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INVOLVEMENT OF Na^+/Ca^{2+} EXCHANGER (NCX) IN GLUTAMATE-INDUCED NEUROPROTECTION IN SH-SY5Y DIFFERENTIATED CELLS SUBJECTED TO HYPOXIA/REOXYGENATION (H/R) INJURY.

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INDEX

1. INTRODUCTION

Degeneration and death of neurons are the main processes responsible for the clinical manifestation of different neurodegenerative diseases like stroke, Alzheimer and Parkinson's disease[1]. Stroke is the second leading cause of death, dementia and disability that it is characterized by a variety of data cases among countries[2]. The World Health Organization (WHO) defines stroke as "rapidly developed signs of focal (or global) disturbance of cerebral function lasting longer than 24 hours (unless interrupted by death), with no apparent nonvascular cause"[3]. The 2018 Heart Disease and Stroke Statistics update of the American Heart Association (AHA) has reported that, although there has been considerable reduction in stroke risks and stroke outcomes, the racial and geographic disparities remain significant, with African Americans and residents of the south-eastern United States undergoing the greatest excess disease burden. According to data from the Centers for Disease Control and Prevention 2015 Behavioral Risk Factor Surveillance System, 2.6% of non-Hispanic whites, 4.1% of non- Hispanic blacks, 1.5% of Asian/Pacific Islanders, 2.3% of Hispanics (of any race), 5.2% of American Indian/Alaska Natives, and 4.7% of other races or multiracial people had a history of stroke[4]. It is important to highlight that ischemia is a heterogeneous group of conditions that share similarities in the biological response, but difference in the system of perfusion. Brain damage depends on the severity and the duration of ischemia. Notably, upon the restoration of the blood flow and the concomitant reoxygenation, brain is susceptible to an exacerbation of the tissue damage[5]. Given that scenario, there is a great and grown interest in developing new strategies to mitigate ischemia-reperfusion injury.

1.1 General features of cerebral ischemia

Ischemia is a pathological condition characterized by blood supply and oxygen deprivation, which generates a mismatch between energy consumption and energy production, leading to complex energy-dependent cellular damage[6]. The brain is responsible for 20–25% of total body oxygen consumption, constituting the highest metabolic activity per unit weight of any organ. Its distinctive physiological properties make this organ extremely sensitive to the loss of blood flow[7]. This

phenomenon results in excessive neuron depolarization, release of excitatory neurotransmitters, reduced re-uptake of neurotransmitters from extracellular space and accumulation of Ca^{2+}[6].Restoration of oxygenated blood flow, although necessary to re-establish delivery of oxygen and nutrients to support cell metabolism, may induce pathogenic processes that exacerbate the ischemic injury, thus producing a further damage known as ischemic/ reperfusion (I/R) injury. This phenomenon is represented by a sum of ischemic damage plus that evoked by reperfusion, which can active immunological and molecular pathways and it can be manifested as different types of cell death, such as necrosis and apoptosis. Within the ischemic territory, the region supplied by end arteries undergoes rapid death and is referred to as the core. Surrounding the core is a variable area of the brain called the penumbra, which it is viable for several hours and can be salvaged by restoration of flow. If reperfusion is not established, the penumbra is gradually recruited into the core[5,8].

1.2 Pathophysiology of cerebral ischemic injury

The pathophysiology of cerebral ischemia is complex, and it consists of a wide variety of cellular processes. Clinically, the most common event is focal cerebral ischemia (called ischemic stroke), which results from occlusion of an artery in the brain and it accounts for more than 80% of all strokes. Unless rapidly reversed, the occlusion of a major artery usually produces tissue infarction that affect parts of the brain exhibiting a non-selective loss of all cells including neurons, astrocytes, oligodendrocytes, microglia and endothelial cells. The size and location of these infarcts are important to determine the long-term functional deficits resulting from ischemic stroke. The differences in the severity of the ischemia in the core and penumbra mean that different mechanisms contribute to cell death. Treatment with a thrombolytic agent to reverse arterial occlusion within the first 3 h following stroke onset provides the only approach in routine clinical use for limiting the acute effects of this disorder in humans. Unfortunately, only a small proportion of those affected by stroke are currently treated with thrombolysis. Spontaneous reversal of arterial occlusion occurs

within the first 6 h in approximately 17% of ischemic stroke patients and in approximately 40–50% by 4 days[8].

Mitochondria have been implicated as central players in the development of ischemic cell death both through impairment of their role in generating ATP for neural cell function and as key mediators in cell death pathways. Considering that during ischemia neurons become unable to maintain cellular function and homeostasis, energy failure is the central event in the pathophysiology of ischemia. The deficit of oxygen affects oxidative phosphorylation and ATP production, leading to dysfunction of ATP-dependent ion transport pumps and to ionic imbalances[6,9]. A compensatory increase in anaerobic glycolysis for ATP production leads to the accumulation of hydrogen ions and lactate, resulting in intracellular acidosis (pH to <7.0) and inhibition of mitochondrial fatty acid and residual energy metabolism. Thus, the impaired ATP synthesis rate is the main cause of the imbalances in ionic state across cellular membranes, largely due to the inability of ATP-dependent pumps to function. In detail, at cellular level there is initially an increased K^+ efflux related to an increased osmotic load due to the accumulation of metabolites and inorganic phosphate. A substantial decline in ATP leads to Na^+/K^+-ATPase inactivation, resulting in a further decline of K^+ and an increase in Na^+ influx. The mechanisms underlying the increased Na^+ early in ischemia are failure to extrude Na^+ via the Na^+/K^+-ATPase and Na^+ influx via Na^+/H^+ exchange, Na^+-HCO_3– cotransport (NBC) and the voltage-gated Na^+ channel. Since Na^+ efflux via the Na^+/K^+-ATPase is attenuated during ischemia, activation of other mechanisms leads to increased intracellular Na^+. Both the Na^+/H^+ exchanger and the cotransporter (NBC) might also contribute to Na^+ influx[10,11]. As a consequence of the intracellular Na^+ concentration increase, Na^+/Ca^{2+} exchanger (NCX), which will be discuss further in detail, is induced to operate in reverse mode resulting in intracellular Ca^{2+} overload as the cell tries to extrude Na^+.Moreover, in addition to disruptions in ionic homeostasis, cellular systems are overwhelmed by an increase in reactive oxygen species (ROS) production and oxidative stress, which can result in further defective Ca^{2+} handling[11,12]. Simultaneously, depolarization of presynaptic terminals leads to massive release of the excitatory neurotransmitter glutamate. Glutamate activates N-methyl-d-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) glutamate receptors, resulting in membrane depolarization and influx of Ca^{2+} and Na^+. Entry of Ca^{2+}

via voltage-sensitive Ca^{2+} channels and release from endoplasmic reticulum (ER) via stimulation of metabotropic glutamate receptors cause an increase in intracellular Ca^{2+} to levels that are toxic to the cell[6].

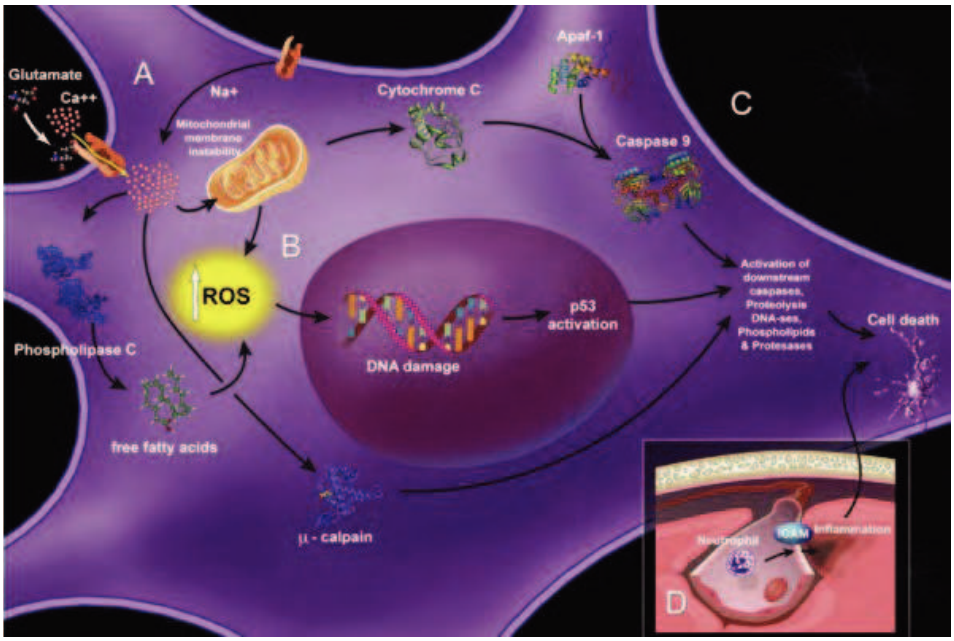

Fig. 1.1. Cell-signaling cascades and mechanisms of ischemia. Glutamate-mediated sodium and calcium channel activation leads to depolarization and activation of cascades leading to apoptosis, targeted by inhibitors of ligand binding or direct blockade of the channels (A). Production of reactive oxidative species (ROS), (B), can lead to DNA damage and trigger downstream cell-death mechanisms. Administration of free radical scavengers has been attempted to thwart this. Administration of neurotrophic and vasoactive agents may modify the extracellular matrix (C) and has shown promise in the treatment of cerebral infarcts in rats. Finally, neutrophil-mediated inflammatory changes have been associated with brain injury in ischemia (D), and antibodies designed to target cell surface receptors on neutrophils and endothelial cells have been trialed in order to prevent infiltration of these cells into the ischemic bed and subsequent downstream oxidative damage[13].

1.3 Pathophysiology of cerebral reperfusion injury

Reperfusion remains the main therapeutic approach to ischemia. Even though it is essential to salvage ischemic tissue restoring oxygen and nutrients delivery and removing ischemic metabolites, it can induce pathogenetic processes that exacerbate injury due to ischemia *per se* and produce tissue injury in distant organs. Restoration of blood flow leads to restoring of aerobic ATP synthesis, increasing of extracellular pH by washing out accumulated H^+, Ca^{2+} overload, opening of the

mitochondrial permeability transition pore (mPTP), endothelial dysfunction, low production of nitric oxide and oxidative stress, migration of inflammatory cells, disruption of membranes and induction of apoptotic and necrotic signals[7,14]. The major deficits in mitochondrial ATP production in severely ischemic core tissue create conditions that ensure the development of necrotic death in all cells within a few hours of stroke onset. This tissue provides no prospects for promoting cell survival unless there is early reperfusion. The milder metabolic deficits in the penumbra allow longer survival of the cells and provide opportunities for protective interventions. Nonetheless, in the absence of reperfusion or other treatments, metabolic changes arising from the limited availability of substrates for oxidative metabolism and probably also from progressive deterioration of mitochondrial function again contributes to the demise of cells in this region. Other mitochondrial changes, including induction of the permeability transition and the release of proteins that trigger apoptosis, have also been implicated in tissue damage, particularly in the reperfused penumbra[8]. The recovery of pH, oxidative stress and Ca^{2+} overload can induce the abrupt opening of the mPTP, a large conductance pore in the inner mitochondrial membrane, which dissipates mitochondrial membrane potential, leads to release of the enzyme cytochrome c and strongly contributes to apoptosis and necrosis. This causes a rapid decline in the intracellular ATP concentrations, which causes the disruption of ionic and metabolic homeostasis and activation of degradative enzymes and ultimately results in irreversible cell damage and necrotic death. Oxidative stress has been strongly implicated as an important factor in the development of both necrosis and apoptosis in focal ischemia, particularly when there is reperfusion. Mitochondria are a major site of production of superoxide in normal cells and probably contribute to increased oxidative stress in ischemic and post-ischemic brain. The effects of some pharmacologic therapeutic interventions, which have been primarily examined in focal cerebral ischemia, indicate that irreversible cellular damage is not determined during the ischemic period, but can develop during reperfusion, especially during delayed reperfusion. Thus, therapeutic interventions initiated up to several hours after 2 to 3 hours of induced focal ischemia may be able to substantially reduce infarction volume, not only in the perifocal regions, but also in brain tissue that has formed parts of the severely disturbed ischemic core[15].

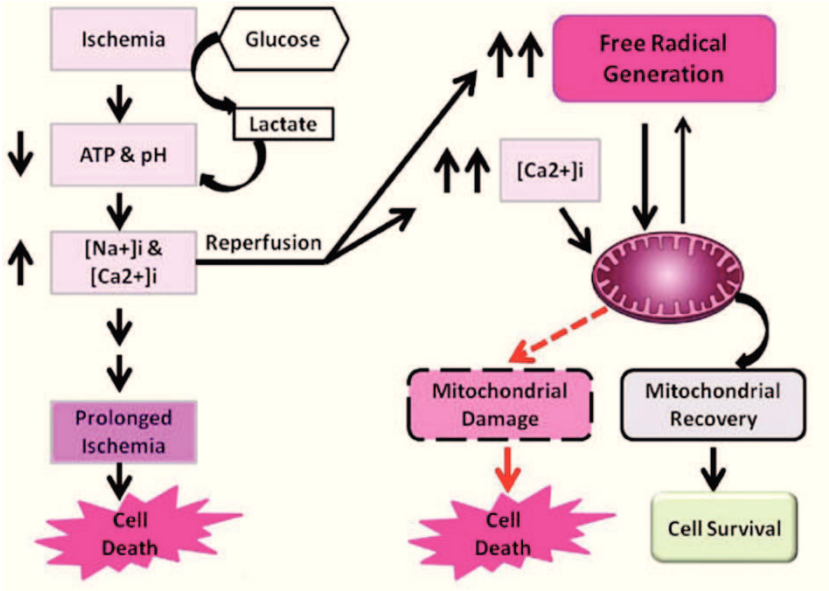

Fig. 1.2. Main events of ischemia reperfusion injury. Ischemia decreases energy content, acidifies intracellular and extracellular environments and alters ionic homeostasis. Reperfusion exacerbates tissue damage producing more ROS, opening of mPTP, inducing Ca^{2+} uptake and cell death[16].

1.4 Cerebral calcium handling

Ca^{2+} is a universal second messenger that it is involved in many physiological processes of eukaryotic cells, including control of metabolism, motility processes, exocytosis, fertilization, muscle contraction, transcription of numerous genes and process of programmed cell death. In neurons it plays an essential role because of its participation in the transmission of the depolarizing signal and contributes to synaptic activity. Thus, neuronal life depends on the precise temporal and spatial regulation of the Ca^{2+} signals. Under physiological conditions, resting levels of cytosolic Ca^{2+} are maintained at submicromolar concentrations by different mechanisms, which involves the coordinated action of the systems that handle Ca^{2+} movements and the activity of different types of channels. Ion channels, exchangers, and pumps at both the plasma membrane and the membranes of mitochondria, endoplasmic reticulum, Golgi apparatus, and nucleus contribute to the regulation of Ca^{2+} cellular homeostasis that, together with the action of G protein-coupled receptors, Ca^{2+} binding proteins, and transcriptional networks, orchestrate neuronal Ca^{2+}-regulated processes. This regulation is important for neuronal plasticity and underlie critical neuronal functions, such as learning and memory. Several families of proteins intrinsic to membranes are implied in controlling Ca^{2+}

concentration within cells, transporting Ca^{2+} across membrane boundaries and/or mediating its passage across them down the steep concentration gradient (about 10,000-fold) between the extracellular ambient and the cell interior, and between the lumen of intracellular Ca^{2+}-storing organelles and the cytosol. These proteinaceous systems are established of various types of channels such as ATPases, exchangers (mostly NCX), and a complex electrophoretic system that mediates the import of Ca^{2+} across the inner mitochondrial membrane into the matrix. They differ in transport mechanism, affinity for Ca^{2+}, and total transport capacity, and thus offer/provide the multiplicity of responses necessary for the efficient operation of the Ca^{2+} signaling system[17]. The key role of Ca^{2+} in synaptic transmission was first established in 1960s by Katz and Miledi, who demonstrated that Ca^{2+} triggers the neurotransmitter release from presynaptic terminals. This mechanism was studied in many "synapses model", including giant squid axon synapses and rat cerebellar parallel fiber synapses, but characterized in greatest detail in the calyx of Held synapses in the brainstem. The first step of the synaptic transmission is the opening of calcium channels in the membrane of a presynaptic terminal during its depolarization by arriving action potentials, and the increase in intracellular Ca^{2+} at the active zone, which triggers fusion in a few hundred microseconds, or possibly even in less than 100 microseconds. The tight regulation of Ca^{2+} triggers the complex mechanism of stored neurotransmitters liberation. At rest, these transmitters are accumulated in membrane-formed vesicles, which perform complicate movements towards the presynaptic membrane. Finally, the vesicles fuse with the latter and form pores through which the stored transmitter substances are released into the synaptic cleft. Ca^{2+} ions injected into the presynaptic terminal play a key role in all steps of this machinery and they are immediately attacked by mitochondria and endoplasmic reticulum. These structures substantially modify the amplitude and time course of such Ca^{2+} transients and, correspondingly, modify their action on the process of exocytosis. Mitochondria accumulate Ca^{2+} from the cytosol extremely rapidly, by a uniporter mechanism at their inner membrane. The Ca^{2+} uptake by the SERCA pump of the endoplasmic reticulum is much slower and less effective. Correspondingly, the mitochondria, with some delay, release these Ca^{2+} ions back into the cytosol via their NCX[18].

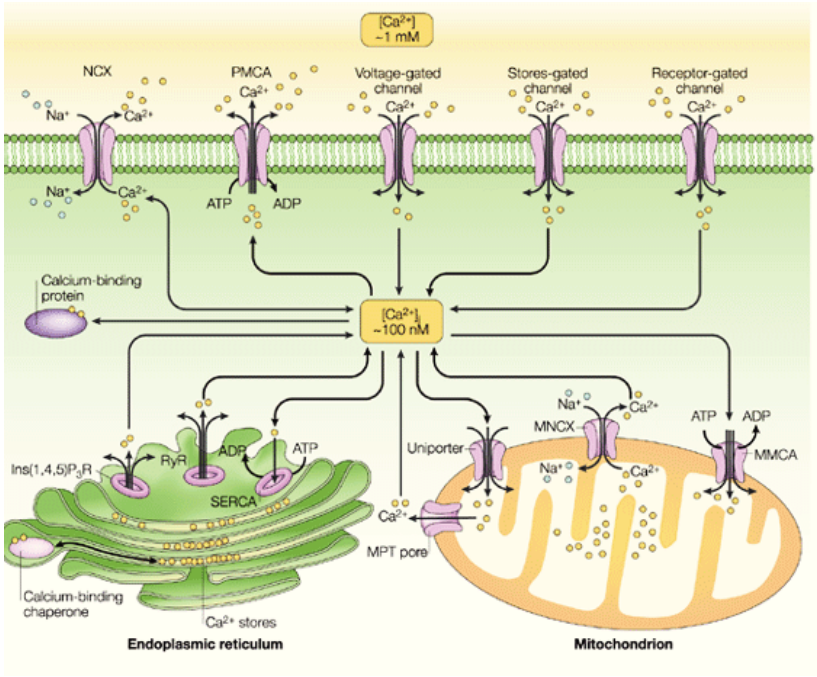

Fig. 1.3. Calcium homeostasis mechanisms. Intracellular calcium concentration is tightly regulated through various channels, pumps and exchangers at different levels[19].

