with control groups. **(C)** HI mice vehicle treated preferentially choose the right arm (P<0.05) rather than left one, this particular result it could be referred to brain asimmetry

5.4.2 HI MICE TREATED WITH NEUROUNINA IMPROVED PERFORMANCE IN ONE-TRIAL OBJECT RECOGNITION TASK

H/I mice 8 weeks old did not differ significantly from naive animals in overall health and appearance, body weight, and core body temperature. Moreover, no apparent alterations in the spontaneous behavior of HI mice were evident in the home cage. To examine spontaneous locomotor activity and response to a novel environment, we randomized the mice in four groups: naïve mice treated with vehicle, naïve mice treated with neurounina, H/I mice treated with vehicle, H/I mice treated with neurounina. Mice were assayed in an open field cage with the objects. The amount of time spent with the novel object compared with the total time spent exploring both objects represents an index of short and long-term memory. During training, animals showed no preference for one object over another and there was no difference between four animals groups in the exploration time, suggesting that the experimental groups were on average equally motivated to explore objects (Fig. 22 B). However, when presented with a novel object, naïve mice treated with vehicle, naïve mice treated with neurounina, but also H/I mice treated with neurounina showed a preference for the new one after 10 minutes or 24 h of retention. In contrast, H/I mice treated with vehicle group showed a significant lower preference for novel objects when compared with other animal groups (Fig. 22 C-D).

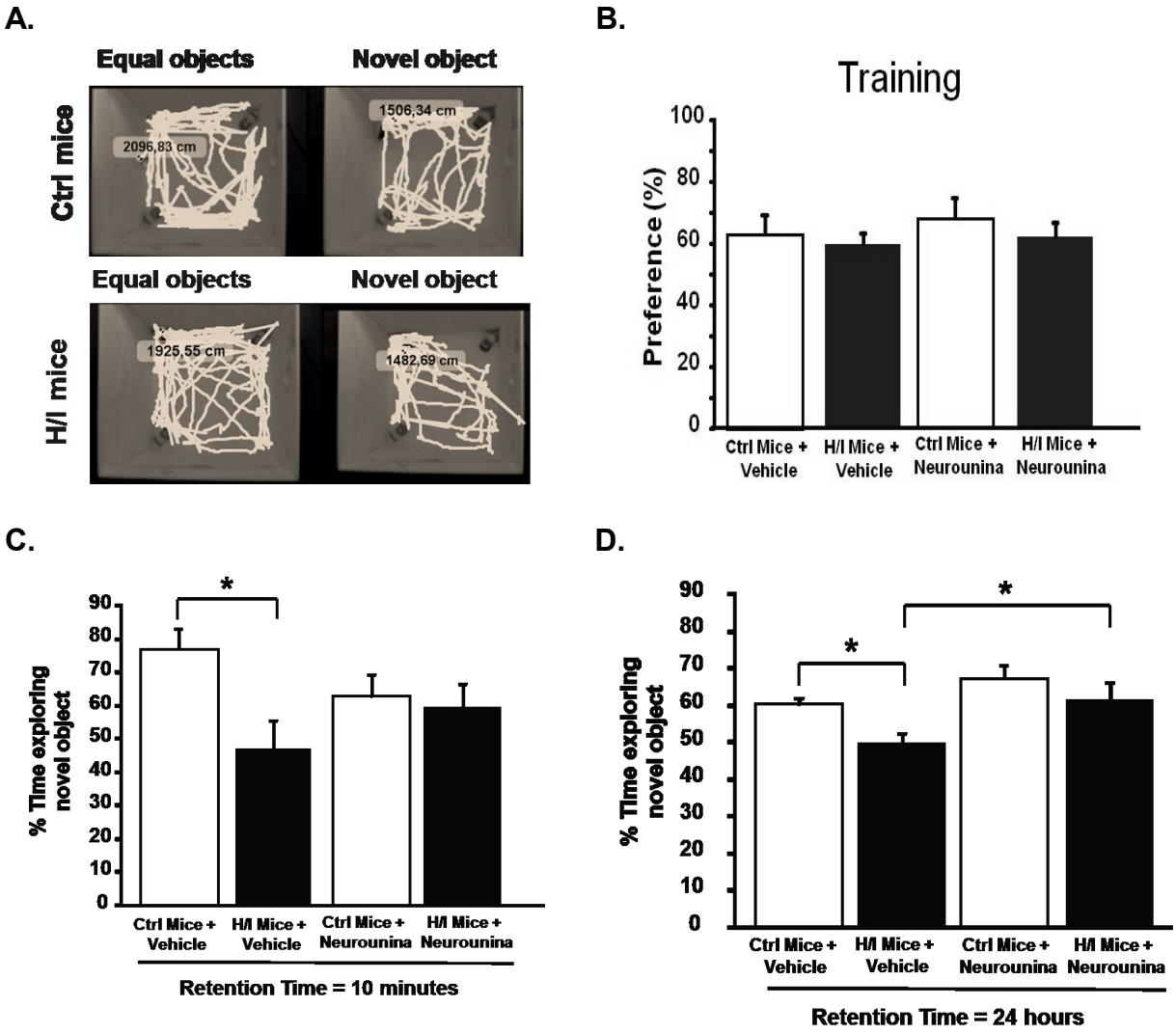
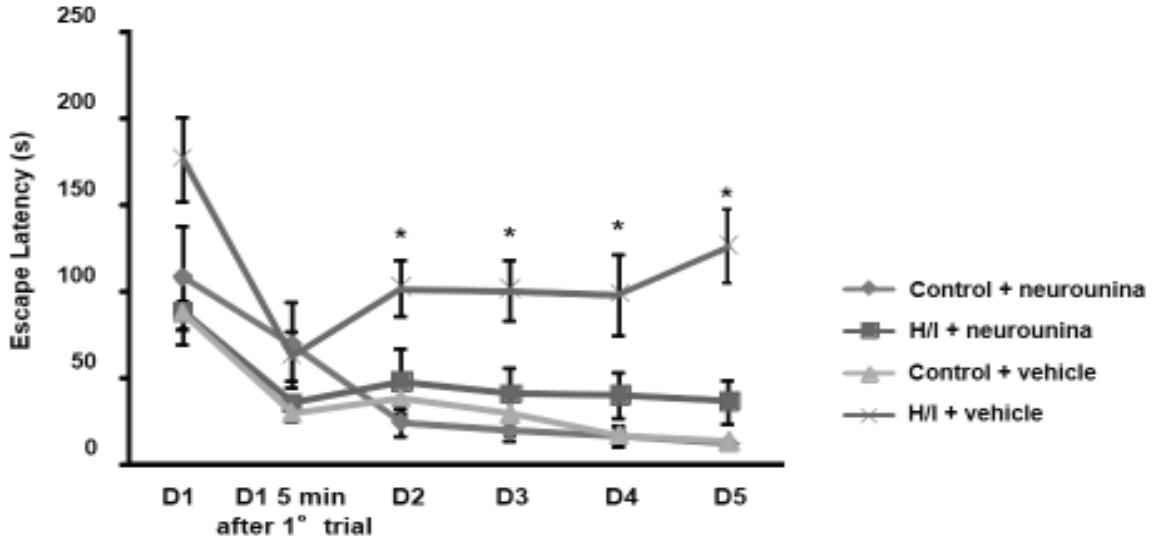


Fig. 22. Percentage of preference in the training phase between two equal objects **(B)**. Percentage of preference in the trial phase of the novel object recognition test 10 minutes **(C)** and 24 h after the training phase **(D).**

5.4.3 HI MICE TREATED WITH NEUROUNINA SHOWED BETTER PERFORMANCE INTO BARNES CIRCULAR MAZE TASK

The learning and memory performance of four groups of animals was also evaluated by means of the Barnes circular maze test, an hippocampus-dependent cognitive task that requires spatial reference memory (Bach et al, 1995; Barnes, 1979; Barnes, 1988). naïve mice treated with vehicle, naïve mice treated with neurounina, but also H/I mice treated with neurounina after the second day showed a significant reduction in the escape latency which progressively decreased until the fifth day (Fig. 23 A). In contrast, H/I mice treated with vehicle did not show any significant changes in escape latencies after the third day and until the fifth day. These escape latencies values were significantly higher from 2^{nd} to 5^{th} days H/I mice treated with vehicle group than those observed in the other three groups (Fig. 23 A). These results showed that H/I mice treated with vehicle did not learn to locate the escape hole during the period of observation (days 1–5), thus suggesting an impairment of the spatial learning and memory performance that is not evident in H/I mice treated with neurounina.

