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REGULATION OF NCX3 EXPRESSION BY HISTONE DEACETYLASES (HDACS) IN CORTICAL NEURONS AND IN BRAIN ISCHEMIA

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Summary

SUMMARY

Na^+- Ca^{2+} exchanger isoform 3 (NCX3) plays a fundamental role in the pathogenesis of stroke damage. Indeed its ablation worsens the experimentally-induced ischemic damage. Interestingly it has been found that NCX3 mRNA and protein are both reduced after stroke. However, the mechanism by which stroke-induced ncx3 gene reduction is still unclear. Notably, in the last decades it has been found that histone deacetylases (HDACs) inhibition by regulating specific neuroprotective genes ameliorates the neurodegeneration that occurs in brain ischemia.

Interestingly, we found that neurons treated with Trichostatin A (TSA), a pan HDACs inhibitor (HDACi), and MC1568, a class II HDACs inhibitor, significantly increased ncx3 promoter activity, whereas MS-275 (class I HDACs inhibitor) had no significant effect. Notably, among the HDACs class II A, we found that when the HDAC4 and HDAC5 isoforms were overexpressed by construct transfection or knocked-down by small interfering RNA (siRNA) transfection, NCX3 mRNA and protein levels were downregulated or increased, respectively. Moreover, experiments of site direct mutagenesis of DREAM (downstream regulatory element antagonist modulator) consensus sequence on ncx3 promoter in MC1568 treated neurons, corroborated that NCX3 downregulation induced by HDACs is achieved by DREAM. Notably, Chromatin Immunoprecipitation (ChIP) assay demonstrated that HDAC4 and HDAC5 binding on ncx3 promoter was significantly increased after transient middle cerebral artery occlusion (tMCAO).

Our findings identify a new epigenetic regulatory mechanism that controls NCX3 gene transcription and demonstrated that HDAC class II A inhibition, by blocking HDAC4 and HDAC5 and modulating the acetylation of ncx3 gene promoter sequence, could be a new therapeutic strategy in stroke treatment.

Introduction

I. INTRODUCTION

I.A. PATHOPHYSIOLOGY OF CEREBRAL ISCHEMIA

Stroke has been described into two main forms: ischemic and hemorrhagic, however the ischemic form is more common than the hemorrhagic. Several drug trials have been directed to find a cure for the ischemic form since the ischemic event represents about the 87% of all the cases of stroke.

Ischemic stroke can be caused by a reduction in the brain blood flow as consequence of a thrombosis, an embolism or a systemic hypo-perfusion.

Within the ischemic core, where the oxygen and glucose reduction delivered by blood vessels is seriously restricted, excitotoxic and necrotic cell death occurs within minutes, whereas in the peripheral ischemic area, the collateral blood flow can buffer injury, leading to different outcome [1].

I.A.1. Excitotoxicity and ionic imbalance

In a resting neuron the cytoplasmic concentration of free Ca^{2+} is approximately around 100 nM, whereas at the extracellular side is estimated to be concentrated 1-2 mM. The intracellular ionic homeostasis of Ca^{2+} is regulated by several mechanisms including specific activation of: receptors, channels, and ionic pumps. The entry of extracellular Ca^{2+} is mediated by the activation of ligand-operated receptors and voltage-gated Ca^{2+} channels. The release of Ca^{2+} from inner stores such as the endoplasmic reticulum is allowed by the stimulation of inositol triphosphate (IP3) receptors and Na^{+} -Ca^{2+} exchanger activation. The extrusion of Ca^{2+} from the cell requires an active transport mediated by the Ca^{2+}-ATPase or Na^{+} -Ca^{2+} exchanger in the plasma membrane. Furthermore, the intracellular Ca^{2+} buffering is regulated by specific target proteins that bind free Ca^{2+} causing a decrease in cytoplasmic free levels as well as by the sequestration of Ca^{2+} into the endoplasmic reticulum through Ca^{2+}-

ATPase or through mitochondria uniport mechanism activation [2,3,4,5]. Thus, energy failure in hypoxic ischemia will cause the accumulation of intraneuronal free Ca^{2+} (Ca^{2+} overload) by enhancing the entry and releasing from the stores, and by interfering with the ATP-dependent extrusion and sequestration of this ion.