1.5 Calcium overload-induced neurotoxicity during I/R

The excessive increase of Ca^{2+} level, which occurs during I/R injury, leads to the over-activation of several deleterious enzymes and signalling processes that impair neuronal function or lead to cell death. One of these events is the neurotoxicity induced by glutamate, the major excitatory neurotransmitter in the mammalian nervous system. It has been recognized that glutamate has a neurotoxic potential since the 1950s.Olney coined the term "excitotoxicity" to describe the process by which glutamate, an excitatory neurotransmitter, elicits toxicity[6]. Generally, this process allows a large inflow of Ca^{2+} and Na^{+} into neurons. Although Ca^{2+} ions are necessary for proper function and survival of neuronal cells, in many pathological states including brain ischemia, Ca^{2+} ions reach critical levels leading to cell injury or death[20]. It has been demonstrated by Sattler et al[21] that the main determinant of Ca^{2+} neurotoxicity is the route by which Ca^{2+} ions gain access to the intracellular space. Some routes of Ca^{2+} entry, such as voltage gated Ca^{2+} channels, did not elicit cell death, whereas others (such as NMDA receptors), were associated with significant Ca^{2+}- dependent toxicity[19]. However, the ionotropic receptors are recognized as being particularly key. There are

three types of ionotropic glutamate receptors: NMDARs, 2-amino-3-(3- hydroxy-5-methylisox-azol-4-yl) propionate receptors (AMPARs), and kainate receptors, each having several subtypes. Activation of ionotropic receptors leads to an enhanced permeability to Na^+, K^+ and/or Ca^{2+} in the associated ion channels[22]. It has been known that exist mechanisms of neurotoxicity that operate independently of, or in parallel with, excitotoxicity. One of such mechanism involves Transient Receptor Potential (TRP) channels. Most of TRPM channels members are permeable of Ca^{2+}. Of these, TRPM2 and TRPM7 have been associated with cell death due to anoxia, ROS and stroke. There is evidence that other TRP family members might participate in survival processes, such as overexpression of TRPC3 and TRPC6 can prevent cerebellar granule neurons death[6]. In ischemic stroke much attention has been also directed to dihydropyridine-sensitive L-type voltage-dependent Ca^{2+} channels (CaV1.2), but blockage of this channel in patients with acute ischemic stroke has shown little benefit[23]. Another major Ca^{2+} extruding system is the plasma membrane Ca^{2+} ATPase (PMCA). In glutamate-mediated neuronal death the PMCA can be internalized after cleavage with caspases, and this contributes to further deregulation of Ca^{2+} concentrations in these cells[24].Ca^{2+} ions may gain access to the neuronal cytoplasm via ion channels or Ca^{2+} transport systems, or through the release of Ca^{2+} ions from intracellular stores. Depletion of calcium ions from the endoplasmic reticulum (ER) has been suggested as an initial signal for ER dysfunction in ischemic neurons. Many studies indicate that a strong release of calcium ions from ER is associated with damage to cells, including damage to neurons after ischemia[6]. Dysregulation of Ca^{2+}-ER homeostasis following ischemia involves two phases: accumulation of Ca^{2+} in ER stores and subsequent release of Ca^{2+}-ER following I/R[25]. Another source of very high cellular calcium ion concentration is mitochondria. It participates both in Ca^{2+} buffering and in the formation of special communications with the ER. The ability of mitochondria to provide ATP to energy-demanding processes is regulated by Ca^{2+}, which is sequestered into the mitochondria matrix via a proton electrochemical gradient generated by the electron transport chain, thus depolarizing the mitochondrial potential. The influx of Ca^{2+} decreases the electrochemical gradient, subsequently reduces ATP synthesis. In addition, aberrations in mitochondrial electron chain functioning can result in excessive ROS production, leading to neurotoxicity[6,17]. Mitochondrial Ca^{2+} overload and dysfunction, due to excessive Ca^{2+} entry through

over-activated glutamate receptors, is a crucial early event in the excitotoxic cascade that follows ischemic or traumatic brain injury. In isolated mitochondria, Ca^{2+} overload can evoke sustained mPTP opening and cytochrome c release. Opening of the mPTP causes a massive swelling of mitochondria coupled with collapse of the mitochondrial membrane potential. Blocking permeability transition prevents apoptotic neurodegeneration and consequently, suggests a potential strategy for neuroprotection[26,27].

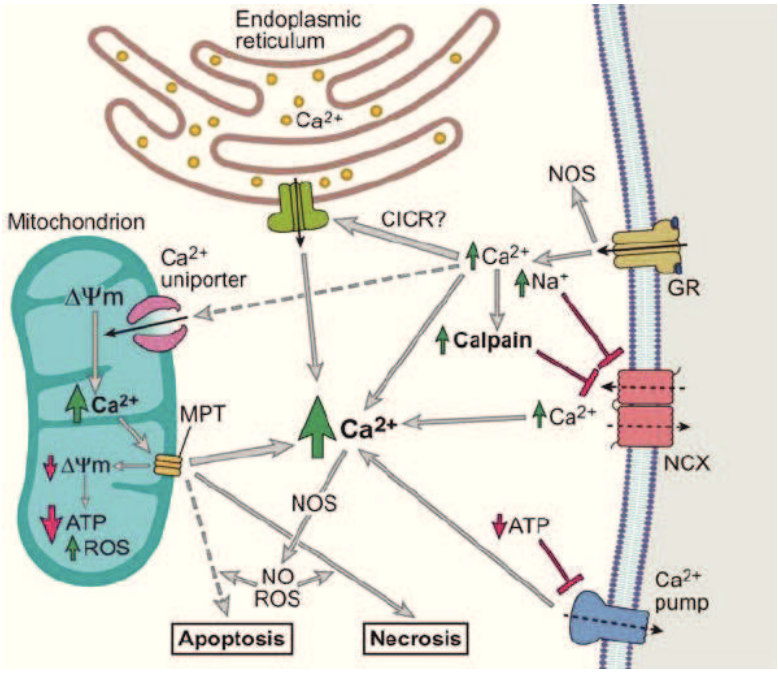

Fig. 1.4. Calcium overload during cell injury. Excess of calcium entry during cell injury induces disfunctions of cell activities, such as decrease of mitochondrial membrane potential, ATP level, ROS production and lead to cell death[22].

1.6 The sodium calcium exchanger (NCX)

The Na^+/Ca^{2+} exchanger (NCX) is a bi-directional membrane ion transporter that catalyze electrogenic exchange of 3 Na^+ ions and 1 Ca^{2+} ion across the plasma membrane. It has emerged as key player in controlling ionic homeostasis at both the plasma membrane and mitochondrial levels[28,29], particularly when overall Ca^{2+} levels are high[30,31]. Depending on electrochemical gradients and membrane potential, NCX functions in either the Ca^{2+}-efflux (forward) or the Ca^{2+}-influx (reverse) mode[30,32,33]. NCX belongs to a multigene family comprising three isoforms named NCX1,

NCX2, and NCX3, which are encoded by separate genes (SLC8A1, SLC8A2, SLC8A3) and their splice variants are expressed in a tissue-specific manner to fulfil physiological demands in excitable and non-excitable tissues and the expression profile change during the development[30,31,34]. Genomic analyses revealed that an additional gene, SLC8A4, is present in teleost, amphibian, and reptilian species, but that this gene has been secondarily and independently lost in mammals and birds. At the post-transcriptional level, at least 17 NCX1 and 5 NCX3 proteins are generated through alternative splicing of the primary nuclear SLC8A1 and SLC8A3 transcripts, whereas no splice variants have been identified for SLC8A2. These variants result from a combination of six small exons (A, B, C, D, E, and F)[31] located on a restricted region of the large intracellular loop-f. All splice variants include a mutually exclusive exon, either A or B in order to maintain an open reading frame[35]. Excitable tissues contain exon A, whereas kidney, stomach, and skeletal muscle tissues comprise NCX with exon B. NCX1 is universally distributed, practically in every mammalian cell including the brain, heart, kidney, skeletal muscle, eye and blood cells, although the NCX1 splice variants are selectively expressed in a tissue-specific manner. NCX2 is predominantly expressed in the brain and spinal cord, but it can also be found in the gastrointestinal tract and kidney tissues. NCX3 is predominantly expressed in the brain and skeletal muscle, but it can also be found in osseous tissue and the immune system[36]. Although all three members arise from separate genes on different chromosomes, they share high amino acid identity (about 70%), especially in the hydrophobic regions[34]. The molecular weight of eukaryotic NCX typically consists of 930-970 amino acids with a molecular mass of 110 kDa. Biochemical analyses over the years have indicated the presence of 9 transmembrane segments (TMSs). However, major advancement in understanding the structural basis has only recently been achieved by solving the crystal structure of a prokaryotic homologue of the exchanger, which revealed the presence of 10 α-helical TMSs rather than the 9 TMS proposed for NCX1[36]. The NH_2-terminal portion of the loop, between TMS5 and TMS6, has a 20-amino acid domain rich in hydrophobic and basic amino acids and is called the XIP (exchange inhibitor peptide) region. This region is an auto-inhibitory sequence, which is associated with the Na^+ inactivation process and it can bind several peptide mimetic compounds. In this regard, recent research showed that a new cell penetrating peptide called P1 is capable to blocks the auto inhibitory XIP domain and

enhances NCX activity[33]. Around the central zone of the loop in the NH_2-terminal portion, there is a sequence of 135 amino acids containing highly acidic residues (two zones of three aspartyl each) that bind $[Ca^{2+}]_i$ with high affinity; this region is responsible for the $[Ca^{2+}]_i$ dependent or allosteric regulation of the exchanger[37].

Mitochondria are involved in Ca^{2+} homeostasis and signalling, as well as in cell death, aging and diseases. Minelli et al[28] demonstrated that a large fraction of neuronal and glial mitochondria in adult rat hippocampus and frontal cortex express high levels of NCX1–3 isoform proteins, suggesting that all three exchangers are likely to give an important contribution to maintain Ca^{2+} homeostasis in excitable and non-excitable CNS cell types in situ. The subcellular localization patterns of NCX1–3 in neurons and astrocytes support their possible involvement in synaptic transmission and neuroprotection[28]. In addition, NCX-mediated Ca^{2+} release may play prominent role in mitochondria located in neuronal dendrites and astrocytic processes with the high variability of NCXs expression patterns in organelles located in different cell types and in different subcellular domains[29]. A major physiological role of mitochondrial NCX isoform (NCX mito) in neuronal physiology is its participation in the process of cytosolic Ca^{2+} buffering by mitochondria; it can transport the Ca^{2+} ions captured by mitochondria when $[Ca^{2+}]_c$ increases in response to extracellular signals back to the cytosol. This transport activity contributes to delaying $[Ca^{2+}]_c$ recovery to baseline levels, giving rise to a shoulder in $[Ca^{2+}]_c$ after membrane depolarization or the activation of plasmamembrane receptors[38].

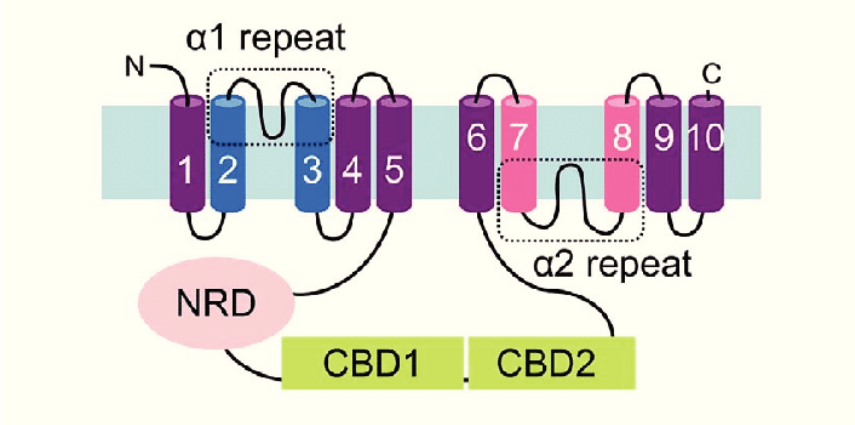

Fig. 1.5. The molecular structure NCX. The molecule presences 10α-helical TMSs, two conserved α-repeats regions within TM helices 2–3 and 7–8, and a P-loop domain. The Ca^{2+}-binding domains (CBD) and the predicted Na^+ regulatory domain (NRD) regulate exchanger activity[23].

	NCX1		NCX2		NCX3	
	Number of profiles	Profiles per mesh	Number of profiles	Profiles per mesh	Number of profiles	Profiles per mesh
Cerebral cortex						
Dendrites/spines	618 (54.7%)	25.9 ± 7	789 (51.7%)	33 ± 4.4	869 (58.6%)	37 ± 6.3
Axon fibers/terminals	20 (1.8%)	0.8 ± 1.2	53 (3.5%)	2.7 ± 2.9	65 (4.4%)	2.9 ± 2.8
Glial processes	388 (34.3%)	16.1 ± 9	523 (34.3%)	22 ± 4.9	490 (33%)	20.3 ± 4.3
Unidentified	104 (9.2%)	4.3 ± 2.3	161 (10.5%)	6.6 ± 2.5	60 (4%)	2.6 ± 1.4
Total labelled profiles	1130 (100%)	47.1 ± 11.4	1526 (100%)	63.7 ± 14.1	1484 (100%)	61.9 ± 16.1
Hippocampus						
Dendrites/spines	868 (40.4%)	36.4 ± 19	889 (51%)	37 ± 8.5	890 (57.1%)	36.6 ± 6
Axon fibers/terminals	38 (1.8%)	1.75 ± 2	51 (2.9%)	2 ± 1.4	83 (5.3%)	3.4 ± 2.9
Glial processes	1162 (54.1%)	48.7 ± 32	732 (42%)	30.5 ± 9	463 (29.7%)	19.3 ± 9.5
Unidentified	80 (3.7%)	3.5 ± 2.6	72 (4.1%)	3 ± 1.1	124 (7.9%)	5.1 ± 2.1
Total labelled profiles	2148 (100%)	89.3 ± 23.5	1744 (100%)	72.5 ± 18.2	1560 (100%)	65 ± 15.6

For each NCX isoform, numbers and relative percentages (in parentheses) of labelled profiles as well as numbers of labelled profiles per mesh (average ± S.D.) are reported.

Table 1. Quantitative analysis of NCX1–3-labelled neuropilar profiles sampled from parietal cortex and hippocampus[28].

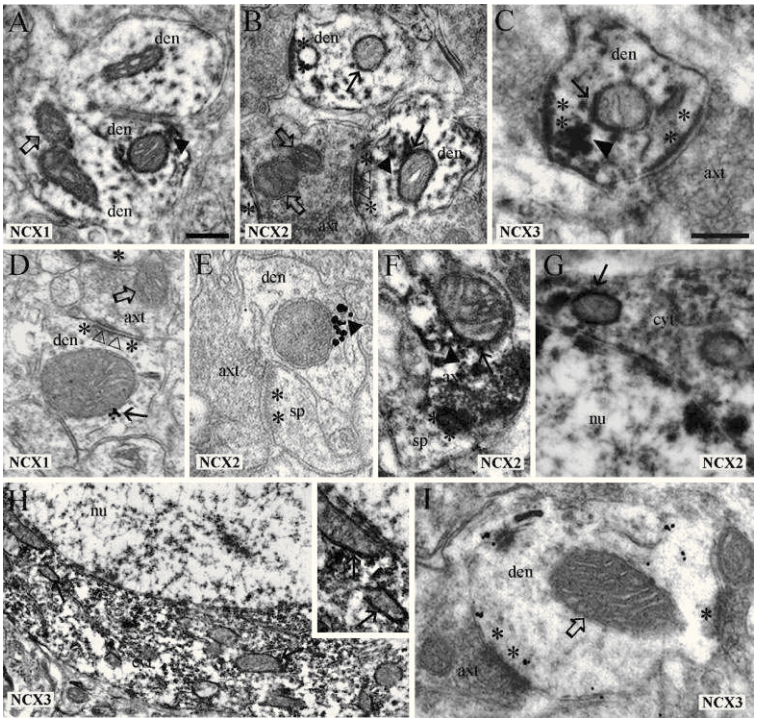

Fig. 1.6. NCXs labelling patterns in neuronal mitochondria (A–I). (A and D) NCX1-ir mitochondria (arrows) in distal dendrites (CA1 Stratum radiatum). (B and E) NCX2-ir mitochondria (arrows) in hippocampal (B) and neocortical (E) dendrites. (C) NCX3-ir mitochondria in neocorticaldistal dendrite (arrow). (F) NCX2-positive mitochondrion in a CA1 axon terminal. (G and H) NCX2 and NCX3-ir mitochondria (arrows) in a cell body (from CA1 pyramidal cell layer); enlarged in the inset in H, two labelled organelles (arrows) near nuclear envelope. (I) NCX3-ir in neocortical distal dendrite with an unlabelled mitochondria (open arrow). In B and D dendrites are contacted by axon terminals forming asymmetric junction (triangles). In A and E, note the labelling bridging plasmalemma and mitochondrial profile. Open arrows indicate unlabelled mitochondria in dendrites (A and I) and axon terminals (B and D). With asterisks the postsynaptic specializations are indicated and the arrowheads show the labelling between mitochondria and plasma membrane. Axt, axon terminal; den,

dendrite; nu, nucleus; cyt, cytoplasm; sp, dendritic spine. Immunoperoxidase reaction in A–C, F, G and H and silver-enhanced immunogold in D, E and I. Calibration bars: in A, 0.25 µm for A, B, D, F, I; in A, 0.5 µm for in set in H; in C, 0.25 µm for C and E; in C, 0.5 µm for G; in C, 1 µm for H[29].