A.



B.

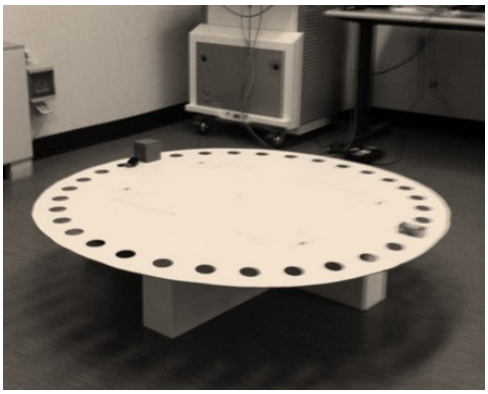
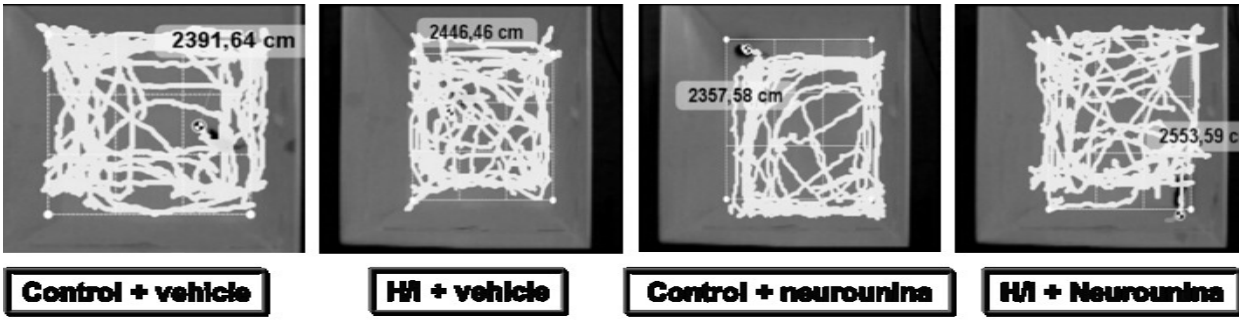


Fig. 23. (A) Animals of all four groups were test daily. Day 1 the trial was repeated 5 minutes after training as proof of working. **(B)** Barnes apparatus.

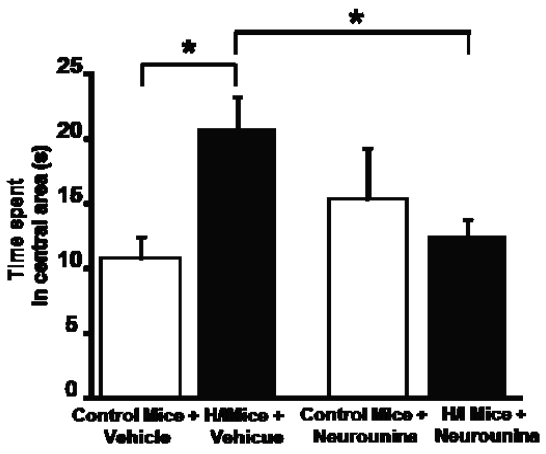
5.4.4 NEUROUNINA REVERTS THE DISINHIBITED BEHAVIOR OF HI MICE IN OPEN FIELD MAZE

Mice examined in an open field test, in order to study whether the reduction of NCX affected anxiety related behavior. So the time and the distance spent by animals in the center of the arena was compared with that spent in the region close to the walls. As this time is related to the size of the central region considered, we evaluated the exploration time in central region having a perimeter equivalent to 1/3 of the original arena (Fig. 24 A). The time spent in the central area by HI mice treated with vehicle differs significantly from the time spent by the other three animal groups considered, showing a disinhibited behavior profile (Fig. 24 B-C). There were no significant difference in the experimental groups for their locomotor and exploratory behavior (Fig. 24 D). Moreover, the defecation score was not significantly different between the four groups (data not shown).

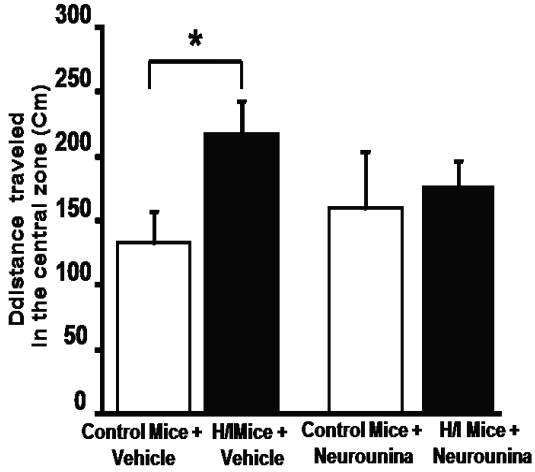
A.



B.



C.



D.

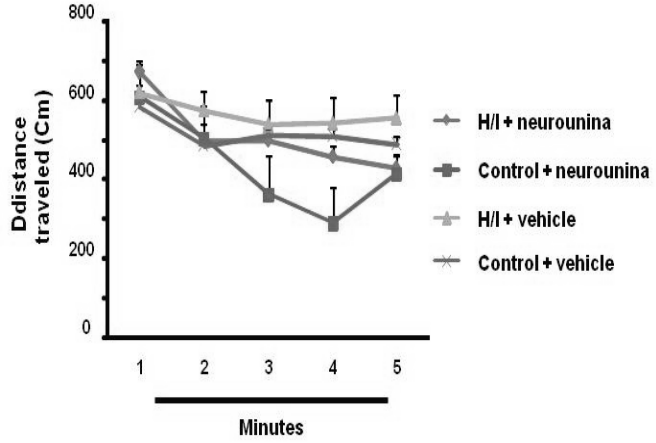


Fig. 24. Locomotor activity and anxiety levels. Time spent and distance traveled was measured using an automated tracking device. **(A)** images of the used apparatus **(B)** Quantification of the time spent by animals in the central area as anxious behavior test. The time spent in the central area by H/I mice treated with vehicle differs significantly from the time spent by the other three animal groups considered, showing a disinhibited behavior profile. **(C)** distance travalled in the central area H/I mice treated with vehicle traveled more in the central area than other three animal groups. **(D)** Locomotor activity of the experimental groups in the open field test.

5.5.5 NEUROUNINA REVERTS THE DISINHIBITED BEHAVIOR OF HI MICE IN ELEVATED ZERO MAZE

In order to verify whether the reduction of NCX affected anxiety related behavior and if the results obtained in the open field were confirmed the animals were assayed in zero maze test. The time spent by animals in the open arms was recorded and compared in the experimental groups. H/I mice treated with vehicle spent 50% of time more than control mice treated with vehicle in the open arms, confirming a disinhibited behavior of H/I mice. H/I mice treated with Neurounina showed no significant difference when compared with control mice treated with Neurounina (data not shown).

6. DISCUSSION

The results of the present study clearly demonstrated for the first time the neuroprotective role of Na^+ /Ca^{2+} exchanger NCX in neuronal disfunction following neonatal hypoxic ischemic injury.

The neuroprotective role of NCX has been demonstrated in our and other laboratories and several study showed that NCX activation is desirable in several pathological disorders such as Alzheimer (Pannaccione et al, 2012; Sokolow et al, 2011), multiple sclerosis (Kurnellas et al, 2007) and stroke (Molinaro et al, 2008; Pignataro et al, 2004). However, although several studies showed the occurrence of cellular ionic imbalance during neonatal HI (Friedman & Haddad, 1993; Nabetani et al, 1997; Vannucci et al, 2001), nobody investigated the role of NCX in this pathology. Therefore, starting from our studies in adult animals, we hypothesized that the two NCX brain isoforms, NCX1 and NCX3, could be involved in the pathogenesis of ischemic lesion also after neonatal HI. Indeed, we have previously demonstrated that in homozygous ncx3−/− mice subjected to MCAO, an increased brain damage occurs (Molinaro et al, 2008). In addition, the silencing of NCX1 and NCX3 expression by RNA interference increases cerebellar granule neurons vulnerability to Ca2+ overload and excitotoxicity (Bano et al, 2005; Secondo et al, 2007). Moreover, the vulnerability to chemical hypoxia of baby hamster kidney (BHK) cells overexpressing NCX1 or NCX3 considerably increases when either NCX1 or NCX3 is silenced (Secondo et al, 2007). Further, ischemic rats treated with NCX1 or NCX3 antisense display a remarkable enlargement of the infarct volume (Pignataro et al, 2004). In addition, it has been recently shown that among the three NCX brain isoforms, NCX1 and NCX3 represent new molecular effectors involved in the