Blood flow interruption in the brain causes deprivation of oxygen and glucose that are used as energetic substrates. Furthermore, impairment in energy production increases glutamate release via membrane depolarization and subsequent voltage gated Ca^{2+} channels activation. It also interferes with the re-uptake of glutamate (primarily into astrocytes), leading to its abnormal accumulation into synapses [2]. After ischemia, the energetic depletion decreases the function of Na^{+}/K^{+}-ATPase and Ca^{2+}-ATPase, which are important plasma membrane ionic pumps found in neurons. A failure in energy production induces membrane depolarization, release of potassium into the extracellular space and entry of sodium and calcium into the cells [6]. The membrane depolarization is also responsible for the activation of the N-methyl-D-aspartate receptors (NMDA receptors). The heteromeric NMDA receptors are highly permeable to Ca^{2+}, as well as Na^{+} and K^{+}. NMDA receptors can be fully activated under plasma membrane depolarization thus abolishing its Mg^{2+} - induced inhibition. Since a brief (>3 min) activation of NMDA receptors is sufficient to trigger neuronal death, the activation of NMDA receptors has been proposed as a primary cause of neuronal death after the focal cerebral ischemia that is accompanied by the transient (~30- 60 min) elevation of extracellular glutamate [7,8]. The Ca^{2+} influx through NMDA receptors mediates the rapidly-triggered NMDA neurotoxicity, while Na^{+} influx contributes to the swelling of the neuronal cell body [9]. The entry of Ca^{2+} via NMDA receptors activation contributes further to the Ca^{2+} overload following hypoxic-ischemic injury, in fact NMDA antagonists prevent the entry and accumulation of Ca^{2+} in neurons exposed to oxygen and glucose deprivation (OGD), an hypoxic-ischemic condition in vitro [10,11].

Prolonged increases in intracellular Ca^{2+} levels lead to catabolic processes and irreversible neuronal death in the ischemic core through multiple mechanisms that involve the activation of Ca^{2+}- binding effector proteins such as calcium dependent proteases, lipases and DNAses [12].

I.A.2. Oxidative and nitrosative stress

Oxidative stress has been strongly implicated in triggering both necrosis and apoptosis processes in case of focal ischemia, above all when followed by reperfusion [13,14]. In physiological conditions, mitochondria are the major producer of superoxide. As the ischemia occurs, mitochondria probably contribute to increase oxidative stress in ischemic and post-ischemic brain [15].

The increase of intracellular calcium, sodium and adenosine diphosphate (ADP) induces mitochondria to produce the deleterious reactive species of oxygen (ROS), which consequently leads to cellular macromolecules (mainly lipids and proteins) destruction. ROS are involved in the activation of signaling mechanisms resulting in apoptotic cell death [16]. Moreover, the high production of superoxide and nitric oxide (NO) affects protein structure and leads to peroxynitrate levels increases. Thrombolytic therapy is effective just during the 4,5 hours after the occurrence of stroke. The reason for this limited therapeutic window is found in the surge of production of free radicals during the delayed reperfusion phase. The oxygen that newly perfuses tissue is responsible for the second wave of oxidative and nitrosative stress that increases the risk of brain hemorrhage and edema.

I.A.3. Apoptosis

Emergent evidence suggests a role for caspases during neuronal death after brain ischemia [17]. The caspases are a family of proteases that include in their catalytic site the cysteine-containing pentapeptide motif

QACXG (X being R, Q or G) and share the feature of an aspartate residue at the N-terminal end of the substrate cleavage site [18,19,20,21,22]. There is an huge evidence that during ischemic injury caspases are activated in the brain. In fact, up-regulation and activation of caspase-3 was found to precede neuronal death.

In the rat brain during transient global ischemia, the activation of caspase-3 induces the cleavage of inhibitor of caspase-activated deoxyribonuclease (ICAD), thus resulting in the apoptotic degradation of DNA by caspase-activated deoxyribonuclease (CAD) [23]. Deoxyribonuclease activity resulting from transient focal ischemia in the rat could be prevented by inhibitors of caspase-3-like activity [24].