1.6.1 Role of NCX in neuronal I/R injury

In the central nervous system (CNS) NCX plays a relevant role in maintaining intracellular Na^+ and Ca^{2+} homeostasis under different neurophysiological and neuropathological conditions. Of note, its expression in neurons is high, especially in regions where there is a large traffic of Ca^{2+} across the plasma membrane: at synapses and at growth cones[33]. In addition to neurons, NCX is also expressed in glial cells, playing many crucial roles, including the regulation in Na^+ and Ca^{2+} homeostasis in astrocytes, neurotransmitter release, oligodendrocyte maturation, and myelin formation[34]. In the brain, unlike other tissues, NCX is present in the three different gene products NCX1, NCX2 and NCX3 with a distinct distribution pattern in different brain regions. In neurons under physiological conditions, it is generally accepted that the NCX is primarily involved in removing Ca^{2+} from the cytosol in cells that require extracellular Ca^{2+} for their physiological activities. However, because NCX can also function in Ca^{2+} entry mode under pathophysiological conditions such as cerebral ischemia, the question has been raised as to whether NCX activity is neuroprotective or neurodamaging during ischemia[31,34,39]. NCX subtypes may carry variable roles in ischemic injury and the mode of action of each subtype may vary in ischemia and reperfusion states exerting different roles during *in vitro* and *in vivo* anoxic conditions leading to a new paradigm in the pathogenesis of ischemic damage[40]. Thus, NCX subtype-specific strategies, which should be based on appropriate timing of administration, have become an alternative therapeutic approach to limit the severity of ischemic injury[34]. Ischemic neurons deprived of oxygen and glucose rapidly deplete ATP, resulting in cell depolarization and glutamate release. Anaerobic glycolysis and ATP degradation produce H^+, activating NCX and causing Na^+ influx. Under anoxic condition the activity of the Na^+/K^+ ATPase is impaired resulting in a decrease of Na^+ efflux. The overstimulation of glutamate receptors (NMDA/AMPA) results in Na^+ and Ca^{2+} influx into the neurons and depending on the duration and severity of the excitotoxic event, acute or delayed neuronal death follows. The processes involved in

Ca^{2+}-mediated neuronal death include activation of calpains, endonucleases, nitric oxide synthase and free radical production, phospholipases and mitochondrial dysfunction. The rise in Na^{+}_{i} is also catastrophic, causing cellular swelling and cytoskeletal dysfunction and inducing the Na^{+}/glutamate carrier to operate in the opposite direction, allowing excess release of glutamate and increasing excitotoxicity. During ischemia NCX operates in reverse mode and contributes to Ca^{2+} overload. The reverse mode activity of NCX may have two opposing roles in ischemic injury: 1) exacerbating ischemic insult by increasing Ca^{2+}_{i} and 2) ameliorating neuronal damage by reducing Na^{+}_{i}[34,39]. In neuronal models of I/R, the role of the different NCX isoforms has been widely investigated in *in vitro* and *in vivo* studies, however it still remains controversial and incompletely elucidated. Recently it has been demonstrated that NCX activity can be strategic for neuroprotection trough different mechanisms. The brain complexity and the presence of all three NCX isoforms make difficult to find strategies for H/R neuroprotection. The majority of *in vivo* studies using the focal cerebral ischemia model indicate that blocking NCX activity is neurodamaging while increasing NCX activity is neuroprotective[39,41]. Pignataro et al.[42] showed that in a model of permanent focal cerebral ischemia, the reduction in NCX1 and NCX3 protein expression causes an increase in the ischemic infarct, suggesting the importance of the NCX forward mode of operation in extruding Ca^{2+} in the ischemic brain[42]. This hypothesis was supported by the observed increase in NCX1 and NCX3 activity responsible for Ca^{2+} cycling from ER and mitochondria leading to neuroprotection[41]. In this scenario, a promising selective compound able to stimulate the activity of NCX1 and to prevent neuronal degeneration *in vitro* and *in vivo* models of ischemia has been synthesized[43]. By contrast, Matsuda et al[44] demonstrated that administration of SEA0400, a selective inhibitor of reverse-mode NCX1, during reperfusion phase significantly reduced the infarct volume in the cortex after 24 h of reperfusion that followed 2 h of ischemia[44]. These findings suggest that NCX activity depends on the specific pathological context within which the exchanger works, therefore, tight regulation of its activity is critical for the brain.

1.7 Neuroprotective strategies against I/R injury

The pathophysiology of brain ischemia is complex, and a therapeutic strategy capable of activating neuroprotective signalling network and/or simultaneously counteracting multiple mediators of I/R injury would likely have the greatest efficacy in mitigating ischemic death. It is well known that the extent of cell dysfunction, injury, and/or death is influenced by both the magnitude and the duration of ischemia. In light of this fact, revascularization and restoration of blood flow as soon as possible remains the main therapeutic approach to ischemia. In a variety of organ systems including the brain, heart and kidneys as in the cases of stroke, myocardial infarction and acute kidney injury, respectively, it has been demonstrated that restoration of vascular supply often paradoxically results in pathogenetic processes initiating additional lethal damage to the tissues. Restoration of blood flow during the first 30 to 60 min in rats and mice greatly limits the size of the infarcts and can completely block cell loss, indicating that the development of irreversible damage in neurons and other cells in the core tissue is delayed. Even with ischemic periods up to 3 h, during the first 2 h following reperfusion there is often near-complete recovery of phosphocreatine and of the adenylate energy charge. The recovery of these metabolic parameters requires the presence of intact and functional cells that at least partially regain the complex metabolic activities and control processes required to meet energy demands. With further perfusion following longer ischemic periods, the core tissue typically exhibits a secondary decline in energy-related metabolites that is most likely associated with final progression to the death of many cells. The timing of these changes is broadly consistent with the finding in human stroke that damage can be reduced, and function improved if flow is restored within 3 h and perhaps after longer periods[8]. Mitochondrial respiratory function is generally completely or near-completely restored in samples prepared from core and penumbral tissue within the first hour following reperfusion, but then declines at later times. Interestingly, this secondary deterioration in mitochondrial respiration apparently develops earlier than the changes in energy-related metabolites when assessed in the same ischemic model, suggesting that the delayed alterations in mitochondrial function are an early step in the development of irreversible cell dysfunction and possibly a contributor to this process[8]. Since the stroke treatments are extremely

limited and reperfusion could be harmful for brain injury, there is a need for the development of novel neuronal ischemic therapies that can make the brain more resistant to ischemic death. In line with these observations, ischemic preconditioning and ischemic postconditioning represent two promising strategies in modulating ischemic damage[45]. The concept of ischemic preconditioning was introduced first in the heart[46] and later on in the brain[46-48]. Several *in vivo* and *invitro* studies have demonstrated that neurons exposed to a brief period of sublethal stimuli develop resistance to subsequently more prolonged and lethal anoxic insults[45,48]. Considering the extreme complexity of preconditioning strategy, the molecular mechanisms underlying the ischemic tolerance are not completely elucidated[48]. Several events are involved in this process, such as activation of protein kinase, induction of transcription factors, alterations in cellular energy metabolism, preservation of mitochondrial functions, improve capacity to preserve cellular ionic and pH homeostasis. Unlike ischemic precondition, postcondition strategy is a relative new concept of neuroprotection[45,49]. It is characterized by repetitive series of brief interruptions of reperfusion applied immediately after ischemia. Experimental and clinical settings demonstrated that repeated cycles of brief reperfusion and re-occlusion were able to reduce the infarct size after cardiac ischemia[45,50,51]. In addition, ischemic postconditioning attenuate neuronal damage in rodent models of spinal cord[52], and focal[49,53] and global[54,55] ischemic injury.

1.8 Energy metabolism

The mammalian brain contains essentially two different metabolic compartments: the glial and the neuronal. These two compartments differ in their utilization of particular substrates, their metabolic products, and the presence of characteristic enzymes. The glial compartment uses glucose or acetate as its main substrates, is characterized by the presence of glutamine synthase, and produces glutamine, which is transferred to the neuronal compartment and metabolized. The neuronal compartment uses glucose and glial glutamine as its main substrates, is characterized by the presence of high glutaminase and GABA decarboxylase activities, and releases glutamate and GABA, which are transferred to the glial compartment and metabolized. In addition, glial lactate has been proposed

recently as an important substrate for the neuronal compartment, at least in neural cell cultures of fetal or neonatal origin during neuronal activation. The metabolisms of neurons and glial cells interact closely, competing for glucose as a primary substrate, and using the glutamate, glutamine, and GABA produced in the other cell type as an alternative substrate for oxidation in the tricarboxylic acid (TCA) cycle. Thus, several substrates are available to neurons and glial cells in situ, allowing to compensate deficits in one substrate with increased utilization of others[56]. Glucose is the obligatory energy substrate of the adult brain. Nevertheless, under particular circumstances the brain has the capacity to use other blood-derived energy substrates, such as ketone bodies during development and starvation or lactate during periods of intense physical activity. Glucose enters cells trough specific glucose transporters (GLUTs) and it is phosphorylated by hexokinase (HK) to produce glucose-6-phosphate. As in other organs, glucose 6-phosphate can be processed via different metabolic pathways, the main ones being glycolysis, the pentose phosphate pathway (PPP), and glycogenesis (in astrocytes only). Neurons are predominantly oxidative whereas glycolysis predominates in astrocytes. In terms of energy cost and delivery, neurons consume 75%–80% of the energy produced whereas the rest is used for glia-based processes. Neurons energy utilization is related to the activity of ionic pumps that reestablish the electrochemical gradients dissipated by signaling, namely action potentials and synaptic potentials. Recent evidence confirms that the majority of the energy used by neurons for signaling appears to be consumed at the synapse, including postsynaptic potentials, presynaptic vesicle recycling. *In vitro* and *in vivo* biochemical and imaging studies converges to indicate that the major energetic burden of the nervous system is localized at the synapse and that molecular mechanisms must exist to couple synaptic activity to local energy delivery. Over the last three decades, evidence has accumulated pointing at astrocytes as key cells for the coupling between synaptic activity and energy metabolism[57].

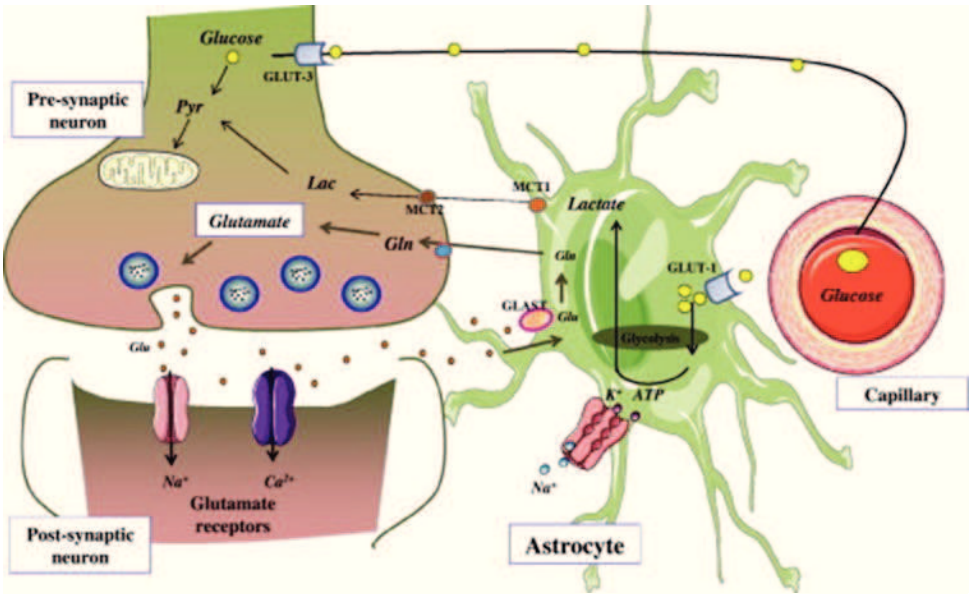

Fig. 1.7. Schematic representation of glucose and glutamate transport, metabolism and secretion by astrocytes and neurons. The glutamate/glutamine cycle is tightly coupled to glucose oxidation in astrocytes, which then release lactate to be taken up by neurons and be oxidized. Lac: lactate; Pyr: pyruvate; Glu: glutamate; MCT: monocarboxylate transporter; GLAST; glutamate/aspartate transporter; GLUT: glucose transporter; GS: glutamine synthetase; Gln: glutamine[58].

1.8.1 Energy metabolism impairment during cerebral I/R

During pathological conditions, such as ischemic stroke, the mismatch between energy consumption and energy production leads to complex energy-dependent cellular damage. Mitochondria have been implicated as central key players in the development of ischemic cell death both through impairment of their normal role in generating much of the ATP for neural cell function and as key mediators in cell death pathways. In animal models of stroke, the limited availability of glucose and oxygen directly impairs oxidative metabolism in severely ischemic regions of the affected tissue and leads to rapid changes in ATP and other energy-related metabolites. Indeed, ATP levels are depleted within minutes, and when blood flow is restored, it triggers irreversible post-ischemic mitochondrial dysfunction if it does not occur within a certain time frame. In addition, a secondary energy depletion occurs with reperfusion. This event is accompanied by the reduction of glucose utilization, despite normal blood flow; thus, ATP depletion during reperfusion may be not related to glucose availability. Moderate alterations in these metabolites develop in the penumbral tissue, associated with near normal glucose use but impaired oxidative metabolism. Glucose oxidation is markedly

decreased due both to lower energy requirements in the post-ischemic tissue and limitations on the mitochondrial oxidation of pyruvate. A secondary deterioration of mitochondrial function subsequently develops that may contribute to progression to cell loss. Mitochondrial release of multiple apoptogenic proteins has been identified in ischemic and post-ischemic brain, mostly in neurons [8].Energy dysfunction induces adaptations in cerebral metabolism, including the utilization of alternative energy sources. For example, intracellular amino acids (e.g., glutamate and glutamine) can be used as metabolic fuel to recover from mitochondrial failure[8,59].

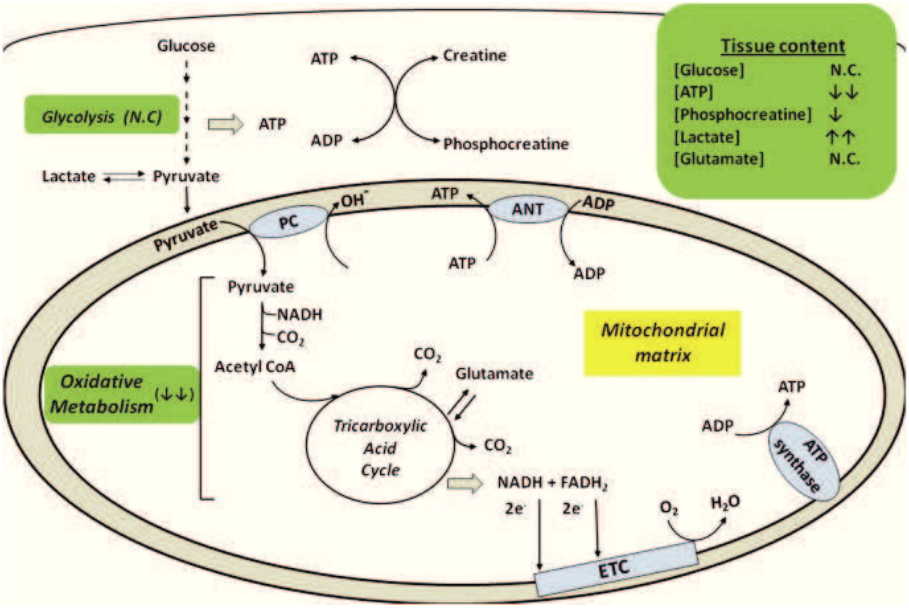

Fig. 1.8. The major alterations of brain energy metabolism induced in the penumbra within the first 2 h of stroke onset. In animal models of stroke, most cells in the penumbra remain viable during this initial period of focal ischemia and can survive if there is early restoration of blood flow or treatments that block other cellular changes critical for cell death. A single arrow indicates a moderate change and a double arrow a large change. N.C., no significant change from control values; ANT, adenine nucleotide translocase; ETC, electron transport chain; PC, pyruvate carrier[8].

1.9 Glutamate as a metabolic substrate

The brain contains huge amounts of glutamate (about 5–15 mmol per kg wet weight depending on the region), but only a small fraction of this glutamate is normally present extracellularly. The cells take up glutamate that it is may use for metabolic purposes (protein synthesis, energy metabolism, ammonia fixation) or as transmitter[60]. Glutamate is the major excitatory amino acid in the CNS. It

has been known for several years that, under pathological conditions such as ischemia, glutamate has a neurotoxic role and it can participate in inducing cell death by its excessive release, reduced uptake or altered receptor function. Indeed, dysfunction of glutamatergic signaling is related to many debilitating diseases, and therefore proper coordination and fidelity of release, activation, and reuptake of this neurotransmitter is paramount for total system homeostasis[60,61]. The failure of clinical trials of several neuroprotective strategies, most of them targeting the excitotoxicity cascade, suggest that glutamate may have a dual and time-dependent effect in ischemic settings: it may be involved in the early neurodestructive phase, however, when physiological conditions are restored and oxygen flow is reintroduced, it may contribute to cells functional recovery through its known physiological functions. Under energy-depleted conditions, alternative energy source could be used to face the brain bioenergetic imbalance. Among them, glutamate is an abundant substrate with the potential to be used by several fuel-providing mechanisms to rescue the cell from energy impairment. Tight control of glutamate handling in the brain is crucial to the maintenance of mitochondrial integrity. Indeed, beyond its roles as a building block for proteins and a major neurotransmitter, glutamate is a key compound in cellular metabolism, linking carbohydrate and amino acid metabolism via the TCA cycle[62]. It is worth noting that under energy-depleted conditions, the stimulation of glutamate conversion to alpha-ketoglutarate is able to restore ATP levels by supporting continuous TCA cycling trough the activity of glutamate dehydrogenase (GHD) and aspartate aminotransferase (AAT). Recently, Kim et al[59] demonstrated that treatments with GDH activator enhanced the utilization of glutamate as an energy source in neurons and astrocytes. In particular, mitochondrial GDH activation leads to increase of alpha-ketoglutarate influx into a TCA cycle thus resulting in a reduction of extracellular glutamate release[59].

It is well established that glutamate has very different roles in astrocytes and neurons in brain. The compartmentation of and regulation of glutamate and glutamine metabolism in astrocytes and neurons in brain is complex. There is evidence for multiple subcellular compartments of glutamate in these cells. Astrocytes and neurons both distinguish between ‘‘exogenous’’ glutamate taken up from the extracellular milieu and ‘‘endogenous’’ glutamate formed via glutaminase from glutamine. Interestingly, the enzymes used to convert exogenous and endogenous glutamate into alpha-

ketoglutarate and thereby mediate entry into the TCA cycle are different in astrocytes and neurons. Glutamate taken up by astrocytes has many possible metabolic fates including direct use for glutamine formation, participation in the purine nucleotide cycle, incorporation into proteins or the tripeptide glutathione, and metabolism via the TCA cycle for energy which can lead to formation of aspartate, lactate, or alanine. Neurons and synaptic terminals are characterized by a lot of number of mitochondria and a high rate of oxidative metabolism. Therefore, energy metabolism and neurotransmission are closely linked through glutamate. Glutamate metabolism in neurons is modulated by several factors including glucose, amino acids, fatty acids, and the ketogenic diet. Recent evidence suggests that cerebellar neurons preferentially use glutamate, rather than glutamine, for energy[62].