neuroprotective mechanisms elicited by “ischemic preconditioning” and "ischemic postconditioning" (Pignataro et al, 2012; Pignataro et al, 2013; Pignataro et al, 2011). More recently, we demonstrated that a newly synthesized compound, neurounoina, by activating NCX in the brain, exerts a remarkable neuroprotective effect in mice subjected to tMCAO (Molinaro et al, 2013). Indeed, it was demonstrated that neurounina displays a potent and reversible stimulatory effect on NCX1 and NCX2 in both forward and reverse modes of operation, with an estimated EC50 in the low nanomolar range. Neurounina exerted a remarkable neuroprotective effect in both in vitro and in vivo experimental models of cerebral ischemia (Molinaro et al, 2013), possibly by enhancing NCX activity. In particular, cortical neurons exposed to neurounina displayed a higher resistance to neuronal damage induced by 3 hours of OGD or 3 hours of OGD followed by 21 hours of reoxygenation as compared with vehicle-treated neurons. It is noteworthy that the results obtained in vivo showed that the intraperitoneal administration of neurounina in single doses ranging from 0.003 to 30 mg/kg significantly reduced infarct volume.

Our results clearly show that a remarkable neuroprotective effect could be achieved also in a model of neonatal hypoxia ischemia. Indeed, neurounina, intraperitoneally administered 2 hours after ischemia induction, determines smaller lesions in the ipsilateral hippocampus of mice subjected to HI compared to vehicle treated animals, this was reflected by better preserved hippocampal morphology and a reduced tissue loss. Since neurounina does not affect NCX3, the neuroprotection seen in our model has to be ascribed only to NCX1. A possible explanation of this effect resides in the fact that neonatal hypoxia–ischemia, although in some aspects different from adult ischemia, sets in motion a cascade of metabolic events which culminate in brain damage. The role of calcium (Ca^{2+}) as a trigger for irreversible cell injury in hypoxia–ischemia has been the subject of wide investigation (Farber, 1981; Hossmann et al,

1983; Siesjo, 1981). During and following hypoxia–ischemia, altered ion homeostasis and membrane depolarization lead to a voltage-dependent influx of Ca^{2+} ions into cells. Additional influx occurs as a consequence of activation of glutamate receptor, Ca^{2+} ion channels and, more importantly, of NCX in the reverse mode. An increase in cytosolic Ca^{2+}, in turn, causes phospholipase activation with the accumulation of free fatty acids, futile cycling of ions across mitochondria, and subsequent uncoupling of oxidative phosphorylation, among other metabolic perturbations investigation (Raichle, 1983).

The onset and magnitude of the calcium increase depend on multiple inherent and environmental factors, including the duration (short vs. long) and severity (mild vs. severe) of insult, the age of animals (young vs. old), the region of the brain (hippocampus, cortex vs. other regions such as thalamus, striatum, dorsal vagal neurons, locus coeruleus, cerebellar granule cells), and even the type of cells (neurons vs. glial cells). For example, there is a minor or moderate increase in cytosolic Ca2+ (decades to hundreds nanomolar) with short or transient hypoxia/ischemia, but a large sustained increase in [Ca2+]i (up to 35μM) in severe and prolonged hypoxia/ischemia (Chen et al, 1999; Diarra et al, 1999; Martinez-Sanchez et al, 2004; Nakamura et al, 1999; Silver et al, 1997; Silver & Erecinska, 1990; Tymianski et al, 1993; Uematsu et al, 1988; Zhang & Lipton, 1999).

In addition, it is well known that young animals show a much less extension and longer latency (up to five times longer) of Ca2+ rise in neurons than those of the mature ones during hypoxia/ischemia (Friedman & Haddad, 1993; Nabetani et al, 1997; Vannucci et al, 2001). This could further justify long term protection effect of neurounina and, more in general, of other potential NCX activator in this pathology. Another interesting aspect that has to be underlined, is the involvment of NCX in the long term effect of neonatal HI, in fact it is well known that also in absence of obvious

neurologic deficits in the newborn period, long-term functional impairments may be present. In the current study, we speculate that NCX activity is reduced at hyppocampal level after HI as a result of protein downregulation occurring in adult mice previously subjected to neonatal HI. In fact, the impairment of intracellular Na+, ([Na+]I) and Ca2+, ([Ca2+]I) homeostasis persists in adulthood, because NCX1 and NCX3 protein expression in hippocampus of mice subjected to neonatal HI was drastically reduced starting seven days after injury. Notably, the expression levels of these two proteins were not restored anymore after an HI insult, thus causing a permanent impairment of ionic homeostasis in the HI hippocampus mice. Therefore, we tested neurounina in adult mice (8 weeks of age) that were injured in neonatal period, in order to demonstrate that impairment of ionic homeostasis could be one of the reason of behavioral disabilities. With this treatment we tried to balance the reduced expression of NCX1 with this NCX activator. The results obtained with neurounina confirmed the protective effect also in this condition. In fact, HI vehicle treated mice showed deficit in working memory; this impairment was reverted by treating these mice with neurounina. Similar results were obtained in tests examining spatial, learning and long term memory. Indeed, all these processes were impaired in HI mice and were reverted by treatment with neurounina.

Few researchers have addressed the issue of behavioral problems in children with a history of neonatal HIE. In some of these behavioral studies, hyperactivity was noticed and was more often present in children with moderate neonatal HIE, but not in children with mild neonatal HIE (Marlow et al, 2005; Moster et al, 2002; Robertson & Finer, 1988). In addition, one study that used parent's observations of their child's behavior found more problems related to tractability, aggression, passivity and anxiety in a mixed group of children with HIE compared to a control group (Moster, Lie et al. 2002). In our experiments we found a situation more similar to that of

children with a moderate neonatal HIE, in fact we found a disinhibited behavior of the HI mice in the open field test and zero maze test, in which the animals were not afraid by the new environment and in which they showed an hyperactive behavior. Interestingly, neurounina was able to revert also this behavior rendering animals more similar to healthy mice.

The significant role of NCX1 and NCX3 in the evolution of the developmental brain injury and in spatial learning and memory processes in adult mice injured in the neonatal period, emerged from the present study. Overall, the findings of the present thesis work represents an important milestone in defining short and long term role of NCX in the pathology of neonatal HIE.

7. AKNOWLEDGEMENTS

I would like to deeply thank all the people I worked with, during the three years of my PhD experience: first of all Main Chief of the Pharmacology Unit in the Neuroscience Department, Prof. Lucio Annunziato, than my tutor Prof. Giuseppe Pignataro and all the colleagues that contributed to this work of thesis, Dr. Antonio Vinciguerra, Dr. Ornella Cuomo, Dr. Serenella Anzilotti. I would like to thank Prof. Klas Blomgren and his lab group for their hospitality at Astrid Lindgren hospital in Karolinska Institute.

8. REFERENCES

Annunziato L, Molinaro P, Secondo A, Pannaccione A, Scorziello A, Pignataro G, Cuomo O, Sirabella R, Boscia F, Spinali A, Di Renzo G (2009) The Na(+)/Ca(2+) Exchanger: A Target for Therapeutic Intervention in Cerebral Ischemia. *Contemp Neurosci***:** 65-87

Annunziato L, Pignataro G, Di Renzo GF (2004) Pharmacology of brain Na+/Ca2+ exchanger: from molecular biology to therapeutic perspectives. *Pharmacol Rev* **56:** 633-654

Avkiran M (2001) Protection of the ischaemic myocardium by Na+/H+ exchange inhibitors: potential mechanisms of action. *Basic Res Cardiol* **96:** 306-311

Azzopardi DV, Strohm B, Edwards AD, Dyet L, Halliday HL, Juszczak E, Kapellou O, Levene M, Marlow N, Porter E, Thoresen M, Whitelaw A, Brocklehurst P (2009) Moderate hypothermia to treat perinatal asphyxial encephalopathy. *N Engl J Med* **361:** 1349-1358

Bach ME, Hawkins RD, Osman M, Kandel ER, Mayford M (1995) Impairment of spatial but not contextual memory in CaMKII mutant mice with a selective loss of hippocampal LTP in the range of the theta frequency. *Cell* **81:** 905-915