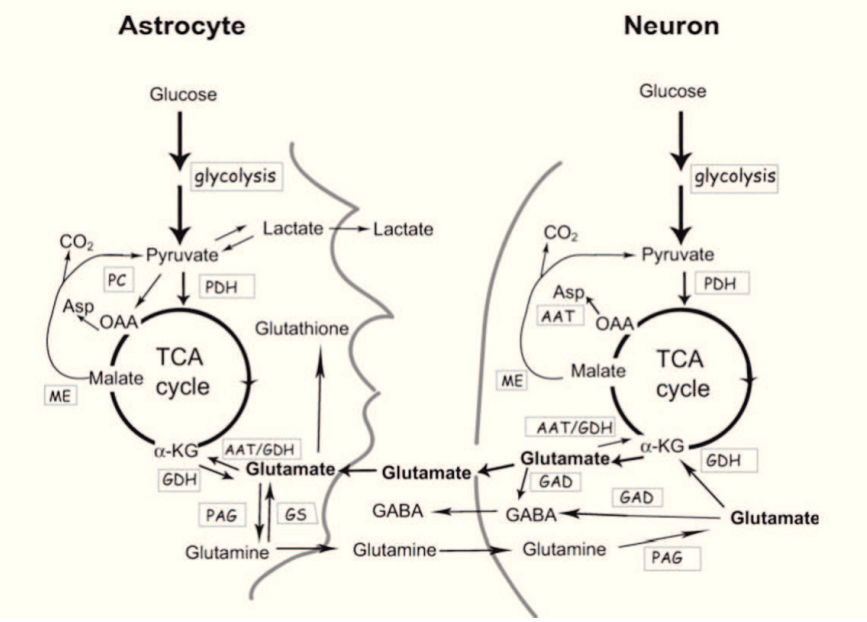

Fig. 1.9. The metabolism of glutamate and glutamine in astrocytes and neurons in brain. Different metabolic fates of glutamate in astrocytes and neurons[62].

1.9.1 Glutamate receptors proteins

The distribution of glutamate is tightly regulated and is highly sensitive to changes in the energy. The rapid turnover of glutamate contributes to keep low its extracellular concentration. Glutamate exerts a signaling role by acting on glutamate receptors, which are located on the surface of the cells. Three different families of glutamate receptor proteins have been identified with molecular cloning.

One family of glutamate receptors is activated by the glutamate analogue N-methyl-D-aspartate (NMDA) and these receptors (NR1, NR2A, NR2B, NR2C and NR2D) are collectively referred to as NMDA-receptors. Another family of receptors is activated by AMPA and by kainate. The NMDA and AMPA/kainate receptors are all glutamate gated ion channels (conducting only Na^+ or both Na^+ and Ca^{2+}) and are collectively referred to as ionotropic glutamate receptors. The AMPA receptors open readily upon glutamate exposure but desensitize quickly and are of low affinity. In contrast, the NMDA receptors have much higher affinities and are slowly inactivating. To be activated they need both glutamate binding and an already depolarized membrane. The third family of glutamate receptors consists of G-protein coupled receptors, the so-called metabotropic receptors (mGluR1–8)[60].

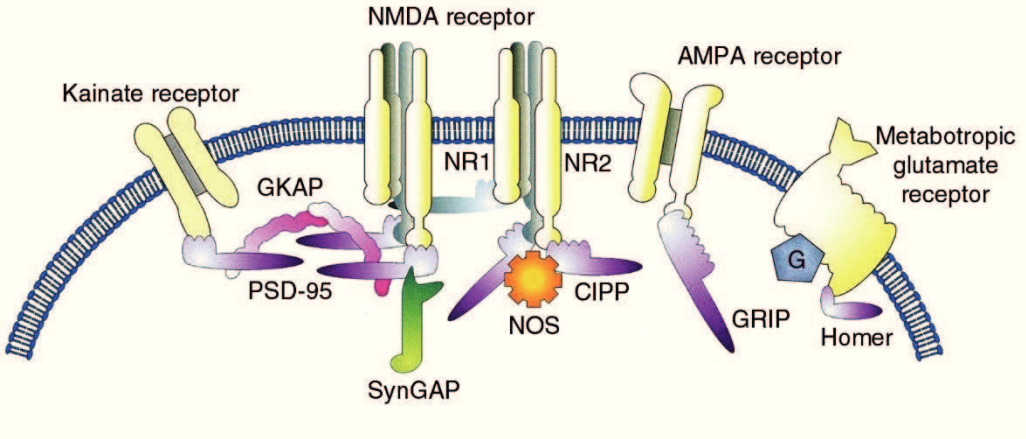

Fig. 1.10. Glutamate receptors. The glutamate distribution is tightly regulated through different receptors located on the surface of the cells: ionotropic receptors (NMDA, AMPA and kainite) and metabotropic receptors (mGluR)[24].

1.10 Role of excitatory amino acid transporters (EAATs)

The cells in the brain express several proteins able to transport glutamate. Some of these are found in the plasma membranes and some are found intracellularly. Excitatory amino acid transporters (EAATs) are secondary active, electrogenic transport systems that couple the accumulation of glutamate in the cytoplasm to downhill movement of co-transported ions along their concentration gradient. Alteration of these ion gradients, such as during anoxic depolarization, halts or even reverses transport of glutamate and can contribute to excitotoxic conditions. It has been known for

several years that EAATs regulate glutamatergic neurotransmission and the mechanism by which they accomplish this process is by a dynamic coupling of bioenergetics of the transport process and the localization of the transporters themselves. The consequence of this coupling is the creation of complex spatiotemporal profiles for extracellular glutamate[61,63]. The solute carrier 1 (SLC1) family of neurotransmitter transporters is comprised of several solute carriers including the excitatory amino acid transporter (EAATs). The initial cloning of a glutamate carrier in the SLC1 family was performed in 1992 with the isolation of a 60 kDa protein from rat brain termed the glutamate/aspartate transporter (GLAST). One month later, glutamate transporter 1 (GLT-1) from rat, and excitatory amino acid carrier 1 (EAAC1) from rabbit were both cloned. All of EAATs were described as Na^+ and K^+ dependent that accumulate glutamate and L- or D-aspartate. Subsequently two novel human isoforms were cloned from the cerebellum and retina, EAAT4 and EAAT5, respectively. Human isoforms of GLAST, GLT-1, and EAAC1 were also cloned and renamed EAAT1-3 to denote their human species of origin. EAATs 1 and 2 are found predominantly in astrocytes while EAAT3, EAAT4, and EAAT5 are neuronal. It has been demonstrated that EAATs transport 1 glutamate with 3 Na^+, 1 H^+, and the counter transport of 1 K^+[61]. During normal physiological conditions, it is unlikely that reverse transport occurs frequently enough to have a physiological consequence, yet during anoxic depolarization it has been suggested that glutamate efflux mediated by reversal of the EAAT transport cycle can mediate excitotoxic conditions. Under ischemic conditions, however, in which the Na^+ and K^+ concentrations and transmembrane voltage are disturbed, a significant amount of glutamate is released through an EAAT mediated reverse transport process. Thus, the bioenergetics of glutamate transport tightly controls the rates and amount of neurotransmitter removal from the synaptic space. Because cessation of excitatory neurotransmission is dependent on the efficient removal of extracellular glutamate, the bioenergetics of EAAT transport contributes to the termination of glutamatergic neurotransmission. EAATs can modulate the concentration of synaptic glutamate to some degree but also act to control the temporal profile of postsynaptic receptor activation and diffusion to perisynaptic regions as it binds and unbinds rapidly to a high number of carriers. The neuronal isoforms have several roles such as cysteine transport for glutathione synthesis and glutamate uptake for GABA synthesis, but the

prevailing hypothesis is that glial isoforms mediate the bulk of glutamate transport and have the greatest degree of influence on extrasynaptic receptor activation[61]. Since EAATs transports glutamate using the favorable Na^+ gradient[64,65], their activity result in transmembrane Na^+ influx, and eventually to stop unless specific mechanisms that preserve the Na^+ gradient are activated. Under physiological conditions, the main Na^+ efflux system in the mitochondrial matrix is the Na^+/H^+ exchanger (NHE), however its role in glutamate dependent influx pathway is presumably trifling, since H^+ is co-transported with Na^+ while glutamate is transported by EAATs[66]. Recently some studies suggested that the antiporter enzyme Na^+/K^+-ATPase may regulate glutamate uptake via EAATs, maintaining the Na^+ and K^+ ion gradients across the membrane[67]. However, a variable but significant component of the Na^+ dependent glutamate transport activity is resistant to Na^+/K^+-ATPase inhibition, suggesting that the Na^+ gradient across the membrane may be sustained through a different mechanism[68].

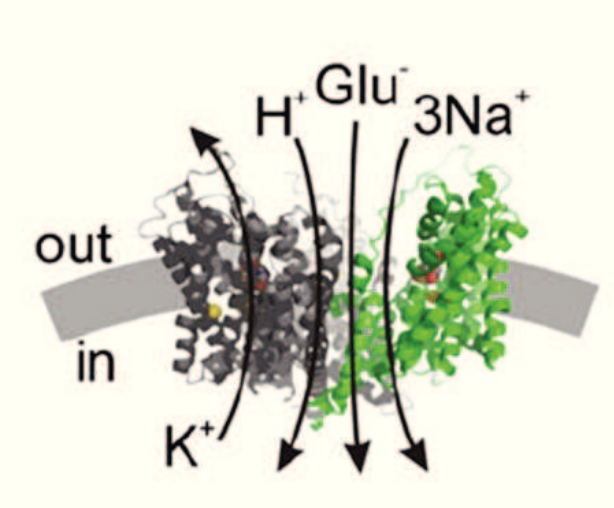

Fig. 1.11. Stoichiometry of EAATs. Glutamate transport by the EAATs is coupled to the cotransport of 3 Na^+ and 1 H^+ followed by the countertransport of 1 K^+[69].

1.11 NCX and EAATs interaction

Brain astrocytes express NCX exchanger, which, upon $[Na^+]_i$ elevation, turns into the reverse mode, pumping Na^+ ions out of the cell in exchange for Ca^{2+}. It has been already proposed that NCX acts as a safety factor maintaining transmembrane Na^+ gradients, and thus supporting the operation of Na^+/glutamate transporter. Therefore, NCX is specifically involved in supporting glutamate entry

trough EAATs[65]. Previous morphologic observations showed that both EAAT[60] and NCX[28] localized in the terminal processes of astroglial cells. Specifically, it has been reported a physical and functional interaction between a specific subtype of NCX, namely NCX1, and EAAT3[70,71]. Their physical association, disclosed by their co-localization, coimmunoprecipitation and mutual activity dependency, emphasizes the high selectivity of the interaction, which represents a novel and complementary mechanism enhancing energy metabolism to meet the increased demand both in neuronal and cardiac model. Such interaction occurs at both the plasma membrane and mitochondrial levels[70,71] where these proteins cooperate to promote the entry of glutamate into the cytoplasm and then into mitochondria, where glutamate serves as a fuel for ATP synthesis[72]. The cooperative interaction between NCX1 and EAAC1 is essential to sustain brain energy metabolism, cooperation that can be especially relevant when ATP production is critically compromised, such as in ischemia. In fact, during an ischemic insult, cells massively release glutamate, which in turn can lead to cell death immediately after the ischemia, but it might also be essential for the recovery of metabolic functionality in later stages[73]. Effectively, glutamate can participate in the recovery of energy production being used as an intermediary metabolite for ATP synthesis, especially when the oxygen tension is not so low as to abolish the oxidative metabolism, as observed in the ischemic penumbra and in the poststroke recovery phases[70,71].

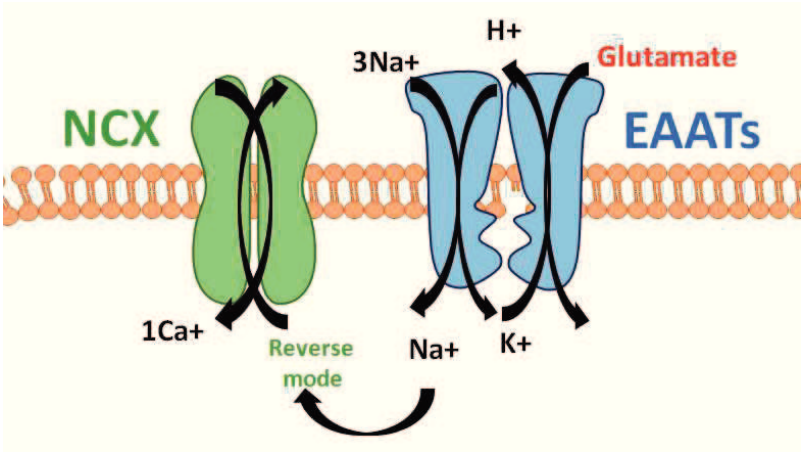

Fig. 1.12. Coordinated activity between NCX and EAATS. The reverse mode of NCX maintains the transmembrane Na^+ gradients supporting effective operation of glutamate uptake through EAATS (Thesis of Maiolino Marta).

1.12 Aim of the thesis

Brain ischemia continues to be among the most frequent causes of debilitating disease and death worldwide[7]. This pathological condition is characterized by blood supply and oxygen deprivation, which generate a mismatch between energy consumption and energy production, leading to complex energy-dependent cellular damage[6]. Under such circumstances, oxidative phosphorylation and ATP production are insufficient to sustain normal metabolic activities, causing dysfunction of ATP-dependent ion transport pumps and ionic impairment[9]. Although reperfusion remains the main therapeutic approach to ischemia, it can induce pathogenetic processes initiating additional lethal damage to the tissues[7,14] and giving rise the so-called "ischemia/reperfusion (I/R) injury"[5,8]. The cerebral bioenergetics imbalance produced by I/R induces metabolic adaptions including the utilization of alternative energy sources such as glutamate, glutamine and GABA[56,74]. Among them, glutamate is an abundant substrate with the potential to be used by several fuel-providing mechanisms to rescue the cell from energy impairment[62]. Recent evidences show that, under energy-depleted conditions, glutamate can be used as a metabolic intermediate and converted to alpha-ketoglutarate which fuels TCA cycle enhancing ATP production and improving cell viability[59,75]. Noteworthy, it has been demonstrated, by using different cell models (neurons, astrocytes and cardiac cells), that under physiological conditions, glutamate supplementation induces a rise of ATP levels through a mechanism that involves NCX and EAATs[70,71]. In addition, it has been reported a functional and physical interaction between NCX1/EAAT3 at both plasma membrane and mitochondrial levels[70,71]. NCX1/EAAT3 interaction promote the uptake of glutamate into the cytoplasm and then into mitochondria where glutamate serves as metabolic fuel for ATP synthesis. On the basis on these findings, the present study aimed at:

- Investigating whether glutamate supplementation during the reoxygenation phase could ameliorate the energy state of the cell and therefore protect against H/R injury.
- Exploring the involvement of NCX1 and EAAT3 in the glutamate protective response.

For these purposes, it has been used, as an *in vitro* model, SH-SY5Y human neuroblastoma cells differentiated with retinoic acid (RA) to a neuron-like state.

If confirmed, such results might pave the way for a change in the classical view of glutamate as a detrimental factor to a cell survival factor. In addition, the investigation of glutamate effects on H/R injury and the hypothetical involvement of NCX1/EAAT3 in such response could open new horizons for the development of new disease-modifying therapeutic strategies for brain ischemia.

2. MATERIALS AND METHODS

2.1 Cell Culture

Human neuroblastoma cell line SH-SY5Y[76] was obtained from American Type Culture Collection (ATCC CRL-2266). SH-SY5Y cells were cultured in 75-ml vented culture flasks using Eagle's Mini-mum Essential Medium/Nutrient Mixture Ham's F-12 (1:1) media supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cell culture medium was replaced every 2 days. The cells were maintained in a humidified incubator at 37 °C and 5% CO_2.

Differentiation into neuron-like cells was achieved by treatment with 10 μM all-trans-retinoic acid (RA) that was added to the cell culture medium every 3 days for 1 week prior to perform the experiments[77,78].

For LDH and NCX activity assays, differentiated cells (4×10^5 cells/well) were cultured onto 6-well plates, whereas for ATP assay differentiated cells (2.5×10^4 cells/well) were cultured onto 96-well plates.

2.2 Silencing of NCX1, NCX3 and EAAT3

RNA interference (RNAi) was performed as previously described[79] with minor modifications. Specifically, silencing of NCX1, NCX3 and EAAT3 was performed with HiPerfect Transfections Kit (Qiagen) according to the manufacturer's instruction by using FlexiTube small interference RNA (siRNA) for NCX1 (Qiagen, Hs_SLC8A1_9), FlexiTube siRNA for NCX3 (Qiagen Hs_SLC8A3_7), FlexiTube siRNA for EAAT3 (Qiagen Hs_SLC1A1_3). The validated irrelevant Allstars siRNA (Qiagen) was used as a negative control. Target sequence of the FlexiTube NCX1 siRNA was Hs_SLC8A1_9 (5'-CAGGCCATCTTCTAAGACTGA-3'), of the NCX3 siRNA sequences was Hs_SLC8A3_7 (5'-ACCATTGGTCTCAAAGATTCA-3'), and of the EAAT3 siRNA was Hs_SLC1A1_3 (5'-GAGGACTGTTCTAACTAGTAA-3'). The transfection protocol as follows: SH-SY5Y cells (90.000 cell/well) were differentiated with 10 μM RA in 12-well plates. After 7 days, SH-SY5Y were incubated 48 h with 2.3 ml of MEM/F-12 media containing 100 μl of MEM/F-12

(without FBS and antibiotics), 12 µl of HiPerfect Transfection Reagents and 80 nM of each siRNA oligonucleotide (each well). 30 h after transfection, SH-SY5Y were subjected to H/R protocol. Cells were then tested for vi-ability, ATP content, NCX1 activity or protein expression. The yield of RNA silencing was assessed by Western blot analysis by using specific antibodies.