Back SA, Craig A, Luo NL, Ren J, Akundi RS, Ribeiro I, Rivkees SA (2006) Protective effects of caffeine on chronic hypoxia-induced perinatal white matter injury. *Ann Neurol* **60:** 696-705

Bano D, Young KW, Guerin CJ, Lefeuvre R, Rothwell NJ, Naldini L, Rizzuto R, Carafoli E, Nicotera P (2005) Cleavage of the plasma membrane Na+/Ca2+ exchanger in excitotoxicity. *Cell* **120:** 275-285

Barks JD, Liu YQ, Shangguan Y, Li J, Pfau J, Silverstein FS (2008) Impact of indolent inflammation on neonatal hypoxic-ischemic brain injury in mice. *Int J Dev Neurosci* **26:** 57-65

Barnes CA (1979) Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat. *J Comp Physiol Psychol* **93:** 74-104

Barnes CA (1988) Spatial learning and memory processes: the search for their neurobiological mechanisms in the rat. *Trends Neurosci* **11:** 163-169

Benders MJ, Bos AF, Rademaker CM, Rijken M, Torrance HL, Groenendaal F, van Bel F (2006) Early postnatal allopurinol does not improve short term outcome after severe birth asphyxia. *Arch Dis Child Fetal Neonatal Ed* **91:** F163-165

Blakemore WF, Franklin RJ (2008) Remyelination in experimental models of toxin-induced demyelination. *Curr Top Microbiol Immunol* **318:** 193-212

Blaustein MP, Lederer WJ (1999) Sodium/calcium exchange: its physiological implications. *Physiol Rev* **79:** 763-854

Boscia F, Gala R, Pannaccione A, Secondo A, Scorziello A, Di Renzo G, Annunziato L (2009) NCX1 expression and functional activity increase in microglia invading the infarct core. *Stroke* **40:** 3608-3617

Boscia F, Gala R, Pignataro G, de Bartolomeis A, Cicale M, Ambesi-Impiombato A, Di Renzo G, Annunziato L (2006) Permanent focal brain ischemia induces isoform-dependent changes in the pattern of Na+/Ca2+ exchanger gene expression in the ischemic core, periinfarct area, and intact brain regions. *J Cereb Blood Flow Metab* **26:** 502-517

Bruce AJ, Boling W, Kindy MS, Peschon J, Kraemer PJ, Carpenter MK, Holtsberg FW, Mattson MP (1996) Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors. *Nat Med* **2:** 788-794

Buser JR, Segovia KN, Dean JM, Nelson K, Beardsley D, Gong X, Luo NL, Ren J, Wan Y, Riddle A, McClure MM, Ji X, Derrick M, Hohimer AR, Back SA, Tan S (2010) Timing of appearance of late

oligodendrocyte progenitors coincides with enhanced susceptibility of preterm rabbit cerebral white matter to hypoxia-ischemia. *J Cereb Blood Flow Metab* **30:** 1053-1065

Cai Z, Hutchins JB, Rhodes PG (1998) Intrauterine hypoxia-ischemia alters nitric oxide synthase expression and activity in fetal and neonatal rat brains. *Brain Res Dev Brain Res* **109:** 265-269

Cai Z, Sigrest T, Hersey K, Rhodes PG (1995) Intrauterine hypoxia-ischemia increases N-methyl-D-aspartate-induced cGMP formation and glutamate accumulation in cultured rat cerebellar granule cells. *Pediatr Res* **38:** 107-112

Calabresi P, Cupini LM, Centonze D, Pisani F, Bernardi G (2003) Antiepileptic drugs as a possible neuroprotective strategy in brain ischemia. *Ann Neurol* **53:** 693-702

Cariou A, Claessens YE, Pene F, Marx JS, Spaulding C, Hababou C, Casadevall N, Mira JP, Carli P, Hermine O (2008) Early high-dose erythropoietin therapy and hypothermia after out-of-hospital cardiac arrest: a matched control study. *Resuscitation* **76:** 397-404

Carloni S, Carnevali A, Cimino M, Balduini W (2007) Extended role of necrotic cell death after hypoxia-ischemia-induced neurodegeneration in the neonatal rat. *Neurobiol Dis* **27:** 354-361

Chahboune H, Ment LR, Stewart WB, Rothman DL, Vaccarino FM, Hyder F, Schwartz ML (2009) Hypoxic injury during neonatal development in murine brain: correlation between in vivo DTI findings and behavioral assessment. *Cereb Cortex* **19:** 2891-2901

Chaudhari T, McGuire W (2008) Allopurinol for preventing mortality and morbidity in newborn infants with suspected hypoxic-ischaemic encephalopathy. *Cochrane Database Syst Rev***:** CD006817

Chen WH, Chu KC, Wu SJ, Wu JC, Shui HA, Wu ML (1999) Early metabolic inhibition-induced intracellular sodium and calcium increase in rat cerebellar granule cells. *J Physiol-London* **515:** 133-146

Choi DW (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1:** 623-634

Dammann O, Kuban KC, Leviton A (2002) Perinatal infection, fetal inflammatory response, white matter damage, and cognitive limitations in children born preterm. *Ment Retard Dev Disabil Res Rev* **8:** 46-50

Dere E, Huston JP, De Souza Silva MA (2007) The pharmacology, neuroanatomy and neurogenetics of one-trial object recognition in rodents. *Neurosci Biobehav Rev* **31:** 673-704

Derrick M, Drobyshevsky A, Ji X, Tan S (2007) A model of cerebral palsy from fetal hypoxia-ischemia. *Stroke* **38:** 731-735

Diarra A, Sheldon C, Brett CL, Baimbridge KG, Church J (1999) Anoxia-evoked intracellular pH and Ca2+ concentration changes in cultured postnatal rat hippocampal neurons. *Neuroscience* **93:** 1003-1016

Dirnagl U, Simon RP, Hallenbeck JM (2003) Ischemic tolerance and endogenous neuroprotection. *Trends Neurosci* **26:** 248-254

Doverhag C, Keller M, Karlsson A, Hedtjarn M, Nilsson U, Kapeller E, Sarkozy G, Klimaschewski L, Humpel C, Hagberg H, Simbruner G, Gressens P, Savman K (2008) Pharmacological and genetic inhibition of NADPH oxidase does not reduce brain damage in different models of perinatal brain injury in newborn mice. *Neurobiol Dis* **31:** 133-144

Drobyshevsky A, Song SK, Gamkrelidze G, Wyrwicz AM, Derrick M, Meng F, Li L, Ji X, Trommer B, Beardsley DJ, Luo NL, Back SA, Tan S (2005) Developmental changes in diffusion anisotropy coincide with immature oligodendrocyte progression and maturation of compound action potential. *J Neurosci* **25:** 5988-5997

Edinger AL, Thompson CB (2004) Death by design: apoptosis, necrosis and autophagy. *Curr Opin Cell Biol* **16:** 663-669

Ehrenreich H, Weissenborn K, Prange H, Schneider D, Weimar C, Wartenberg K, Schellinger PD, Bohn M, Becker H, Wegrzyn M, Jahnig P, Herrmann M, Knauth M, Bahr M, Heide W, Wagner A, Schwab S, Reichmann H, Schwendemann G, Dengler R, Kastrup A, Bartels C (2009) Recombinant human erythropoietin in the treatment of acute ischemic stroke. *Stroke* **40:** e647-656

Eicher DJ, Wagner CL, Katikaneni LP, Hulsey TC, Bass WT, Kaufman DA, Horgan MJ, Languani S, Bhatia JJ, Givelichian LM, Sankaran K, Yager JY (2005a) Moderate hypothermia in neonatal encephalopathy: efficacy outcomes. *Pediatr Neurol* **32:** 11-17

Eicher DJ, Wagner CL, Katikaneni LP, Hulsey TC, Bass WT, Kaufman DA, Horgan MJ, Languani S, Bhatia JJ, Givelichian LM, Sankaran K, Yager JY (2005b) Moderate hypothermia in neonatal encephalopathy: safety outcomes. *Pediatr Neurol* **32:** 18-24

Ennaceur A, Delacour J (1988) A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behav Brain Res* **31:** 47-59

Farber JL (1981) The role of calcium in cell death. *Life Sci* **29:** 1289-1295

Felderhoff-Mueser U, Buhrer C, Groneck P, Obladen M, Bartmann P, Heep A (2003) Soluble Fas (CD95/Apo-1), soluble Fas ligand, and activated caspase 3 in the cerebrospinal fluid of infants with posthemorrhagic and nonhemorrhagic hydrocephalus. *Pediatr Res* **54:** 659-664

Ferriero DM (2004) Neonatal brain injury. *N Engl J Med* **351:** 1985-1995

Filippi L, la Marca G, Fiorini P, Poggi C, Cavallaro G, Malvagia S, Pellegrini-Giampietro DE, Guerrini R (2009) Topiramate concentrations in neonates treated with prolonged whole body hypothermia for hypoxic ischemic encephalopathy. *Epilepsia* **50:** 2355-2361

Follett PL, Deng W, Dai W, Talos DM, Massillon LJ, Rosenberg PA, Volpe JJ, Jensen FE (2004) Glutamate receptor-mediated oligodendrocyte toxicity in periventricular leukomalacia: a protective role for topiramate. *J Neurosci* **24:** 4412-4420

Friedman JE, Haddad GG (1993) Major differences in Ca2+i response to anoxia between neonatal and adult rat CA1 neurons: role of Ca2+o and Na+o. *J Neurosci* **13:** 63-72

Fujioka Y, Hiroe K, Matsuoka S (2000) Regulation kinetics of Na+-Ca2+ exchange current in guinea-pig ventricular myocytes. *J Physiol-London* **529:** 611-624

Furwentsches A, Bussmann C, Ramantani G, Ebinger F, Philippi H, Poschl J, Schubert S, Rating D, Bast T (2010) Levetiracetam in the treatment of neonatal seizures: a pilot study. *Seizure* **19:** 185-189

Gidday JM (2006) Cerebral preconditioning and ischaemic tolerance. *Nat Rev Neurosci* **7:** 437-448

Glass HC, Glidden D, Jeremy RJ, Barkovich AJ, Ferriero DM, Miller SP (2009) Clinical Neonatal Seizures are Independently Associated with Outcome in Infants at Risk for Hypoxic-Ischemic Brain Injury. *J Pediatr* **155:** 318-323

Gluckman PD, Wyatt JS, Azzopardi D, Ballard R, Edwards AD, Ferriero DM, Polin RA, Robertson CM, Thoresen M, Whitelaw A, Gunn AJ (2005) Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: multicentre randomised trial. *Lancet* **365:** 663-670

Goldberg RN, Moscoso P, Bauer CR, Bloom FL, Curless RG, Burke B, Bancalari E (1986) Use of barbiturate therapy in severe perinatal asphyxia: a randomized controlled trial. *J Pediatr* **109:** 851-856

Gonzalez FF, Abel R, Almli CR, Mu D, Wendland M, Ferriero DM (2009) Erythropoietin sustains cognitive function and brain volume after neonatal stroke. *Dev Neurosci* **31:** 403-411

Gonzalez FF, McQuillen P, Mu D, Chang Y, Wendland M, Vexler Z, Ferriero DM (2007) Erythropoietin enhances long-term neuroprotection and neurogenesis in neonatal stroke. *Dev Neurosci* **29:** 321-330

Grow J, Barks JD (2002) Pathogenesis of hypoxic-ischemic cerebral injury in the term infant: current concepts. *Clin Perinatol* **29:** 585-602, v

Gunes T, Ozturk MA, Koklu E, Kose K, Gunes I (2007) Effect of allopurinol supplementation on nitric oxide levels in asphyxiated newborns. *Pediatr Neurol* **36:** 17-24

Hagberg H, Gilland E, Bona E, Hanson LA, Hahin-Zoric M, Blennow M, Holst M, McRae A, Soder O (1996) Enhanced expression of interleukin (IL)-1 and IL-6 messenger RNA and bioactive protein after hypoxia-ischemia in neonatal rats. *Pediatr Res* **40:** 603-609

Hall RT, Hall FK, Daily DK (1998) High-dose phenobarbital therapy in term newborn infants with severe perinatal asphyxia: a randomized, prospective study with three-year follow-up. *J Pediatr* **132:** 345-348

Hanon E, Klitgaard H (2001) Neuroprotective properties of the novel antiepileptic drug levetiracetam in the rat middle cerebral artery occlusion model of focal cerebral ischemia. *Seizure* **10:** 287-293

Hasegawa H, Muraoka M, Matsui K, Kojima A (2003) Discovery of a novel potent Na+/Ca2+ exchanger inhibitor: design, synthesis and structure-activity relationships of 3,4-dihydro-2(1H)-quinazolinone derivatives. *Bioorg Med Chem Lett* **13:** 3471-3475

Hedtjarn M, Mallard C, Hagberg H (2004) Inflammatory gene profiling in the developing mouse brain after hypoxia-ischemia. *J Cereb Blood Flow Metab* **24:** 1333-1351

Herrero AI, Del Olmo N, Gonzalez-Escalada JR, Solis JM (2002) Two new actions of topiramate: inhibition of depolarizing GABA(A)-mediated responses and activation of a potassium conductance. *Neuropharmacology* **42:** 210-220

Hersey K, Hu ZY, Zhang JP, Rhodes PG, Sun GY (1995) In utero hypoxic ischemia decreases the cholinergic agonist-stimulated poly-phosphoinositide turnover in the developing rat brain. *Neurochem Res* **20:** 1477-1482

Hossmann KA, Paschen W, Csiba L (1983) Relationship between calcium accumulation and recovery of cat brain after prolonged cerebral ischemia. *J Cereb Blood Flow Metab* **3:** 346-353

Hu BR, Liu CL, Ouyang Y, Blomgren K, Siesjo BK (2000) Involvement of caspase-3 in cell death after hypoxia-ischemia declines during brain maturation. *J Cereb Blood Flow Metab* **20:** 1294-1300

Iwamoto T, Inoue Y, Ito K, Sakaue T, Kita S, Katsuragi T (2004) The exchanger inhibitory peptide region-dependent inhibition of Na+/Ca2+ exchange by SN-6 [2-[4-(4-nitrobenzyloxy)benzyl]thiazolidine-4-carboxylic acid ethyl ester], a novel benzyloxyphenyl derivative. *Mol Pharmacol* **66:** 45-55

Iwamoto T, Kita S (2006) YM-244769, a novel Na+/Ca2+ exchange inhibitor that preferentially inhibits NCX3, efficiently protects against hypoxia/reoxygenation-induced SH-SY5Y neuronal cell damage. *Mol Pharmacol* **70:** 2075-2083

Iwamoto T, Kita S, Uehara A, Inoue Y, Taniguchi Y, Imanaga I, Shigekawa M (2001) Structural domains influencing sensitivity to isothiourea derivative inhibitor KB-R7943 in cardiac Nna(+)/Ca(2+) exchanger. *Mol Pharmacol* **59:** 524-531

Jacobs SE, Morley CJ, Inder TE, Stewart MJ, Smith KR, McNamara PJ, Wright IM, Kirpalani HM, Darlow BA, Doyle LW (2011) Whole-body hypothermia for term and near-term newborns with hypoxic-ischemic encephalopathy: a randomized controlled trial. *Arch Pediatr Adolesc Med* **165:** 692-700

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

Juul SE, McPherson RJ, Farrell FX, Jolliffe L, Ness DJ, Gleason CA (2004) Erytropoietin concentrations in cerebrospinal fluid of nonhuman primates and fetal sheep following high-dose recombinant erythropoietin. *Biol Neonate* **85:** 138-144

Kaandorp JJ, Benders MJ, Rademaker CM, Torrance HL, Oudijk MA, de Haan TR, Bloemenkamp KW, Rijken M, van Pampus MG, Bos AF, Porath MM, Oetomo SB, Willekes C, Gavilanes AW, Wouters MG, van Elburg RM, Huisjes AJ, Bakker SC, van Meir CA, von Lindern J, Boon J, de Boer IP, Rijnders RJ, Jacobs CJ, Uiterwaal CS, Mol BW, Visser GH, van Bel F, Derks JB (2010) Antenatal allopurinol for reduction of birth asphyxia induced brain damage (ALLO-Trial); a randomized double blind placebo controlled multicenter study. *BMC Pregnancy Childbirth* **10:** 8

Kang TM, Hilgemann DW (2004) Multiple transport modes of the cardiac Na+/Ca2+ exchanger. *Nature* **427:** 544-548

Kassed CA, Herkenham M (2004) NF-kappaB p50-deficient mice show reduced anxiety-like behaviors in tests of exploratory drive and anxiety. *Behav Brain Res* **154:** 577-584