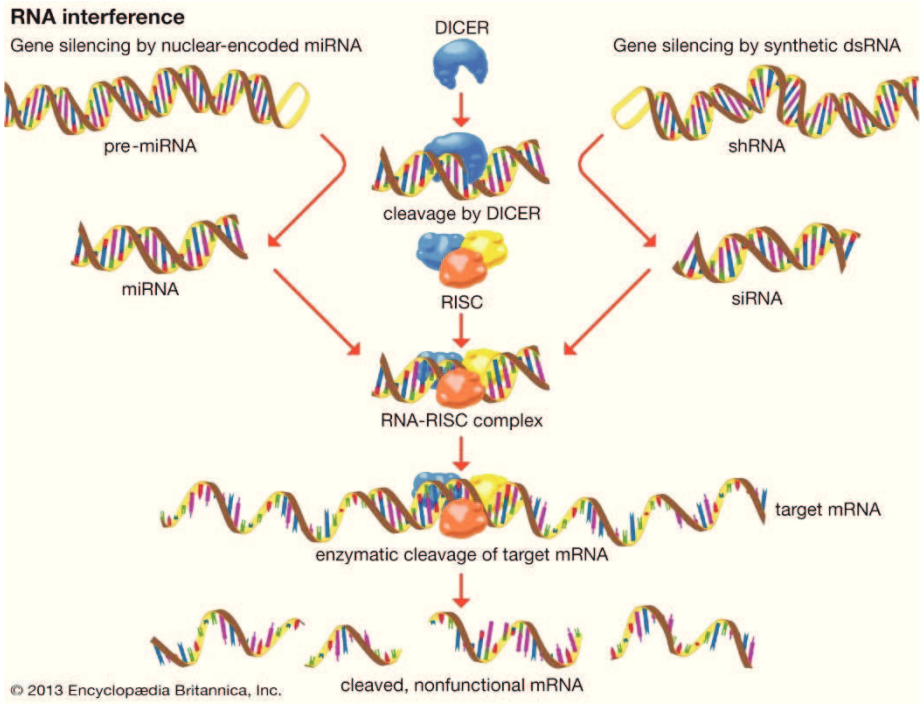

Fig. 2.1. RNA interference. The dsRNA is degraded by a highly conserved cellular RNAse named Dicer into small oligoribonucleotides with a length of about 22 base pairs, including 2-nucleotide long 3´ overhangs. This type of small dsRNA is called small interfering RNA or siRNA. In a second step, siRNA binds to a multimolecular protein complex consisting of several proteins named RISC (RNA Induced Silencing Complex). The double-stranded siRNA molecule is unwound resulting in a ribonucleoprotein particle consisting of the RISC proteins and one siRNA strand. If an mRNA with a sequence complementary to the siRNA moiety is encountered by this complex, the mRNA is cleaved by an RNAse named Slicer and thereby rendered inactive. If the complementarity is not perfect, RISC may only bind to the mRNA which also blocks translation inhibiting expression (Kara Rogers, Encyclopædia Britannica 2017).

2.3 In vitro hypoxia/reoxygenation (H/R) challenge

The cells were subjected to H/R as described earlier[80] with minor modifications. Briefly, an oxygen-glucose deprivation (OGD) buffer containing (in mM): 154 NaCl, 5.6 KCl, 5.0 HEPES, 3.6 $NaHCO_3$ and 2.3 $CaCl_2$ (pH 7.4) was bubbled with 95% N_2–5% CO_2 for 10 min at room temperature. Then,

the culture medium containing 4.5 g/l glucose was replaced with the OGD buffer and the culture plates were put into an airtight chamber gassed with 95% N_2–5% CO_2 for 10 min. After closing all sealable connectors, the chamber was transferred to an incubator and the cells were subjected to hypoxia at 37 °C, 5% CO_2, for 16 h (**Fig. 2.2**). Reoxygenation was initiated by opening the chamber and returning the cells to their normal culture conditions for 24 h before they were used to perform experiments. Preliminary time-course studies showed that a 16 h period of hypoxia followed by 24 h of reoxygenation was optimal to achieve a significant increase in cell death.

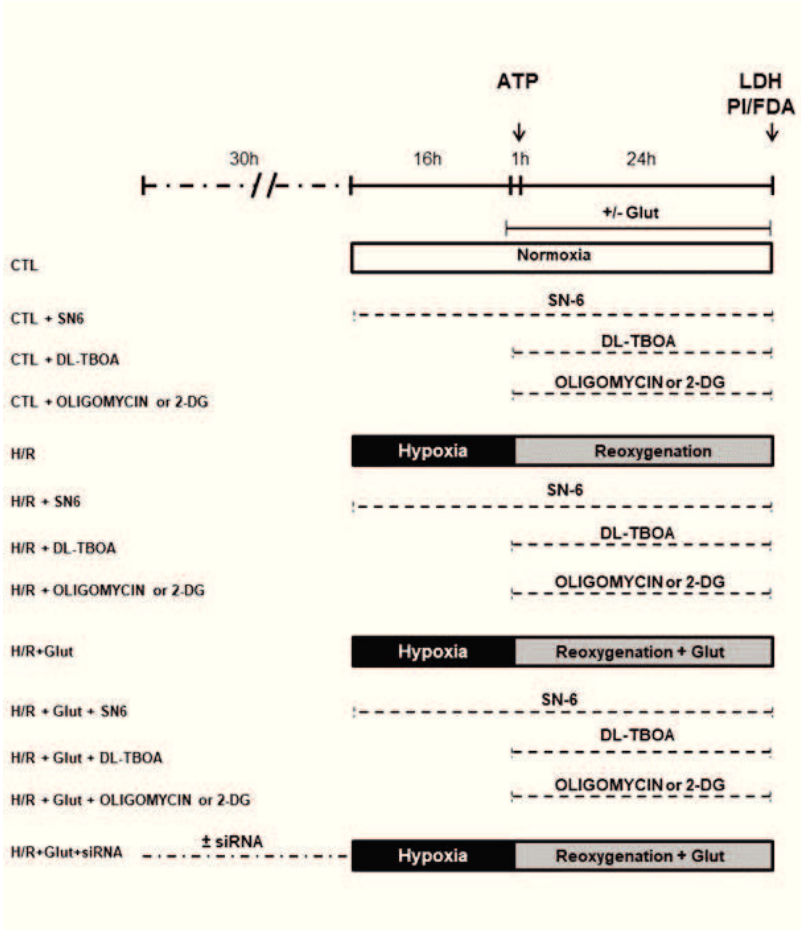

Fig. 2.2. Timeline of the experimental protocol (H/R) in RA-differentiated SH-SY5Y cells. Control groups were incubated under normoxic conditions at 37°C for the entire protocol. Glutamate (0.5 mM) and drugs (DL-TBOA, oligomycin and 2-DG) were administrated during the reoxygenation phase. SN-6 was administrated during the entire H/R protocol. Cell viability (assessed by extracellular LDH measurement) was evaluated at the end of the protocol; ATP levels were evaluated after the first hour of the reoxygenation phase.
CTL= control; H/R= hypoxia/reoxygenation; Glut= glutamate; 2-DG= 2-deoxyglucose.

2.4 Evaluation of cell viability

H/R- induced cell injury was evaluated by measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells in the experimental media[81,82] and by the method of double staining with fluorescein diacetate/propidium iodide (FDA/PI)[82,83]. Briefly, culture medium was collected at the end of the H/R challenge and centrifuged at 250xg for 10 min. Then, 100 µl of the supernatant were transferred to a 96-well plate and incubated with the same volume of reaction mixture (Diaphorase/NAD+ mixture premixed with iodotetrazolium chloride/sodium lactate-Roche Diagnostics, Monza, Italy-) at room temperature in dark environment for 30 min.

LDH activity was assessed by reading the absorbance of the sample medium at 490 nm in a Victor Multilabel Counter plate reader (Perkin Elmer, Waltham, MA, USA).

For FDA/PI staining, cells were differentiated on glass coverslips and subjected to H/R. Afterwards, cells were treated with 36 µM FDA (Sigma, Milan, Italy) and 7 µM PI (Calbiochem., San Diego, CA, U.S.A.) for 10 min at room temperature in PBS. Stained cells were examined immediately with an inverted Zeiss Axiovert 200 microscope (Carl Zeiss, Milan, Italy) and then analyzed. When FDA crosses the cell membrane it is hydrolyzed by intracellular esterases producing a green-yellow fluorescence. Cell damage curtails FDA staining and allows cell permeation by PI that, interacting with nuclear DNA, yields a bright red fluorescence.

2.5 ATP Assay

ATP synthesis was evaluated by using a commercially available luciferase-luciferin system (ATPlite, Perkin Elmer, Waltham, MA) according to the manufacturer's instructions[84]. Cells were differentiated in 96 multiwell plates at the density of 2.5 × 104 cells/well. After 7 days, differentiation medium was removed and cells were first washed twice with PBS, then exposed to different glutamate concentrations (0.5 and 1 mM) in Eagle's Minimum Essential Medium/Nutrient Mixture Ham's F-12 (1:1) without FBS, for 1h at 37°C. For H/R experiments, after being differentiated and subjected to16 h of hypoxia in OGD buffer, the reoxygenation phase was started

by returning the cells to their normal culture conditions. Glutamate and the specific pharmacological/molecular tools were added as described in the specific time schedule (**Fig. 2.2**). After 1 h of reoxygenation, intracellular ATP levels were analyzed with a luminescence counter (Victor Multilabel Counter, Perkin Elmer) and normalized to the respective protein content.

2.6 Antibodies

NCX1 protein was detected by using a commercially available mouse monoclonal IgG antibody[84] (R3F1, Swant, Bellinzona, Switzerland, dilution 1:500). EAAC1/EAAT3 proteinwas detected by using mouse anti-EAAC1/EAAT3 (Chemicon International, CA, USA, dilution 1:1000)[84]. β-actin[81] (1:10000; A5316, Sigma) was used as loading control.

2.7 Western blotting analysis

Differentiated SH-SY5Y cells were lysed using a protein lysis buffer containing (in mM): NaCl, 150; Tris-HCl (pH 7.4), 10; EDTA (pH 8.0), 1; SDS 1%, and a protease inhibitor cocktail mixture (Roche Diagnostics). Protein content was determined by the Bradford method (Bio-Rad, Milan, Italy), using bovine serum albumin as standard. Samples containing equal amounts of protein (30 μg) were boiled in 4X Laemmli sample buffer with 2–mercaptoethanol for 10 min. Proteins were subjected to 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then electro-transferred to polyvinylidine difluoride (PVDF) membranes (Immobilon Transfer Membranes, Millipore Co., Bedford, MA, USA). The membranes were blocked in PBS buffer containing 5% non-fat dry milk for 1 hour at room temperature and then incubated with the appropriate primary antibody overnight at 4°C. Immunoreactions were revealed by incubation with secondary antibody conjugated to horseradish peroxidase (Santa Cruz, CA, USA) (dilution 1:1000), for 1 h at room temperature. An enhanced chemiluminescence detection system (Super Signal West Femto kit, Thermo Scientific, Milano, Italy) was used to detect bound antibodies. Images were captured and stored on a ChemiDoc station (BioRad, Milan, Italy). NCX1 and EAAT3 band densities were analyzed with the Quantity One (Bio-Rad) analysis software and normalized to corresponding β-actin band densities.

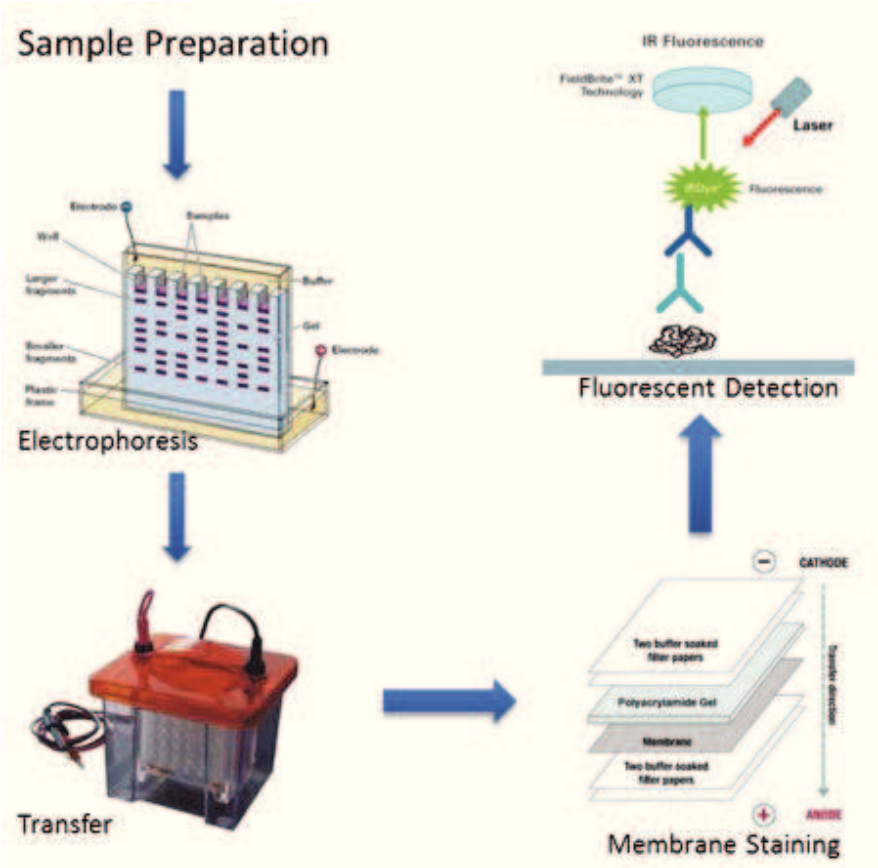

Fig. 2.3. Separation of proteins by SDS-PAGE. SDS. Equal amount of protein samples is loaded into a gel made of polyacrylamide and then an electric field is applied to the gel. The electric field acts as the driving force, drawing the SDS coated proteins towards the anode with larger proteins moving more slowly than small proteins. SDS coated proteins are thus separated.

2.8 Analysis of NCX activity

Solutions

Ca-PSS contained (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 10 glucose, 2 $CaCl_2$, and 20 HEPES, buffered to pH 7.4 with Tris. Na-PSS had an identical composition except that calcium was omitted and 0.1 mM EGTA was included. K-PSS contained (in mM): 140 KCl, 1 $MgCl_2$, 10 glucose, 0.1 $CaCl_2$, and 20 HEPES, buffered to pH 7.4 with Tris.

Experimental protocol

Intracellular Ca^{2+} levels were measured by single-cell computer-assisted videoimaging using LSM 510 confocal system (Carl Zeiss, Milan, Italy)[85]. After being differentiated into neuron-like cells on

25 mm coverslip, SH-SY5Y were subjected to H/R challenge (**Fig. 2.2**). Afterwards, cells were loaded with 4 μM Fluo-4/AM (Molecular Probe, Eugene, OR) in Ca-PSS 0.08% pluronic acid (Molecular Probe) for 40 min in the dark at room temperature. Coverslips were then washed once in Na-PSS and treated for 10 min with 1 μM thapsigargin in Na-PSS. After an additional wash, coverslips were placed into a perfusion chamber mounted onto the stage of an inverted Zeiss Axiovert 200 microscope. NCX activity was evaluated as Ca^{2+} uptake through the reverse mode by switching Na-PSS to K-PSS. Bath solutions were changed with a peristaltic pump and images were acquired every 5 s. Cells and perfusion solutions were maintained at 37°C by using a heated microscope stage and climate box form PeCon GmbH. Excitation light was provided by argon laser al 488 nm and the emission was time-lapsed recorded at 505-530 nm. Analysis of fluorescence intensity was performed off-line after images acquisition, by averaging the fluorescence intensity values within selected areas overlying the cell somata as previously described[81,86].

2.9 Drug and chemicals

SN-6 and DL-TBOA were obtained from Tocris. RNAi kit was purchased from Qiagen. All the other chemicals were of analytical grade and were purchased from Sigma.

2.10 Data analysis

Data were expressed as mean ± S.E.M. Values less than 0.05 were considered to be significant. When comparing two data sets, the Student's t test for unpaired data was used. To compare multiple groups, statistical comparisons were performed by one-way ANOVA followed by Dunnet's post hoc test. Statistical comparisons were carried out using the GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA).

3. RESULTS

3.1 Protective effect of glutamate on H/R-induced cell injury: involvement of NCX1

It was initially developed an *in vitro* model of neuronal H/R by using RA-differentiated SH-SY5Y cells. When cells were subjected to 16 h of hypoxia (H) followed by 24 h of reoxygenation (R) (**Fig. 3.1**), cell damage, as assessed by extracellular LDH levels[81] and FDA/PI double staining[83], was significantly higher compared to that of normoxic control (**Fig. 3.3 a, b**). In order to study whether glutamate attenuates H/R injury, RA-differentiated SH-SY5Y cells were treated with glutamate at the onset of the reoxygenation phase (**Fig. 3.1**). As shown in **Fig. 3 3**, glutamate supplementation significantly attenuated H/R damage. Noteworthy, glutamate at concentration used (0.5 mM) was devoid of detectable toxicity under normoxia (**Fig. 3.3**). To explore the specific contribution of NCX1 to the protection exerted by glutamate supplementation we first used a pharmacological approach. In particular, when cells were exposed to the selective NCX inhibitor 2-[[4-[(4Nitrophenyl) methoxy] phenyl] methyl]-4-thiazolidinecarboxylic acid ethyl ester (SN-6, 1 μM)[87], glutamate supplementation during the reoxygenation phase was wholly ineffective in protecting cells from H/R injury (**Fig. 3.3 a, b**). SN-6 *per se* had no effect on RA-differentiated SH-SY5Y cell viability under normoxia -or when introduced during our H/R protocol in the absence of glutamate (**Figs 3.1 and 3.3 a**)-. The concentration of 1 μM has been chosen because it has been demonstrated to be selective for NCX1 under condition of ATP depletion, as in case of ischemia. To specifically assess the role of NCX in glutamate-induced protection, we used a RNAi-mediated approach to silence either NCX1 or NCX3 expression (**Fig. 3.2 a, b**). Silencing of NCX2 isoform was not performed because under our culture conditions SH-SY5Y cells do not express NCX2 protein, neither when undifferentiated[86] nor after RA-treatment (data not shown). As shown in **Fig. 3.3 c**, silencing of NCX1 significantly prevented the glutamate protection during H/R injury. We also tested whether silencing of NCX3 could be able to abolish the effect of glutamate. The results showed that NCX3 did not interfere with glutamate protection (**Fig. 3.4**), supporting the hypothesis that a functional NCX1 is specifically required for glutamate protection.

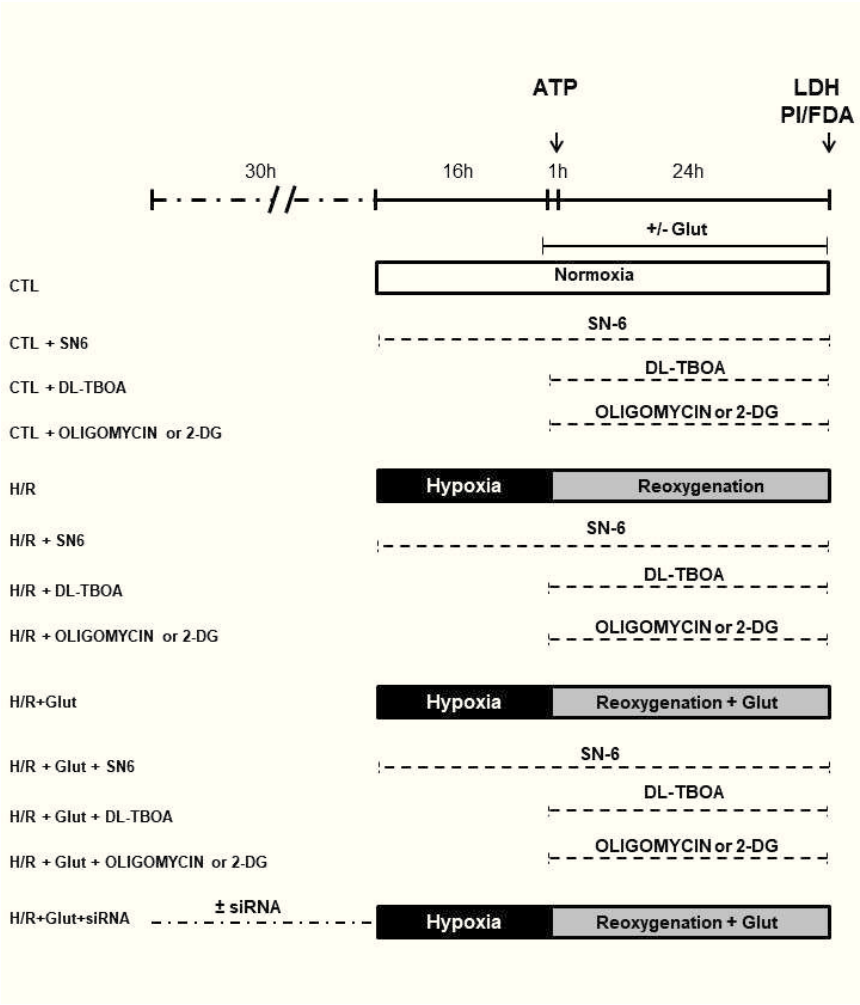

Figure 3.1. Timeline of the experimental protocol (H/R) in RA-differentiated SH-SY5Y cells.

Control groups were incubated under normoxic conditions at 37°C for the entire protocol. Glutamate (0.5 mM) and drugs (DL-TBOA, oligomycin and 2-DG) were administrated during the reoxygenation phase. SN-6 was administrated during the entire H/R protocol. Cell viability (assessed by extracellular LDH measurement) was evaluated at the end of the protocol; ATP levels were evaluated after the first hour of the reoxygenation phase.

CTL= control; H/R= hypoxia/reoxygenation; Glut= glutamate; 2-DG= 2-deoxyglucose.

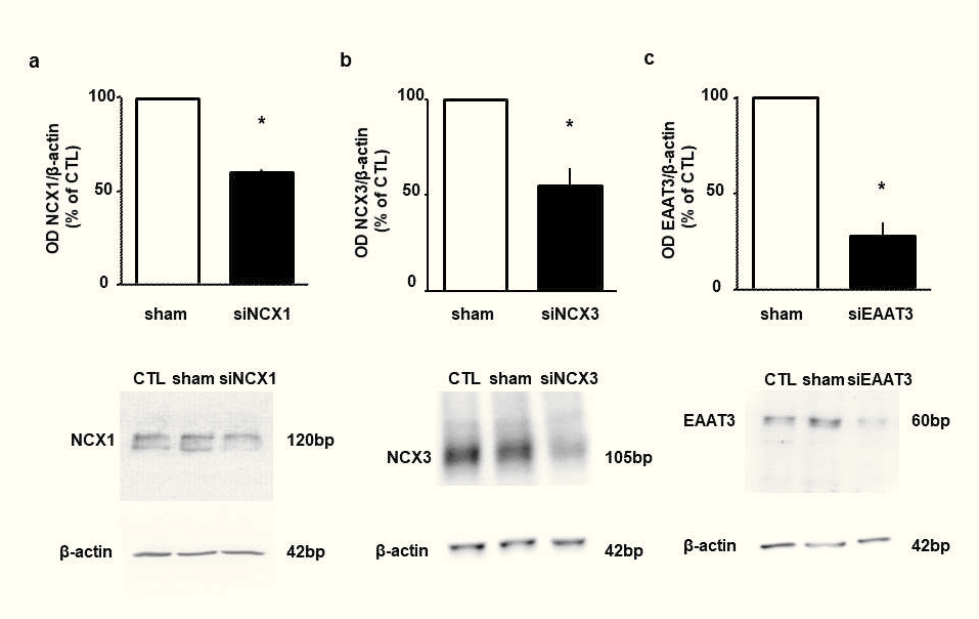

Figure 3.2. Silencing of NCX1 (a)**, NCX3** (b) **and EAAT3** (c) was performed by using HiPerfect Transfection® Kit (Qiagen) and FlexiTube siRNA as described in the "Materials and Methods" section.

CTL=control; sham= siRNA negative control; siNCX1= siRNA for NCX1; siNCX3= siRNA for NCX3; siEAAT3= siRNA for EAAT3.

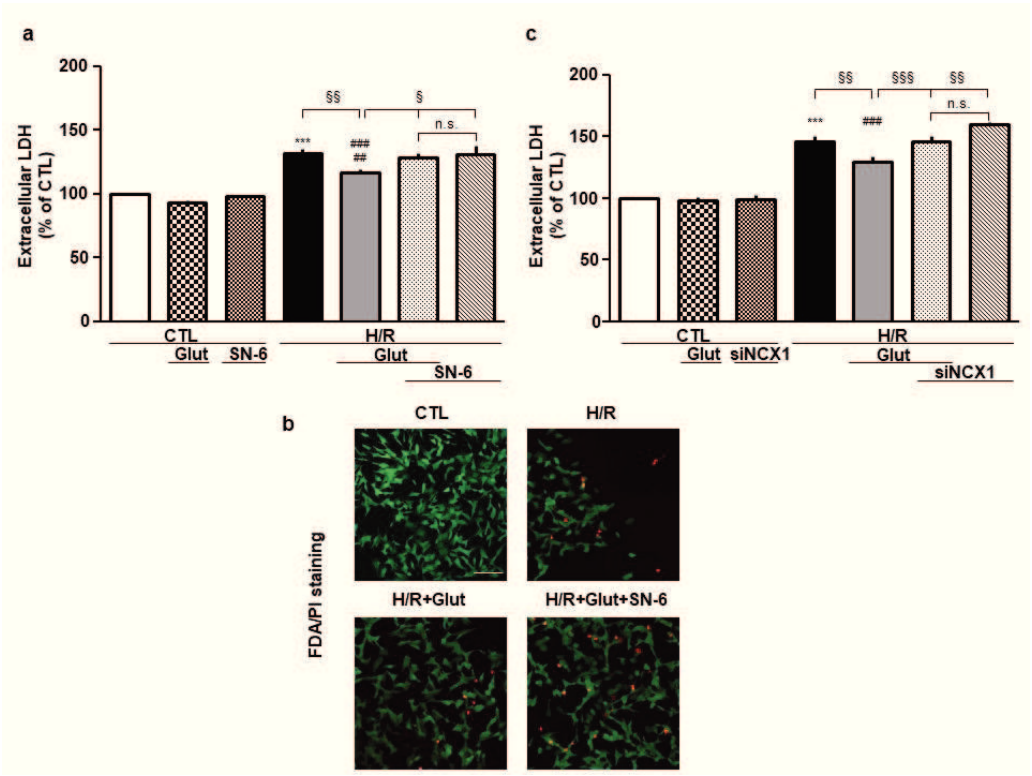

Figure 3.3. Glutamate-induced protection against H/R injury is prevented by NCX1 inhibition.

Extracellular LDH activity measured at the end of the H/R protocol (16 h of hypoxia followed by 24 h of reoxygenation) under different experimental conditions. In each experiment, LDH release was expressed as percentage of the control.

(a) 0.5 mM glutamate was added during the reoxygenation phase, alone or in combination with 1 μM SN-6, according to the time schedule showed in Fig. 1. Each column represents the mean ± S.E.M. of almost 5 independent experiments performed in triplicate. Where unseen, error bars overlap with the histogram outline. Differences among means were assessed by one-way ANOVA followed by Dunnet's post hoc test. F (6, 70) = 29.57. ***p< 0.0001 vs control groups; ###p< 0.0001 vs CTL, CTL + Glut; ##p< 0.001 vs CTL + SN-6; §§p< 0.001, §p< 0.01 vs the indicated groups. The H/R group was not significantly different from the H/R + SN-6 + Glut and H/R+SN-6 groups.

(b) Analysis of cell survival by FDA/PI staining. Images are representative of 3 independent experiments. Scale bar 50 μm.

(c) Silencing of NCX1 was performed 30 h before H/R challenge. Glutamate was added at the onset of the reoxygenation phase. Each column represents the mean ± S.E.M. of almost 5 independent experiments performed in triplicate. Differences among means were assessed by one-way ANOVA followed by Dunnet's post hoc test. F (6, 29) = 44.76. ***p< 0.0001 vs control groups; §§§p< 0.0001, §§p< 0.001 vs the indicated groups. The H/R group was not significantly different from the H/R + siNCX1 + Glut and H/R+ siNCX1 groups.

There was no statistically significant difference between the control groups.

CTL= control; H/R= hypoxia/reoxygenation; Glut= glutamate; siNCX1= siRNA for NCX1; n.s.= not significant.

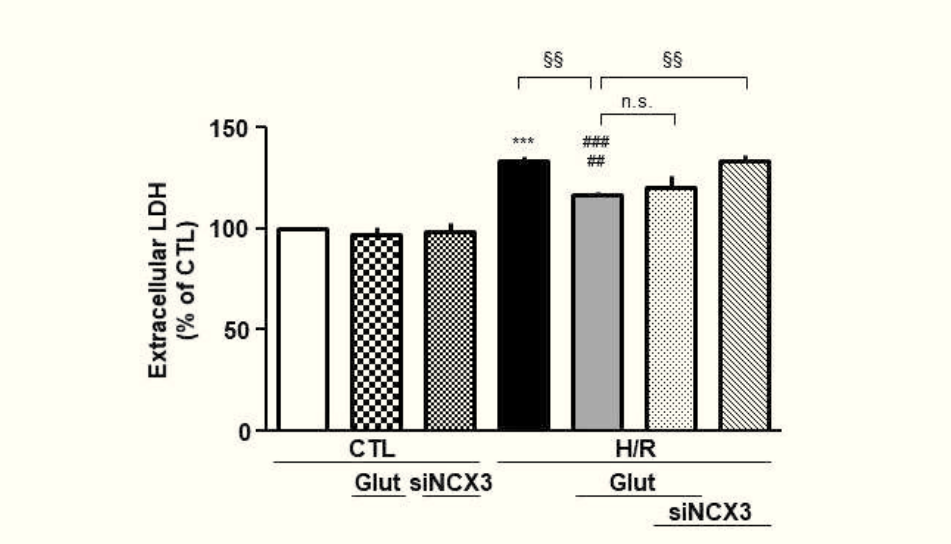

Figure 3.4. Effect of NCX3 silencing on glutamate-induced protection against H/R injury. Extracellular LDH activity measured at the end of the H/R protocol (16 h of hypoxia followed by 24 h of reoxygenation) under different experimental conditions. In each experiment, LDH release was expressed as percentage of the control. Silencing of NCX3 was performed 30 h before H/R challenge. Glutamate was added at the onset of the reoxygenation phase. Each column represents the mean ± S.E.M. of almost 5 independent experiments performed in triplicate. Differences among means were assessed by one-way ANOVA followed by Dunnet's post hoc test. F (6, 32) = 23.45. ***p<0.001 vs control groups; ###p<0.0001 vs CTL+ Glut; ##p<0.001 vs CTL and CTL+siNCX3; §§p<0.001vs the indicated groups. The H/R group was not significantly different from the HR+siNCX3 group.

There was no statistically significant difference between the control groups.

CTL = control; H/R = hypoxia/reoxygenation; Glut = glutamate; siNCX3 = siRNA for NCX3; n.s. = not significant.

3.2 Protective effect of glutamate on H/R-induced cell injury: involvement of EAAT3

It is well known that EAATs mediate the high-affinity uptake of glutamate. We demonstrated that, under both physiological and pathological conditions[84], glutamate entry into cells through EAAT3 is functionally related to NCX1 activity[70,71]. Therefore, once established that a functional NCX1 was required under hypoxic conditions to stimulate a response able to mitigate cell injury, it has been also explored whether EAAT3 activity could also be mandatory required. At first, cells were exposed to H/R protocol in the presence of the non-transportable EAATs blocker DL-threo-β-Benzyloxyaspartic acid (DL-TBOA)[88] at the concentration of 300 μM[84]. In particular, it has been shown that DL-TBOA inhibits ischemia-evoked glutamate release, which has been ascribed to EAATs reverse-mode of action[89]. Since this effect could play a protective role during ischemia[90], DL-TBOA was administered at the onset of the reoxygenation phase (**Fig. 3.1**). Under these experimental conditions, glutamate exposure failed to attenuate H/R-induced cell damage (**Fig. 3.5 a**). DL-TBOA *per se* did not affect cell viability neither under normoxic conditions nor when administered during our H/R protocol (**Figs 3.1 and 3.5 a, b**). Afterwards, in order to specifically assess EAAT3 contribution to the protection exerted by glutamate via NCX1, RNAi-mediated approach to silence EAAT3 was performed (**Fig. 3.2 c**). Silencing of EAAT3 significantly counteracted the beneficial effect of glutamate, supporting that EAAT3 is determinant in the observed glutamate-induced protective response (**Fig. 3.5 c**).

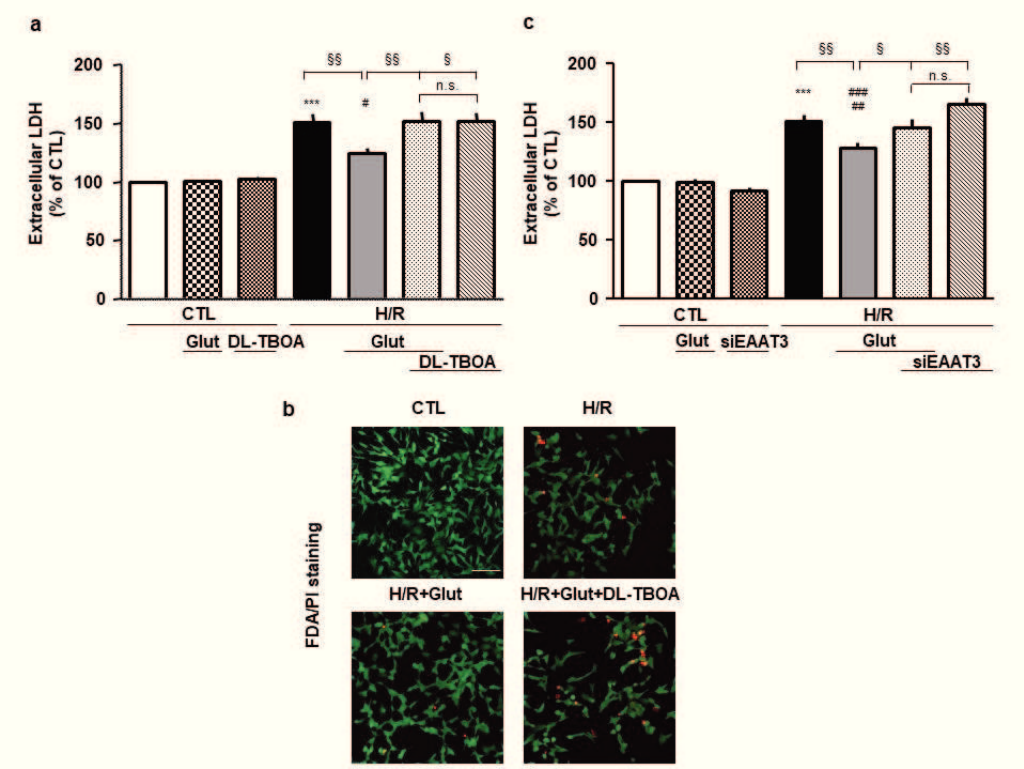

Figure 3.5. Glutamate-induced protection against H/R injury is prevented by EAAT3 inhibition.

Extracellular LDH activity measured at the end of the H/R protocol (16 h of hypoxia followed by 24 h of reoxygenation) under different experimental conditions. In each experiment, LDH release was expressed as percentage of the control.

(a) 0.5 mM glutamate was added during the reoxygenation phase, alone or in combination with 300 μM DL-TBOA, according to the time schedule showed in Fig. 1. Each column represents the mean ± S.E.M. of almost 8 independent experiments performed in triplicate. Where unseen, error bars overlap with the histogram outline. Differences among means were assessed by one-way ANOVA followed by Dunnet's post hoc test. $F(6, 42) = 18.38$. ***$p < 0.0001$ vs control groups; #$p < 0.01$ vs CTL and CTL+ Glut; §§$p < 0.001$, §$p < 0.01$ vs the indicated groups. The H/R group was not significantly different from the H/R+ DL-TBOA+ Glut and H/R+ DL-TBOA groups.

(b) Analysis of RA-differentiated SH-SY5Y cell survival by FDA/PI staining. Images are representative of 3 independent experiments. Scale bar 50 μm.

(c) Silencing of EAAT3 was performed 30 h before the H/R challenge. Glutamate was added at the onset of the reoxygenation phase. Each column represents the mean ± S.E.M. of almost 6 independent experiments performed in triplicate. Differences among means were assessed by one-way ANOVA followed by Dunnet's post hoc test. $F(6, 53) = 25.33$. ***$p < 0.0001$ vs control groups; ###$p < 0.0001$ vs CTL and CTL + siEAAT3; ##$p < 0.001$ vs CTL + Glut; §§$p < 0.001$, §$p < 0.01$ vs the indicated groups. The H/R group was not significantly different from the H/R + siEAAT3 + Glut and H/R + siEAAT3 groups.

There was no statistically significant difference between the control groups.

CTL= control; H/R= hypoxia/reoxygenation; Glut= glutamate; siEAAT3= siRNA for EAAT3; n.s.= not significant.

3.3 Glutamate supplementation and ATP production: role of NCX and EAAT

Under both physiological and pathological conditions glutamate can contribute to cellular energy balance as substrate for anaplerotic reactions, and it was demonstrated that NCX1 provides functional support for both glutamate uptake and ATP synthesis[84]. Therefore, it has been investigated whether the protective effect of glutamate could be ascribed to its potential to sustain ATP synthesis. In an initial set of experiments, RA-differentiated SH-SY5Y were exposed to different concentrations of glutamate (0.1-1 mM) under normoxic conditions for 1 h. The results showed that glutamate concentration of 0.5 and 1 mM induced a significant rise in intracellular ATP levels, in line with our previous findings obtained in undifferentiated SH-SY5Y cells (**Fig. 3.6 a**)[71]. Thus, for the subsequent sets of experiments the lowest concentration of glutamate (0.5 mM) able to significantly increase ATP levels was chosen. It has been further explored glutamate ability to sustain ATP synthesis during the reoxygenation phase, and verified whether the NCX1/EAAT functional coupling could be involved. As expected, during H/R challenge intracellular ATP levels sharply dropped compared to that of the control (**Fig. 3.6 b, c**). When glutamate was given at the onset of the reoxygenation phase, it evoked a partial recovery of intracellular ATP content. Interestingly, SN-6 counteracted this response (**Fig. 3.6 b**) and this finding was confirmed by NCX1 silencing (**Fig. 3.6 c**), suggesting that the exchanger played a crucial role during metabolic recovery. Considering that the interaction between NCX1 and EAAT3 was necessary to partially prevent cell death induced by H/R, we analyzed whether exposure to DL-TBOA could interfere with glutamate-induced ATP synthesis. Likewise, both DL-TBOA and EAAT3 silencing prevented recovery of ATP levels evoked by glutamate (**Fig. 3.7 a, b**). Both SN-6 and DL-TBOA did not affect ATP levels neither under normoxia[71] nor after H/R protocol (**Figs 3.6 b and 3.7 a**).