Kazlauckas V, Schuh J, Dall'Igna OP, Pereira GS, Bonan CD, Lara DR (2005) Behavioral and cognitive profile of mice with high and low exploratory phenotypes. *Behav Brain Res* **162:** 272-278

Kellert BA, McPherson RJ, Juul SE (2007) A comparison of high-dose recombinant erythropoietin treatment regimens in brain-injured neonatal rats. *Pediatr Res* **61:** 451-455

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. *Pediatric Research* **59:** 227-231

Kim J, Kondratyev A, Gale K (2007a) Antiepileptic drug-induced neuronal cell death in the immature brain: Effects of carbamazepine, topiramate, and levetiracetam as monotherapy versus polytherapy. *Journal of Pharmacology and Experimental Therapeutics* **323:** 165-173

Kim JM, Kwon S, Seo HE, Choe BH, Cho MH, Park SP (2009) Long-term effectiveness and tolerability of topiramate in children with epilepsy under the age of 2 years: 4-year follow-up. *J Korean Med Sci* **24:** 1078-1082

Kim JS, Kondratyev A, Tomita Y, Gale K (2007b) Neurodevelopmental impact of antiepileptic drugs and seizures in the immature brain. *Epilepsia* **48 Suppl 5:** 19-26

Kintner DB, Wang Y, Sun D (2007) Role of membrane ion transport proteins in cerebral ischemic damage. *Front Biosci* **12:** 762-770

Kirino T (2002) Ischemic tolerance. *J Cereb Blood Flow Metab* **22:** 1283-1296

Kurnellas MP, Donahue KC, Elkabes S (2007) Mechanisms of neuronal damage in multiple sclerosis and its animal models: role of calcium pumps and exchangers. *Biochem Soc Trans* **35:** 923-926

Lafemina MJ, Sheldon RA, Ferriero DM (2006) Acute hypoxia-ischemia results in hydrogen peroxide accumulation in neonatal but not adult mouse brain. *Pediatr Res* **59:** 680-683

Laptook AR (2009a) Use of Therapeutic Hypothermia for Term Infants with Hypoxic-Ischemic Encephalopathy. *Pediatr Clin N Am* **56:** 601-+

Laptook AR (2009b) Use of therapeutic hypothermia for term infants with hypoxic-ischemic encephalopathy. *Pediatr Clin North Am* **56:** 601-616, Table of Contents

Lee C, Dhalla NS, Hryshko LV (2005) Therapeutic potential of novel Na+-Ca2+ exchange inhibitors in attenuating ischemia-reperfusion injury. *Can J Cardiol* **21:** 509-516

Li Z, Matsuoka S, Hryshko LV, Nicoll DA, Bersohn MM, Burke EP, Lifton RP, Philipson KD (1994) Cloning of the NCX2 isoform of the plasma membrane Na(+)-Ca2+ exchanger. *J Biol Chem* **269:** 17434-17439

Lipton SA (1999) Redox sensitivity of NMDA receptors. *Methods Mol Biol* **128:** 121-130

Lister RG (1990) Ethologically-based animal models of anxiety disorders. *Pharmacol Ther* **46:** 321-340

Little LJ (1861) *A mother's peace offering to American houses*, New-York,: J. A. Gray, printers.

Liu XH, Kwon D, Schielke GP, Yang GY, Silverstein FS, Barks JD (1999) Mice deficient in interleukin-1 converting enzyme are resistant to neonatal hypoxic-ischemic brain damage. *J Cereb Blood Flow Metab* **19:** 1099-1108

Luo J, Sun D (2007) Physiology and pathophysiology of Na(+)/H(+) exchange isoform 1 in the central nervous system. *Curr Neurovasc Res* **4:** 205-215

Ma D, Lim T, Xu J, Tang H, Wan Y, Zhao H, Hossain M, Maxwell PH, Maze M (2009) Xenon preconditioning protects against renal ischemic-reperfusion injury via HIF-1alpha activation. *J Am Soc Nephrol* **20:** 713-720

Ma E, Haddad GG (1997) Expression and localization of Na+/H+ exchangers in rat central nervous system. *Neuroscience* **79:** 591-603

Maiese K, Li F, Chong ZZ (2005) New avenues of exploration for erythropoietin. *JAMA* **293:** 90-95

Malo ME, Li L, Fliegel L (2007) Mitogen-activated protein kinase-dependent activation of the Na+/H+ exchanger is mediated through phosphorylation of amino acids Ser770 and Ser771. *J Biol Chem* **282:** 6292-6299

Marlow N, Rose AS, Rands CE, Draper ES (2005) Neuropsychological and educational problems at school age associated with neonatal encephalopathy. *Arch Dis Child Fetal Neonatal Ed* **90:** F380-387

Martinez-Sanchez M, Striggow F, Schroder UH, Kahlert S, Reymann KG, Reiser G (2004) Na(+) and Ca(2+) homeostasis pathways, cell death and protection after oxygen-glucose-deprivation in organotypic hippocampal slice cultures. *Neuroscience* **128:** 729-740

Matsuda T, Arakawa N, Takuma K, Kishida Y, Kawasaki Y, Sakaue M, Takahashi K, Takahashi T, Suzuki T, Ota T, Hamano-Takahashi A, Onishi M, Tanaka Y, Kameo K, Baba A (2001) SEA0400, a novel and selective inhibitor of the Na+-Ca2+ exchanger, attenuates reperfusion injury in the in vitro and in vivo cerebral ischemic models. *J Pharmacol Exp Ther* **298:** 249-256

Mazarati AM, Baldwin R, Klitgaard H, Matagne A, Wasterlain CG (2004) Anticonvulsant effects of levetiracetam and levetiracetam-diazepam combinations in experimental status epilepticus. *Epilepsy Res* **58:** 167-174

Ment LR, Schwartz M, Makuch RW, Stewart WB (1998) Association of chronic sublethal hypoxia with ventriculomegaly in the developing rat brain. *Brain Res Dev Brain Res* **111:** 197-203

Miller SP, Weiss J, Barnwell A, Ferriero DM, Latal-Hajnal B, Ferrer-Rogers A, Newton N, Partridge JC, Glidden DV, Vigneron DB, Barkovich AJ (2002) Seizure-associated brain injury in term newborns with perinatal asphyxia. *Neurology* **58:** 542-548

Molinaro P, Cantile M, Cuomo O, Secondo A, Pannaccione A, Ambrosino P, Pignataro G, Fiorino F, Severino B, Gatta E, Sisalli MJ, Milanese M, Scorziello A, Bonanno G, Robello M, Santagada V, Caliendo G, Di Renzo G, Annunziato L (2013) Neurounina-1, a novel compound that increases Na+/Ca2+ exchanger activity, effectively protects against stroke damage. *Mol Pharmacol* **83:** 142-156

Molinaro P, Cuomo O, Pignataro G, Boscia F, Sirabella R, Pannaccione A, Secondo A, Scorziello A, Adornetto A, Gala R, Viggiano D, Sokolow S, Herchuelz A, Schurmans S, Di Renzo G, Annunziato L (2008) Targeted disruption of Na+/Ca2+ exchanger 3 (NCX3) gene leads to a worsening of ischemic brain damage. *J Neurosci* **28:** 1179-1184

Moster D, Lie RT, Markestad T (2002) Joint association of Apgar scores and early neonatal symptoms with minor disabilities at school age. *Arch Dis Child Fetal Neonatal Ed* **86:** F16-21

Nabetani M, Okada Y, Takata T, Takada S, Nakamura H (1997) Neural activity and intracellular Ca2+ mobilization in the CA1 area of hippocampal slices from immature and mature rats during ischemia or glucose deprivation. *Brain Res* **769:** 158-162

Nakamura T, Minamisawa H, Katayama Y, Ueda M, Terashi A, Nakamura K, Kudo Y (1999) Increased intracellular Ca2+ concentration in the hippocampal CA1 area during global ischemia and reperfusion in the rat: a possible cause of delayed neuronal death. *Neuroscience* **88:** 57-67

Nakanishi N, Tu S, Shin Y, Cui J, Kurokawa T, Zhang D, Chen HS, Tong G, Lipton SA (2009) Neuroprotection by the NR3A subunit of the NMDA receptor. *J Neurosci* **29:** 5260-5265

Nicoll DA, Hryshko LV, Matsuoka S, Frank JS, Philipson KD (1996) Mutation of amino acid residues in the putative transmembrane segments of the cardiac sarcolemmal Na+-Ca2+ exchanger. *J Biol Chem* **271:** 13385-13391

Nicoll DA, Longoni S, Philipson KD (1990) Molecular cloning and functional expression of the cardiac sarcolemmal Na(+)-Ca2+ exchanger. *Science* **250:** 562-565

Nicoll DA, Ottolia M, Lu L, Lu Y, Philipson KD (1999) A new topological model of the cardiac sarcolemmal Na+-Ca2+ exchanger. *J Biol Chem* **274:** 910-917

Noh MR, Kim SK, Sun W, Park SK, Choi HC, Lim JH, Kim IH, Kim HJ, Kim H, Eun BL (2006) Neuroprotective effect of topiramate on hypoxic ischemic brain injury in neonatal rats. *Exp Neurol* **201:** 470-478

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

O'Brien FE, Iwata O, Thornton JS, De Vita E, Sellwood MW, Iwata S, Sakata YS, Charman S, Ordidge R, Cady EB, Wyatt JS, Robertson NJ (2006) Delayed whole-body cooling to 33 or 35 degrees C and the development of impaired energy generation consequential to transient cerebral hypoxia-ischemia in the newborn piglet. *Pediatrics* **117:** 1549-1559

Ohls RK, Ehrenkranz RA, Das A, Dusick AM, Yolton K, Romano E, Delaney-Black V, Papile LA, Simon NP, Steichen JJ, Lee KG (2004) Neurodevelopmental outcome and growth at 18 to 22 months' corrected age in extremely low birth weight infants treated with early erythropoietin and iron. *Pediatrics* **114:** 1287-1291

Olney JW, Sharpe LG (1969) Brain lesions in an infant rhesus monkey treated with monsodium glutamate. *Science* **166:** 386-388

Palmada M, Centelles JJ (1998) Excitatory amino acid neurotransmission. Pathways for metabolism, storage and reuptake of glutamate in brain. *Front Biosci* **3:** d701-718

Palmer C, Menzies SL, Roberts RL, Pavlick G, Connor JR (1999) Changes in iron histochemistry after hypoxic-ischemic brain injury in the neonatal rat. *J Neurosci Res* **56:** 60-71

Pannaccione A, Secondo A, Molinaro P, D'Avanzo C, Cantile M, Esposito A, Boscia F, Scorziello A, Sirabella R, Sokolow S, Herchuelz A, Di Renzo G, Annunziato L (2012) A new concept: Abeta1-42 generates a hyperfunctional proteolytic NCX3 fragment that delays caspase-12 activation and neuronal death. *J Neurosci* **32:** 10609-10617

Papile LA, Rudolph AM, Heymann MA (1985) Autoregulation of cerebral blood flow in the preterm fetal lamb. *Pediatr Res* **19:** 159-161

Patti G, Tomai F, Melfi R, Ricottini E, Macri M, Sedati P, Giardina A, Aurigemma C, Leporace M, D'Ambrosio A, Di Sciascio G (2013) Strategies of clopidogrel load and atorvastatin reload to prevent ischemic cerebral events in patients undergoing protected carotid stenting. Results of the randomized ARMYDA-9 CAROTID (Clopidogrel and Atorvastatin Treatment During Carotid Artery Stenting) study. *J Am Coll Cardiol* **61:** 1379-1387

Perlman JM (2004) Brain injury in the term infant. *Semin Perinatol* **28:** 415-424

Perlman JM (2007) Pathogenesis of hypoxic-ischemic brain injury. *Journal of Perinatology* **27:** S39-S46

Perlman M, Shah PS (2009) Ethics of therapeutic hypothermia. *Acta Paediatr* **98:** 211-213

Philipson KD, Nicoll DA (2000) Sodium-calcium exchange: a molecular perspective. *Annu Rev Physiol* **62:** 111-133

Pignataro G, Boscia F, Esposito E, Sirabella R, Cuomo O, Vinciguerra A, Di Renzo G, Annunziato L (2012) NCX1 and NCX3: two new effectors of delayed preconditioning in brain ischemia. *Neurobiol Dis* **45:** 616-623

Pignataro G, Cuomo O, Vinciguerra A, Sirabella R, Esposito E, Boscia F, Di Renzo G, Annunziato L (2013) NCX as a key player in the neuroprotection exerted by ischemic preconditioning and postconditioning. *Adv Exp Med Biol* **961:** 223-240

Pignataro G, Esposito E, Cuomo O, Sirabella R, Boscia F, Guida N, Di Renzo G, Annunziato L (2011) The NCX3 isoform of the Na+/Ca2+ exchanger contributes to neuroprotection elicited by ischemic postconditioning. *J Cereb Blood Flow Metab* **31:** 362-370

Pignataro G, Tortiglione A, Scorziello A, Giaccio L, Secondo A, Severino B, Santagada V, Caliendo G, Amoroso S, Di Renzo G, Annunziato L (2004) Evidence for a protective role played by the Na+/Ca2+ exchanger in cerebral ischemia induced by middle cerebral artery occlusion in male rats. *Neuropharmacology* **46:** 439-448

Pintado AJ, Herrero CJ, Garcia AG, Montiel C (2000) The novel Na(+)/Ca(2+) exchange inhibitor KB-R7943 also blocks native and expressed neuronal nicotinic receptors. *Br J Pharmacol* **130:** 1893-1902

Quednau BD, Nicoll DA, Philipson KD (1997) Tissue specificity and alternative splicing of the Na+/Ca2+ exchanger isoforms NCX1, NCX2, and NCX3 in rat. *Am J Physiol* **272:** C1250-1261

Raichle ME (1983) The pathophysiology of brain ischemia. *Ann Neurol* **13:** 2-10

Raoul C, Estevez AG, Nishimune H, Cleveland DW, deLapeyriere O, Henderson CE, Haase G, Pettmann B (2002) Motoneuron death triggered by a specific pathway downstream of Fas. potentiation by ALS-linked SOD1 mutations. *Neuron* **35:** 1067-1083

Reeves JP, Hale CC (1984) The stoichiometry of the cardiac sodium-calcium exchange system. *J Biol Chem* **259:** 7733-7739

Reuter H, Henderson SA, Han T, Matsuda T, Baba A, Ross RS, Goldhaber JI, Philipson KD (2002) Knockout mice for pharmacological screening: testing the specificity of Na+-Ca2+ exchange inhibitors. *Circ Res* **91:** 90-92

Rice JE, 3rd, Vannucci RC, Brierley JB (1981) The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann Neurol* **9:** 131-141

Robertson CM, Finer NN (1988) Educational readiness of survivors of neonatal encephalopathy associated with birth asphyxia at term. *J Dev Behav Pediatr* **9:** 298-306

Rodgers RJ, Cutler MG, Jackson JE (1997) Behavioural effects in mice of subchronic buspirone, ondansetron and tianeptine. II. The elevated plus-maze. *Pharmacol Biochem Behav* **56:** 295-303

Rosenkrantz TS, Diana D, Munson J (1988) Regulation of cerebral blood flow velocity in nonasphyxiated, very low birth weight infants with hyaline membrane disease. *J Perinatol* **8:** 303-308

Ruth V, Widness JA, Clemons G, Raivio KO (1990) Postnatal changes in serum immunoreactive erythropoietin in relation to hypoxia before and after birth. *J Pediatr* **116:** 950-954

Sarnat HB, Sarnat MS (1976) Neonatal encephalopathy following fetal distress. A clinical and electroencephalographic study. *Arch Neurol* **33:** 696-705

Scafidi J, Fagel DM, Ment LR, Vaccarino FM (2009) Modeling premature brain injury and recovery. *Int J Dev Neurosci* **27:** 863-871

Schubert S, Brandl U, Brodhun M, Ulrich C, Spaltmann J, Fiedler N, Bauer R (2005) Neuroprotective effects of topiramate after hypoxia-ischemia in newborn piglets. *Brain Res* **1058:** 129-136

Secondo A, Pannaccione A, Molinaro P, Ambrosino P, Lippiello P, Esposito A, Cantile M, Khatri PR, Melisi D, Di Renzo G, Annunziato L (2009) Molecular pharmacology of the amiloride analog 3-amino-6-chloro-5-[(4-chloro-benzyl)amino]-n-[[(2,4-dimethylbenzyl)-amino]iminom ethyl]-pyrazinecarboxamide (CB-DMB) as a pan inhibitor of the Na+-Ca2+ exchanger isoforms NCX1, NCX2, and NCX3 in stably transfected cells. *J Pharmacol Exp Ther* **331:** 212-221