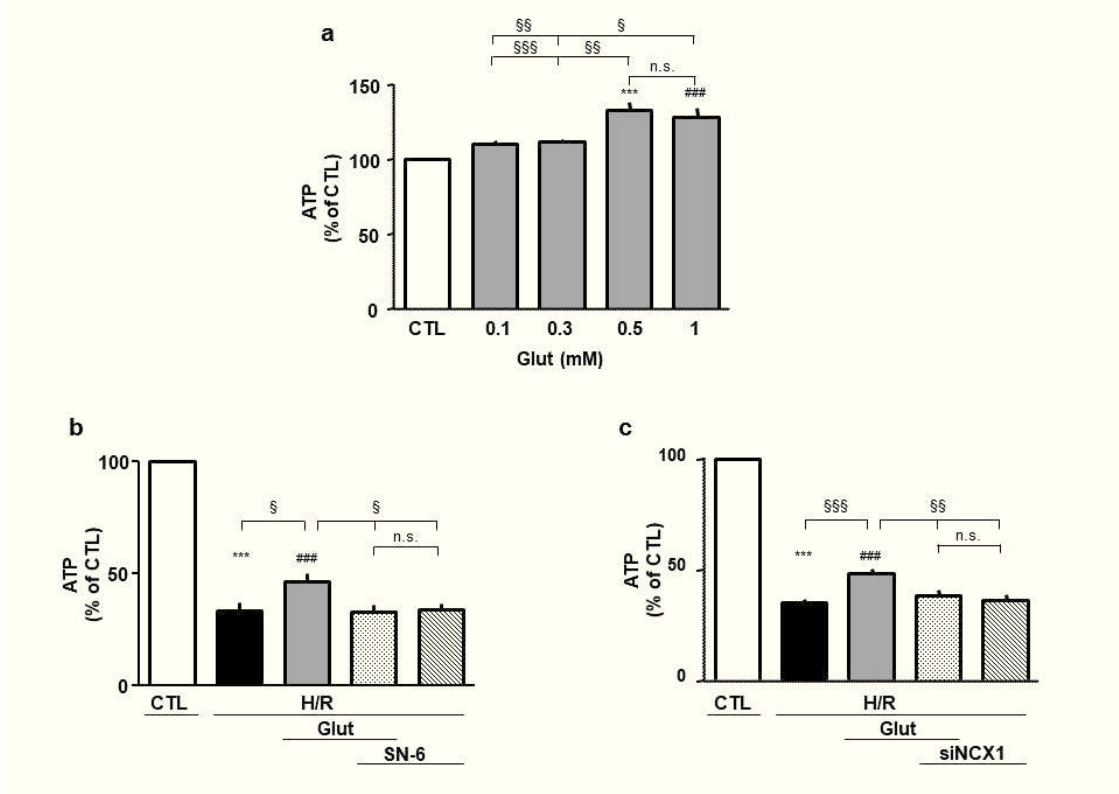

Figure 3.6. Glutamate-induced recovery of ATP synthesis during H/R challenge is prevented by NCX1 inhibition.

(a) Intracellular ATP levels evaluated under normoxic conditions in cells exposed to glutamate (0.1- and 1 mM) for 1 h. ATP levels were normalized to the respective sample protein content and expressed as percentage of the control. Each column represents the mean ± S.E.M. of 6 independent experiments performed in triplicate. Differences among means were assessed by one-way ANOVA followed by Dunnet's post hoc test. F (4, 25) = 11.38. ***p< 0.0001 vs control group; §§p< 0.001, §p< 0.01 vs the indicated groups. Both 0.1 mM and 0.3 mM glutamate concentrations were not significantly different from the control group.

(b) Intracellular ATP levels evaluated under different experimental conditions. 0.5 mM glutamate was added at the onset of the reoxygenation phase, alone or in combination with 1 μM SN-6, according to the time schedule showed in Fig. 1. ATP was monitored after 1 h. ATP levels were normalized to the respective sample protein content and expressed as percentage of the control. Each column represents the mean ± S.E.M. of almost 5 independent experiments performed in triplicate. Differences among means were assessed by one-way ANOVA followed by Dunnet's post hoc test. F (5, 48) = 97.62. ***p< 0.0001 vs control group; §p< 0.01 vs the indicated groups. The H/R group was not significantly different from the H/R + SN-6 + Glut and H/R + SN-6 groups.

(c) Silencing of NCX1 was performed 30 h before H/R challenge. Glutamate was added for the first hour of the reoxygenation phase. ATP levels were normalized to the respective sample protein content and expressed as percentage of the control. Each column represents the mean ± S.E.M. of almost 4 independent experiments performed in triplicate. Differences among means were assessed by one-way ANOVA followed by Dunnet's post hoc test. F (5, 28) = 189.5.

***p< 0.0001 vs control group; §§§p< 0.0001, §§p< 0.001 vs the indicated groups. The H/R group was not significantly different from the H/R + siNCX1 + Glut and H/R + siNCX1 groups.

H/R= hypoxia/reoxygenation; Glut= glutamate; siNCX1= siRNA NCX1; n.s.= not significant.

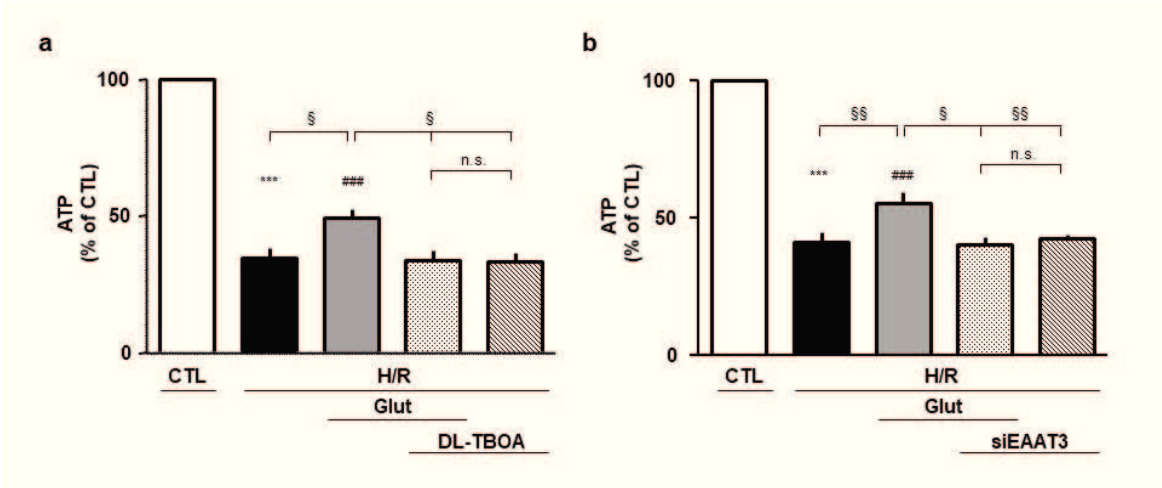

Figure 3.7. Glutamate-induced recovery of ATP synthesis during H/R challenge is prevented by EAAT3 inhibition. Intracellular ATP levels evaluated under different experimental conditions. 0.5 mM glutamate was added at the onset of the reoxygenation phase, alone or in combination with 300 μM DL-TBOA, according to the time schedule showed in Fig. 1. ATP was monitored after 1 h. ATP levels were normalized to the respective sample protein content and expressed as percentage of the control. Each column represents the mean ± S.E.M. of almost 5 independent experiments performed in triplicate. Differences among means were assessed by one-way ANOVA followed by Dunnet's post hoc test. F (5, 23) = 65.12. ***p< 0.0001 vs control group; §p< 0.01 vs the indicated groups. The H/R group was not significantly different from the H/R + DL-TBOA + Glut and H/R +DL-TBOA groups. (b) Silencing of EAAT3 was performed 30 h before H/R challenge. Glutamate was added during the first hour of the reoxygenation phase. ATP levels were normalized to the respective sample protein content and expressed as percentage of the control. Each column represents the mean ± S.E.M. of almost 4 independent experiments performed in triplicate. Differences among means were assessed by one-way ANOVA followed by Dunnet's post hoc test. F (5, 18) = 84.31. ***p< 0.0001 vs control group; §§p< 0.001, §p< 0.01 vs the indicated groups. The H/R group was not significantly different from the H/R + siEAAT3 + Glut and H/R + siEAAT3 groups.

H/R= hypoxia/reoxygenation; Glut= glutamate; siEAAT3= siRNA EAAT3; n.s.= not significant.

3.4 Glutamate effect on cellular bioenergetics following H/R challenge

An increase in ATP levels may occur within the cells by two main bioenergetics pathways: the oxidative phosphorylation and the glycolysis. Therefore, a question has been raised as to whether the

protection induced by glutamate during H/R was related to an improvement of oxidative metabolism or to an enhancement of the glycolytic pathway. It was performed a set of experiments using the ATP synthase inhibitor oligomycin (3 μg/ml) or the inhibitor of glycolytic ATP production 2-deoxyglucose (2-DG, 2 mM)[83]. Data obtained showed that oligomycin counteracted the increase in ATP levels induced by glutamate (**Fig. 3.8 a**), while 2-DG was unable to prevent this effect (**Fig. 3.8 b**). These results supported the hypothesis that in this experimental condition, the mitochondrial oxidative phosphorylation may be the route to produce ATP in the presence of glutamate. Both oligomycin and 2-DG did not affect ATP levels neither under normoxia (data not shown) nor after H/R protocol (**Fig. 3.8**).

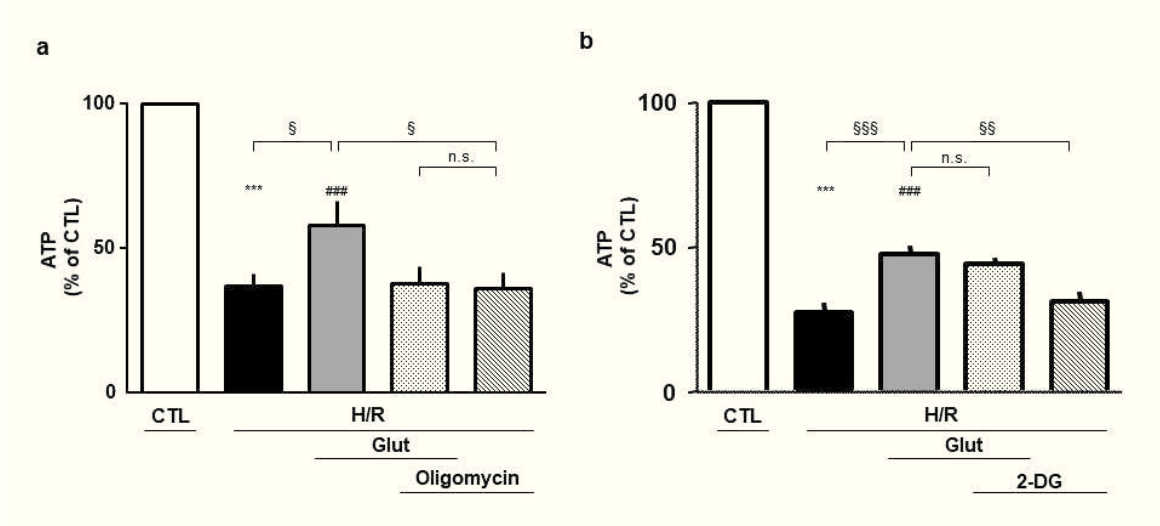

Figure 3.8. Effect of oligomycin and 2-DG on glutamate-induced ATP synthesis in cells subjected to H/R challenge.

(a) Intracellular ATP content evaluated after H/R in the presence of 0.5 mM glutamate, alone or in combination with 3 μg/ml oligomycin, added at the beginning of the reoxygenation phase and maintained for 1 h. ATP levels were normalized to the respective sample protein content and expressed as percentage of the control. Each column represents the mean ± S.E.M. of 5 independent experiments performed in triplicate. Differences among means were assessed by one-way ANOVA followed by Dunnet's post hoc test. F (5, 42) = 27.40. ***p< 0.0001 vs control group; §p< 0.01 vs the indicated groups. The H/R group was not significantly different from the H/R + oligomycin + Glut and H/R + oligomycin groups.

(b) Intracellular ATP content evaluated after H/R in the presence of 0.5 mM glutamate, alone or in combination with 2 mM 2-DG, added at the beginning of the reoxygenation phase and maintained for 1 h. ATP levels were normalized to the respective sample protein content and expressed as percentage of the control. Each column represents the mean ± S.E.M. of 7 independent experiments performed in triplicate. Differences among means were assessed by one-way ANOVA followed

by Dunnet's post hoc test. F (5, 35) = 99.91. ***p< 0.0001 vs control group; §§§p< 0.0001, §§p< 0.001 vs the indicated groups. The H/R group was not significantly different from the H/R + 2-DG group.

H/R= hypoxia/reoxygenation; Glut= glutamate; 2-DG= 2-deoxyglucose; n.s.= not significant.

3.5 Analysis of NCX and EAATs expression following H/R challenge

Once verified that under H/R conditions NCX1 and EAAT3 are critical to support the protective action of glutamate, it was investigated whether the expression of these proteins was modified by H/R challenge. Interestingly, the results showed that NCX1 expression was unmodified, whereas EAAT3 levels were significantly increased (approximately 40% compared to that of the control) (**Fig. 3.9 a, b**).

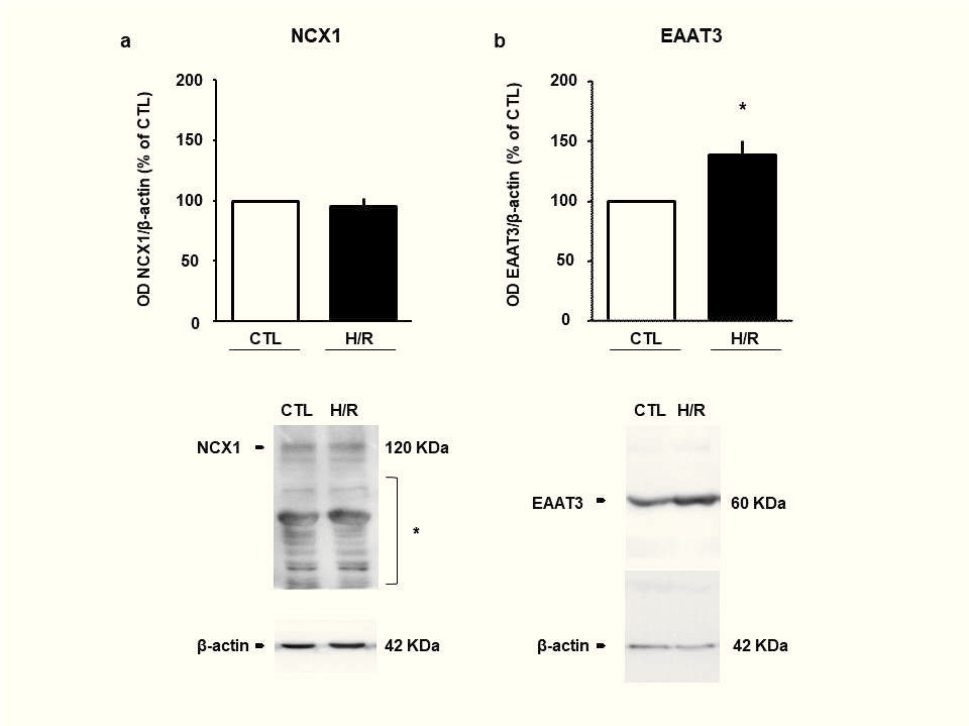

Figure 3.9. NCX1 and EAAT3 expression after H/R challenge.

Quantitative densitometry showing (a) the expression of NCX1 and (b) the Na^+-dependent glutamate transporters EAAT3 in cells exposed to H/R. β-actin was used as loading control. Normalized optical density values are expressed as percentage of the respective control. Each column represents the mean ± S.E.M. of 3 independent experiments. Differences among means were assessed by Student's t-test. (b) *p< 0.05 vs CTL.

Representative western blot images are shown below.

CTL= control; H/R= hypoxia/reoxygenation; asterisk indicates cross reactive bands.

3.6 Analysis of NCX activity alteration following H/R injury

Considering that under ischemic conditions NCX activity can be altered, as a consequence of energy metabolism failure[34], these sets of experiments were performed in order to investigate whether NCX activity could be affected by H/R experimental conditions (**Fig. 3.1**). Exchanger activity was evaluated as Na^+ gradient-dependent Ca^{2+} uptake in Fluo-4 loaded cells, by monitoring fluorescence signals. In particular, it has been analyzed reverse mode activity triggered by a stepwise reduction of extracellular Na^+ (substituted with K^+) at the end of the experimental protocol (see "Materials and Methods" and **Fig. 3.1** for further details). As showed in **Fig. 3.10**, when NCX reverse mode was activated by Na^+ free perfusion, a decrease in fluorescence signal (approximately 50%) occurred in H/R cells compared to the controls. Notably, glutamate exposure during the entire phase of reoxygenation elicited a significant increase in fluorescence signal compared to H/R group. This effect was lost when cells were pre-treated with siNCX1 or siEAAT3.

siRNA treatment did not affect per se Ca^{2+} responses, neither under normoxia nor under H/R conditions (**Fig. 3.10 b, c**). Likewise, fluorescence levels were not altered by glutamate supplementation under normoxic conditions (**Fig. 3.10 d**).