Secondo A, Staiano RI, Scorziello A, Sirabella R, Boscia F, Adornetto A, Valsecchi V, Molinaro P, Canzoniero LM, Di Renzo G, Annunziato L (2007) BHK cells transfected with NCX3 are more resistant to hypoxia followed by reoxygenation than those transfected with NCX1 and NCX2: Possible relationship with mitochondrial membrane potential. *Cell Calcium* **42:** 521-535

Seeger T, Fedorova I, Zheng F, Miyakawa T, Koustova E, Gomeza J, Basile AS, Alzheimer C, Wess J (2004) M2 muscarinic acetylcholine receptor knock-out mice show deficits in behavioral flexibility, working memory, and hippocampal plasticity. *J Neurosci* **24:** 10117-10127

Shalak L, Perlman JM (2004) Hypoxic-ischemic brain injury in the term infant-current concepts. *Early Hum Dev* **80:** 125-141

Shankaran S (2009) Neonatal encephalopathy: treatment with hypothermia. *J Neurotrauma* **26:** 437-443

Shankaran S, Laptook AR, Ehrenkranz RA, Tyson JE, McDonald SA, Donovan EF, Fanaroff AA, Poole WK, Wright LL, Higgins RD, Finer NN, Carlo WA, Duara S, Oh W, Cotten CM, Stevenson DK, Stoll BJ, Lemons JA, Guillet R, Jobe AH (2005) Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. *N Engl J Med* **353:** 1574-1584

Sheldon RA, Sedik C, Ferriero DM (1998) Strain-related brain injury in neonatal mice subjected to hypoxia-ischemia. *Brain Res* **810:** 114-122

Shepherd JK, Grewal SS, Fletcher A, Bill DJ, Dourish CT (1994) Behavioural and pharmacological characterisation of the elevated "zero-maze" as an animal model of anxiety. *Psychopharmacology (Berl)* **116:** 56-64

Siesjo BK (1981) Cell damage in the brain: a speculative synthesis. *J Cereb Blood Flow Metab* **1:** 155-185

Silbereis JC, Huang EJ, Back SA, Rowitch DH (2010) Towards improved animal models of neonatal white matter injury associated with cerebral palsy. *Dis Model Mech* **3:** 678-688

Silver IA, Deas J, Erecinska M (1997) Ion homeostasis in brain cells: differences in intracellular ion responses to energy limitation between cultured neurons and glial cells. *Neuroscience* **78:** 589-601

Silver IA, Erecinska M (1990) Intracellular and extracellular changes of [Ca2+] in hypoxia and ischemia in rat brain in vivo. *J Gen Physiol* **95:** 837-866

Simbruner G, Mittal RA, Rohlmann F, Muche R (2010) Systemic hypothermia after neonatal encephalopathy: outcomes of neo.nEURO.network RCT. *Pediatrics* **126:** e771-778

Singh D, Kumar P, Narang A (2005) A randomized controlled trial of phenobarbital in neonates with hypoxic ischemic encephalopathy. *J Matern Fetal Neonatal Med* **18:** 391-395

Sokolow S, Luu SH, Headley AJ, Hanson AY, Kim T, Miller CA, Vinters HV, Gylys KH (2011) High levels of synaptosomal Na(+)-Ca(2+) exchangers (NCX1, NCX2, NCX3) co-localized with amyloid-beta in human cerebral cortex affected by Alzheimer's disease. *Cell Calcium* **49:** 208-216

Stone BS, Zhang J, Mack DW, Mori S, Martin LJ, Northington FJ (2008) Delayed neural network degeneration after neonatal hypoxia-ischemia. *Ann Neurol* **64:** 535-546

Tan S, Liu YY, Nielsen VG, Skinner K, Kirk KA, Baldwin ST, Parks DA (1996) Maternal infusion of antioxidants (Trolox and ascorbic acid) protects the fetal heart in rabbit fetal hypoxia. *Pediatr Res* **39:** 499-503

Tanabe Y, Masu M, Ishii T, Shigemoto R, Nakanishi S (1992) A family of metabotropic glutamate receptors. *Neuron* **8:** 169-179

Taverna S, Sancini G, Mantegazza M, Franceschetti S, Avanzini G (1999) Inhibition of transient and persistent Na+ current fractions by the new anticonvulsant topiramate. *J Pharmacol Exp Ther* **288:** 960-968

Torrance HL, Benders MJ, Derks JB, Rademaker CM, Bos AF, Van Den Berg P, Longini M, Buonocore G, Venegas M, Baquero H, Visser GH, Van Bel F (2009) Maternal allopurinol during fetal hypoxia lowers cord blood levels of the brain injury marker S-100B. *Pediatrics* **124:** 350-357

Tymianski M, Charlton MP, Carlen PL, Tator CH (1993) Source specificity of early calcium neurotoxicity in cultured embryonic spinal neurons. *J Neurosci* **13:** 2085-2104

Uematsu D, Greenberg JH, Reivich M, Karp A (1988) In vivo measurement of cytosolic free calcium during cerebral ischemia and reperfusion. *Ann Neurol* **24:** 420-428

Unal Cevik I, Dalkara T (2003) Intravenously administered propidium iodide labels necrotic cells in the intact mouse brain after injury. *Cell Death Differ* **10:** 928-929

Van Bel F, Shadid M, Moison RM, Dorrepaal CA, Fontijn J, Monteiro L, Van De Bor M, Berger HM (1998) Effect of allopurinol on postasphyxial free radical formation, cerebral hemodynamics, and electrical brain activity. *Pediatrics* **101:** 185-193

van Landeghem FK, Felderhoff-Mueser U, Moysich A, Stadelmann C, Obladen M, Bruck W, Buhrer C (2002) Fas (CD95/Apo-1)/Fas ligand expression in neonates with pontosubicular neuron necrosis. *Pediatr Res* **51:** 129-135

Vannucci RC, Brucklacher RM, Vannucci SJ (2001) Intracellular calcium accumulation during the evolution of hypoxic-ischemic brain damage in the immature rat. *Brain Res Dev Brain Res* **126:** 117-120

Vannucci RC, Vannucci SJ (2005) Perinatal hypoxic-ischemic brain damage: evolution of an animal model. *Dev Neurosci* **27:** 81-86

Watano T, Harada Y, Harada K, Nishimura N (1999) Effect of Na+/Ca2+ exchange inhibitor, KB-R7943 on ouabain-induced arrhythmias in guinea-pigs. *Br J Pharmacol* **127:** 1846-1850

Welsh FA, Vannucci RC, Brierley JB (1982) Columnar alterations of NADH fluorescence during hypoxia-ischemia in immature rat brain. *J Cereb Blood Flow Metab* **2:** 221-228

White HS, Brown SD, Woodhead JH, Skeen GA, Wolf HH (1997) Topiramate enhances GABA-mediated chloride flux and GABA-evoked chloride currents in murine brain neurons and increases seizure threshold. *Epilepsy Res* **28:** 167-179

Wilkinson DJ (2009) Cool heads: ethical issues associated with therapeutic hypothermia for newborns. *Acta Paediatr* **98:** 217-220

Wirrell EC, Armstrong EA, Osman LD, Yager JY (2001) Prolonged seizures exacerbate perinatal hypoxic-ischemic brain damage. *Pediatr Res* **50:** 445-454

Yu SP, Choi DW (1997) Na(+)-Ca2+ exchange currents in cortical neurons: concomitant forward and reverse operation and effect of glutamate. *Eur J Neurosci* **9:** 1273-1281

Zhang Y, Lipton P (1999) Cytosolic Ca2+ changes during in vitro ischemia in rat hippocampal slices: major roles for glutamate and Na+-dependent Ca2+ release from mitochondria. *J Neurosci* **19:** 3307-3315

Zhou WH, Cheng GQ, Shao XM, Liu XZ, Shan RB, Zhuang DY, Zhou CL, Du LZ, Cao Y, Yang Q, Wang LS (2010) Selective head cooling with mild systemic hypothermia after neonatal hypoxic-ischemic encephalopathy: a multicenter randomized controlled trial in China. *J Pediatr* **157:** 367-372, 372 e361-363

Zona C, Ciotti MT, Avoli M (1997) Topiramate attenuates voltage-gated sodium currents in rat cerebellar granule cells. *Neurosci Lett* **231:** 123-126