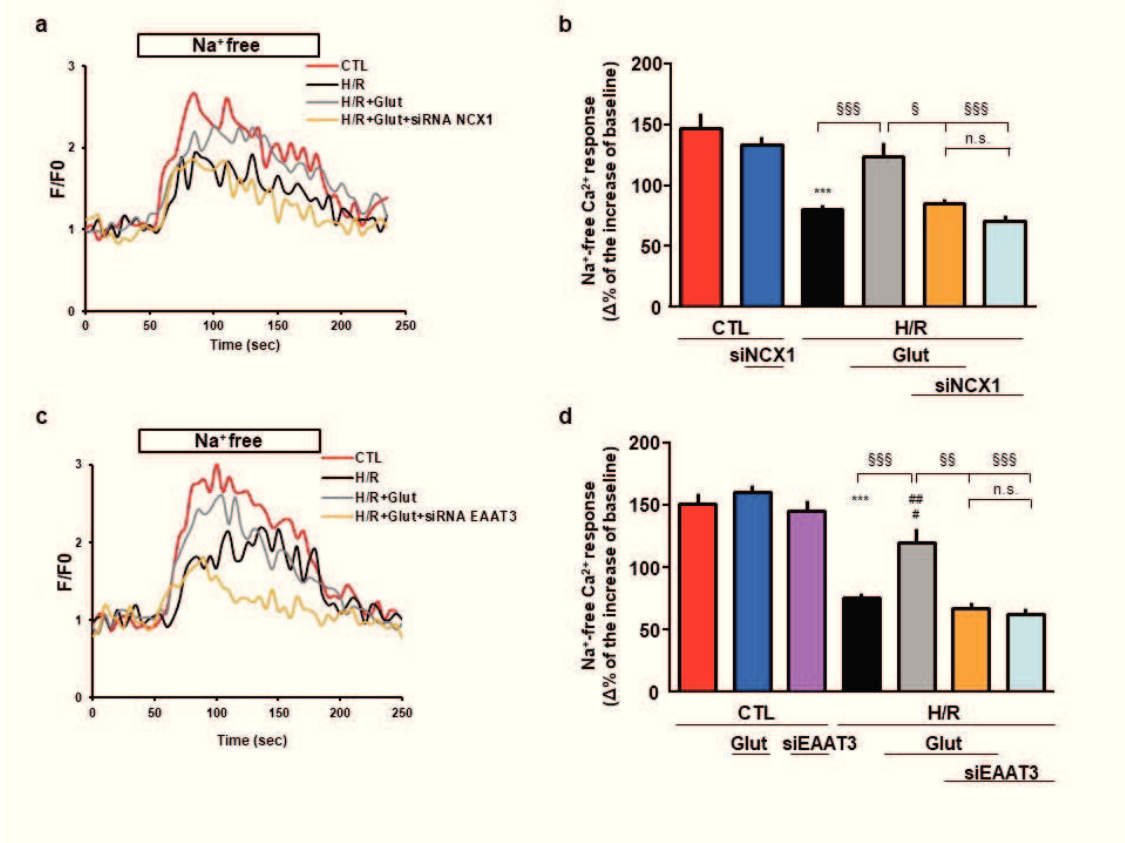

Figure 3.10. Effect of glutamate exposure on NCX activity after H/R challenge.

(a, c) Single trace recordings of Ca^{2+} response to Na^+ free after incubation under normoxic conditions (red line), H/R insult (black line) and H/R protocol with Glut supplementation (0.5 mM) during the reoxygenation phase alone (grey line) or in combination with siRNA (siNCX1 and siEAAT3, orange line). Fluorescence intensity values were normalized to resting fluorescence (F/F0).

(b, d) Measurements were performed at the end of the experimental protocol as described in Fig. 1. NCX1 activity was expressed as Δ% fluorescence increase. The bar plot reports the mean ± S.E.M. of fluorescence increase elicited by Na^+ free pulse. For each experimental group, Δ% values used for the statistical analysis derived from 4 independent experiments and 100-200 cells were recorded in each different session. Differences among means were assessed by one-way ANOVA followed by Dunnet's post hoc test. (b) F (5, 599) = 18.09. ***p< 0.0001 vs control groups; §§§p< 0.0001, §p< 0.01 vs the indicated groups. The H/R group was not significantly different from the H/R + siNCX1 + Glut and H/R + siNCX1 groups. The H/R + Glut group was not significantly different from control groups.

(d) F (6, 830) = 32.10. ***p< 0.0001 vs control groups; ##p< 0.001 vs CTL + Glut; #p< 0.01 vs CTL; §§§p< 0.0001, §§p< 0.001 vs the indicated groups. The H/R group was not significantly different from the H/R + siEAAT3 + Glut and H/R + siEAAT3 groups. The H/R + Glut group was not significantly different from CTL + siEAAT3.

CTL= control; H/R= hypoxia/reoxygenation; Glut= glutamate; siNCX1= siRNA NCX1; siEAAT3= siRNA EAAT3; n.s.= not significant.

4. DISCUSSION

4.1 Rationale

Stroke remains one of the most important cause of disability and death worldwide. Despite all the advances in the acute treatment of ischemic strokes, the proportion of patients who qualify for such medical and endovascular interventions is small and the outcomes are not good even for many who undergo acute therapies[91]. The mechanisms of ischemic stroke are variable, complex and multifactorial in nature[14]. Several studies have made great effort to better understand the underlying mechanisms of ischemia-induced tissue damage for almost two centuries, with the hope for developing therapies to limit the devastating health and economic outcomes characterized by reductions in organ-specific blood flow[7]. Consequently, stroke prevention remains one of the most important targets in the fields of neurology, cardiology, vascular medicine, and geriatrics medicine[91]. Evidences supporting the concept that reperfusion could paradoxically induce and exacerbate tissue injury and necrosis induced by ischemia provided a major impetus for research because this component of tissue injury is amenable to therapeutic intervention. Despite years of intensive investigation, we are still far away from thoroughly understanding the underlying mechanisms of I/R[7]. It has been reported that interventions in the area of metabolic substrate enhancement improve post-ischemic recovery reducing consistently the extent of the tissue injury. In particular, under I/R conditions the ability of neurons and glial cells to obtain energy from substrates other than glucose contributes importantly to cellular survival. The amino acids glutamate, GABA, and glutamine are available as substrates in the extracellular fluid and may be used as alternative fuels by astrocytes or neurons under glucose deprivation[56]. Although glutamate is the major excitatory neurotransmitter, which is involved in brain ischemia excitotoxicity, it plays a crucial role in brain energy metabolism, which is of fundamental importance to neuronal functions and survival[62,71,92]. It can fuel respiration and improve the anaplerotic refilling of TCA cycle intermediates[75], thus inducing ATP synthesis[62]. In line with these findings, it has been previously demonstrated that under both normoxia and cardiac and neuronal H/R conditions, glutamate can sustain cell metabolism increasing ATP levels[70,71,84]. In

particular, glutamate, at the concentration used 500 μM[70,71], has no evident toxic effect on RA-differentiated SH-SY5Y cells.

4.2 Protective effect of glutamate on H/R-induced cell injury: involvement of NCX1

Taken together, these data showed for the first time that glutamate -given at the onset of the reoxygenation phase- can positively affected cell viability in an *in vitro* model of RA-differentiated SH-SY5Y human neuroblastoma cells subjected to H/R injury. The protective action of glutamate likely depends on its ability to sustain oxidative metabolism by stimulating ATP synthesis. Notably, this protective effect strictly relies on NCX1 activity. Further evidences that NCX1 activity is essential for glutamate protection was obtained by using pharmacological blockade of NCX1 with SN-6 and RNAi approach with siRNA against NCX1. Its inhibition during the entire H/R protocol prevented the beneficial effects of glutamate in terms of cell protection (**Fig. 3.3**), partial recovery of ATP production through the stimulation of oxidative phosphorylation (**Fig. 3.6**) and normalization of NCX reverse mode activity (**Fig. 3.10 a, b**).During brain ischemia a marked elevation in extracellular glutamate occurs, as a consequence of the overstimulation of glutamate receptors, especially NMDA receptors (NMDARs)[92]. However, all the clinical trials of NMDAR antagonists for stroke and traumatic brain injury surprisingly showed a lack of efficacy of these compounds[73]. These results suggest that glutamate may be involved in the early neurodestructive phase after ischemic injury[73], but when the physiological conditions are restored, glutamate may cease its detrimental role and participate in cell recovery through its known physiological functions. In line with these findings, Dhawan et al showed that delayed stimulation of NMDAR, after focal ischemia, ameliorates long-terminal functional outcomes improving significantly somatosensitory and cognitive activities. They hypothesized that the mechanisms underlying this effect are related to the stimulation of neuron plasticity and reorganization rather than prevention of early neuronal death[93]. It has been known that metabotropic glutamate receptors (mGluR1 and mGluR5) are involved in ischemia-induced neurotoxicity[94,95]. Interestingly, several studies have reported that activation of

mGluR1 in neurons can also protect against a broad range of diseases in the CNS including brain ischemia[96-99]. In particular, protection exerted by mGluR1 occurs mainly at the level of free radical generation induced by oxidative stress and by activation of the Pi3kinase/Akt pathway[97,100-102].

Although the role of the NCX isoforms has been widely studied by using different I/R neuronal models, it still remains controversial and incompletely elucidated. On the one hand, in a model of permanent focal cerebral ischemia, the knocking down of NCX1 and NCX3 causes a remarkable increase in the ischemic lesions, suggesting the importance of the NCX forward mode of operation in extruding Ca^{2+} in the ischemic brain[42]. On the other hand, the inhibition of NCX1 reverse-mode activity, by SEA0400 administration during reperfusion, induced a significantly reduction of the infarct volume in the cortex after 24 h of reperfusion that followed 2 h of ischemia[44]. These findings suggest that NCX activity depends on the specific pathological context within which the exchanger works, therefore, tight regulation of its activity is crucial for brain activity. Here, it has not been observed a significant role of NCX during the entire H/R protocol. Interestingly, NCX became crucial for the functional support of glutamate -induced protection. In particular, when cells were subjected to H/R challenge the viability was dramatically affected and glutamate supplementation induces a partial but significant cell recovery. Since H/R causes a dramatic fall in the intracellular ATP content, as a consequence of the energy imbalance[103], it has been investigated whether glutamate could fuel oxidative metabolism and stimulate ATP synthesis, thereby improving cell viability. As shown in **Figs. 3.6 b, c, 3.7**, when cells were subjected to H/R protocol, ATP levels dramatically dropped. Exposure to glutamate for the first hour of the reoxygenation caused a significant rise (50%) in the intracellular ATP content, compared to H/R, suggesting that the positive effect exerted by glutamate may rely on its ability to support cell metabolism. Additionally, pharmacological inhibition with SN-6 abolished glutamate effects. RNAi approach demonstrated that NCX1 was specifically involved in such glutamate-induced protection.

4.3 Protective effect of glutamate on H/R-induced cell injury: involvement of EAAT3

Glutamate can get access to the mitochondrial matrix through aspartate/glutamate carriers, a component of malate/aspartate shuttle. Recently, it has been proposed an alternative and innovative pathway, whereby EAAT3 and NCX1 cooperate in order to favor glutamate entry into the cytoplasm and then into mitochondria[70,71,84]. Here, it has been demonstrated the involvement of EAATs in such metabolic response and that its inhibition with DL-TBOA blocked the beneficial effects of glutamate, likely by hampering glutamate entry. Specifically, RNAi approach showed that EAAT3 is the isoform involved in this pathway (**Figs.3.5, 3.7, 3.10**). These results suggest that NCX1 and EAAT3 might play a critical role in the glutamate-induced ATP synthesis under both physiological and pathological conditions.

The main interest in EAAT3/EAAC1 arises from previous studies[70], in which immunoprecipitation experiments performed on rat brain extracts, demonstrated a specific link between NCX1 and EAAC1, without any involvement of the glial glutamate transporters GLT-1 and GLAST[70]. Glutamate turnover is actually dependent on glial compartment[104,105] and, in this context, GLT-1 is the main transporter responsible for the modulation of extracellular glutamate levels[60,104,106]. Considering that neuron model used in this study only recapitulates neuronal functions, it is not possible to exclude that in *in vivo* systems GLT-1 may contribute to the metabolic response evoked by glutamate in ischemic settings. Collectively, the data presented here indicated that glutamate may attenuate neuronal damage in an *in vitro* setting of H/R and that NCX1 and EAAT3 have a central role. In relation to these findings, an increase in EAAT3 expression occurs, in line with previous reports showing a specific rapid increase in EAAC1 expression after transient focal ischemia in rat pyramidal neurons of the hippocampal CA1 region and of the parietal cerebral cortex[107]. This result may reflect the peculiar role of EAAT3/EAAC1 in subserving a neuroprotective mechanism in which the uptake of glutamate and its fate within cells play a major role. In this respect, it has been reported that EAAC1 knockout reduces the brain tolerance in a murine model of focal brain ischemia[108]. Although the authors ascribe such vulnerability to impaired cysteine uptake by

EAAC1[109]-with a consequent lack in antioxidant defenses-they fail to identify any significant change in glutathione levels between knockout and wild-type mice. This finding suggests that the absence of EAAT3/EAAC1 may likely worsen the focal brain ischemia outcomes because of the impaired glutamate entry, which may negatively affect neuronal metabolism. On the other hand, the peculiar activity of EAAT3/EAAC1 as cysteine transporter cannot be overlooked, since other results supported its role in maintaining the redox state of neurons[110-112]. In particular, in neurons from EAAC1 knockout animals, pre-treatment with the membrane-permeant prodrug N-acetyl cysteine[110-112] counteracts the increased vulnerability to I/R-induced oxidative stress. Therefore, it is not possible to exclude such a role for EAAT3/EAAC1 also in our experimental model. Further *in vivo* studies are needed to better elucidate the balance among the glutamate/cysteine transport activity of EAAT3 in ischemic settings. Glutamate also serves as the precursor for the synthesis of GABA, and its uptake through EAAT3 may be relevant to this purpose[104,113]. In particular, it has been showed that inhibition of glutamate uptake into GABAergic neurons decreases the amount of GABA release thus inducing post-synaptic inhibitory currents generated by GABA release[104]. Since SH-SY5Y cells can synthesize and release this inhibitory neurotransmitter[114], the observed neuroprotective effect of glutamate may be partially relied on its conversion to GABA. Its production by SH-SY5Y is far less than that observed in brain areas enriched in GABAergic neurons[114], therefore, experimental model presented here-not entirely reflecting *in vivo* CNS complexity-does not allow to further clarify this issue.

4.4 Glutamate fuels oxidative phosphorylation stimulating ATP synthesis

To find the source responsible for glutamate-induced ATP generation, both oxidative phosphorylation and glycolysis processes were investigated. Exposure to oligomycin counteracted the glutamate-induced increase in ATP levels, whereas the presence of 2-DG was ineffective in blocking ATP synthesis (**Fig. 3.8 a, b**). These results suggested the possibility that the increase in intracellular ATP content induced by exogenous glutamate might depend on its ability to fuel the mitochondrial oxidative phosphorylation. The hypothesis of glutamate as an alternative energy

source in metabolism-compromised states is in line with recent findings that try to metabolically "rehabilitate" the excess of glutamate released during the early phase of ischemia. It has been shown that the correction of hypoxia by supplemental oxygen during ischemic brain injury can induce the expression of the glutamate-metabolizing enzyme "glutamate oxaloacetate transaminase" (GOT)[115]. This enzyme can metabolize otherwise extracellular neurotoxic glutamate by enabling utilization of this amino acid as a metabolic fuel to support survival in the face of ischemia-induced hypoglycemia[75]. As a transaminase, GOT catalyzes the transfer of the amino group from glutamate to oxaloacetate, to generate aspartate and alpha-ketoglutarate, which fuels the TCA cycle and sustains cell viability. The increased GOT activity has been indirectly demonstrated through the rise in intracellular aspartate levels. This point could be a matter of concern, raising the question of a possible aspartate neurotoxicity through the NMDAR activation. However, since GOT overexpression attenuated stroke lesion volume, decreased neurodegeneration, and improved post stroke sensorimotor function[75], the excess of aspartate could be mainly retained in the cytosol, where it is metabolically recycled[116] without causing significant toxicity. The modulation of the glutamate dehydrogenase (GDH) enzyme has also been proposed. GDH is a TCA cycle enzyme that converts glutamate to alpha-ketoglutarate improving overall bioenergetics. Under energy-depleted conditions (i.e., I/R and oxygen-glucose deprivation, OGD), the modulation of GDH activity toward the utilization of glutamate seems to enhance ATP synthesis and to positively affect neuronal survival[59]. One possible explanation is that the activation of GDH may increase the influx of alpha-ketoglutarate in the TCA cycle, thereby reducing the extracellular glutamate release. Collectively, this approach fits into this novel scenario, which reveals the potentiality of glutamate to be transformed into a neuronal "survival factor".

4.5 Glutamate limits the H/R-induced suppression of NCX activity

The hypothetical impact of the H/R challenge on the reverse-mode activity of the exchanger was explored. Surprisingly, NCX reverse-mode activity was significantly reduced and glutamate exposure tended to normalize the global exchanger activity. Regarding the decrease in the exchanger

reverse-mode activity, several mechanisms may come into play, including the removal of the exchanger from the extracellular surface by endocytic pathways[117,118] and the compensatory action possibly taking place among the different isoforms[119]. Nevertheless, the increase in the intracellular ATP levels induced by glutamate ultimately represents an upstream signal that tends to restore the overall cellular homeostasis. The partial recovery of NCX activity induced by glutamate supplementation was specifically counteracted by RNAi directed against NCX1 and EAAT3 (**Fig. 3.10**). These results confirmed that the glutamate effects were mediated by the NCX1-EAAT3 interplay. Since the reverse-mode activity of NCX is essential to maintain Na^+ driving force for allowing the entry of glutamate, the reduction in the exchanger reverse-mode activity could strongly affect the ability of EAAT3 to efficiently uptake glutamate. This effect in turn may reduce the amount of glutamate that is metabolically available and may explain the partial recovery of both ATP production and cell viability. However, other mechanisms may also explain the partial efficacy of glutamate, including a slight excitotoxic effect mediated by NMDAR[120,121] and/or the activation of an NMDAR-independent oxidative response[122]. To confirm these hypotheses, further investigations are needed.

4.6 Conclusion

In conclusion, data presented in this study disclose a new and beneficial role of glutamate - administered during the entire reoxygenation phase- in improving cell viability in an *in vitro* neuronal model of H/R. Glutamate supplementation enhance oxidative metabolism through the crucial involvement of NCX1/EATT3 pathway[123].

The present findings are supported by the following evidences:

- Glutamate partial protects cell survival fueling the oxidative phosphorylation, leading to an increase in ATP content in RA-differentiated SH-SY5Y cells.
- Inhibition of NCX1 and EAAT3 by using pharmacological tools, SN-6 and DL-TBOA, and siRNA approach abolish the protective effects of glutamate in RA-differentiated SH-SY5Y cells.

These findings validate that the defensive glutamate action relied on the activity of NCX1 that supported EAAT3 in a mandatory way. As already stated in previous reports[70,71,84], it has been proposed that by extruding Na^+, the reverse-mode activity of the exchanger is essential for maintaining the Na^+-driving force for effective glutamate uptake. This uptake in turn tends to bring Ca^{2+} into mitochondria and to increase ATP, likely through enhancement of mitochondrial dehydrogenases activity[72]. Collectively, these evidences suggest that glutamate supplementation during the reoxygenation phase significantly attenuated H/R damage and that its ability to possibly serve as a "survival factor" relies, at least in part, on its metabolic utilization that fuels the oxidative phosphorylation, stimulating ATP synthesis. Remarkably, glutamate protective effect strictly depends on the coordinated activity of NCX1 and EAAT3, which, in a complementary fashion, orchestrate its uptake and intracellular utilization. These findings could be helpful in the refinement of further *in vivo* studies, which could pave the way for a change in the classical view of glutamate as detrimental factor and for the development of new therapeutic target.

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