Focal brain ischemia was reported to increase levels of mRNA and protein of several procaspases, including -3, -6, and -8 [25,26,27], and to cause activation of caspases-3 [27] and -8. The activation of caspase-8 that has been documented in a few studies of experimental brain ischemia [27, 28] suggests that receptor-mediated activation of caspases could contribute to ischemic brain damage. The release of tumor necrosis factor alpha (TNF-α), by ischemic neurons, glia, activated microglia and infiltrating inflammatory cells could stimulates many receptors linked to caspases [29,30,31]. The mitochondrial pathway of programmed cell death involves the release of cytochrome c, procaspase-9, and apoptotic protease activating factor 1 (Apaf-1) from the mitochondrial intermembrane space and a series of subsequent biochemical interactions that include the activation of caspase-9 and lead to the activation of caspase-3. Mitochondrial release of cytochrome c after brain ischemia is caused by the action of Bax (bcl-2-like protein 4) and other pro-apoptotic members of the B-cell lymphoma 2 (Bcl-2) family [32,21,33]. The death of neurons after brain ischemia is preceded by an increase in the level of Bax [34,35,36,37,38,39]. After transient focal brain ischemia, Bax translocates rapidly from the cytosol to the mitochondria, where it interacts with the mitochondrial adenine nucleotide translocator and the voltage-dependent

anion channel. The timing and regional distribution of the translocation of Bax coincides with the mitochondrial release of cytochrome c and caspase-9.

I.A.4. Inflammation

Inflammation plays an important role in the pathogenesis of ischemic stroke and other forms of ischemic brain injury [40]. Among the various types of leukocytes, neutrophils are the first to infiltrate ischemic brain (30 min to a few hours after focal cerebral ischemia). Infiltrating neutrophils release several pro-inflammatory mediators stored in granules and vescicles, such as inducible nitric oxide synthase (iNOS) and matrix metalloproteinases (MMPs) [41], thus triggering inflammation and injury. Microglia could be rapidly activated within few minutes in response to cerebral ischemia [42,43]. The inflammatory mediators released from the activated microglia lead to cell damage and death.

By contrast, microglia can also produces the transforming growth factor β1 (TGF-β1), which acts as a neuroprotective molecule [42]. These dual and opposite functions may be referred to the different activation time of microglia since data suggested that early activation is detrimental and later activation is beneficial [44].

After ischemic stroke, astrocytes proliferate and differentiate, a condition known as astrogliosis. Also the astrocytes release several inflammatory mediators [45,46] such as TNF-α, a potent pro-inflammatory cytokine, that was found upregulated in the brain after both permanent [47] and transient middle cerebral occlusion (pMCAO and tMCAO) [48].

Matrix Metalloproteinase (MMPs), a family of zinc-dependent proteolytic enzymes, are normally as a pro- or inactivated forms and can be activated by the removal of amino-terminal pro-peptides. Their constitutive expression is low, but can be upregulated by many pathogenic events, including stroke. MMPs play important roles in brain injury after stroke. Among various MMPs, MMP-9 is most closely implicated in cerebral

ischemia [49]. MMP-9 was upregulated in brain tissue and also in serum of patients with acute ischemic stroke [50]. It was associated with blood brain barrier (BBB) disruption, edema development, and hemorrhagic transformation of ischemic stroke [51,52].

I.B. EPIGENETICS

Epigenetics is a branch of molecular biology that investigates heritable changes in genic expression without alterations in the original DNA sequence. Among the various epigenetic modifications, three major categories have been described such as DNA methylation, histone posttranslational modifications, and noncoding RNAs (ncRNAs) which include microRNAs (miRNA), small noncoding RNAs (sncRNA), and long noncoding RNAs (lncRNA) [53].

I.B.1. Nucleosome

Chromatin is the complex of DNA wrapped around histonic proteins found in the eukaryotic nuclei. The functional unit of chromatin, the nucleosome, represents the primitive structure allowing the packing of all the entire genome **(Figure 1-A)**. Each nucleosome is composed of an octamer of histonic proteins and consists of two copies of each histones (H2A, H2B, H3 and H4) wrapping a segment of 147 base pairs long DNA [54]. Histones, enriched in basic amino acids, are proteins which structure is made of a globular domain and an N- terminal tail protruding from the nucleosome. Although the histones are classified among the most evolutionarily conserved proteins, they represent the most variable in terms of posttranslational modifications.

The N- terminal tails of histones are usually targets for various covalent posttranslational modifications, including acetylation, phosphorylation,

methylation, sumoylation and ubiquitination **(Figure 1-B)**. The specific combinations in posttranslational modifications generate a sort of "histone code". The role of these modifications is found in their particular combinatorial pattern since they decode for a selective chromatin affinity to the associated proteins, which determine whether the chromatin is active (relaxed state) or silent (condensed state) [55]. Thus, histone code influences the structure and pattern of chromatin condensation and consequently it has been found involved in the gene regulation [56].

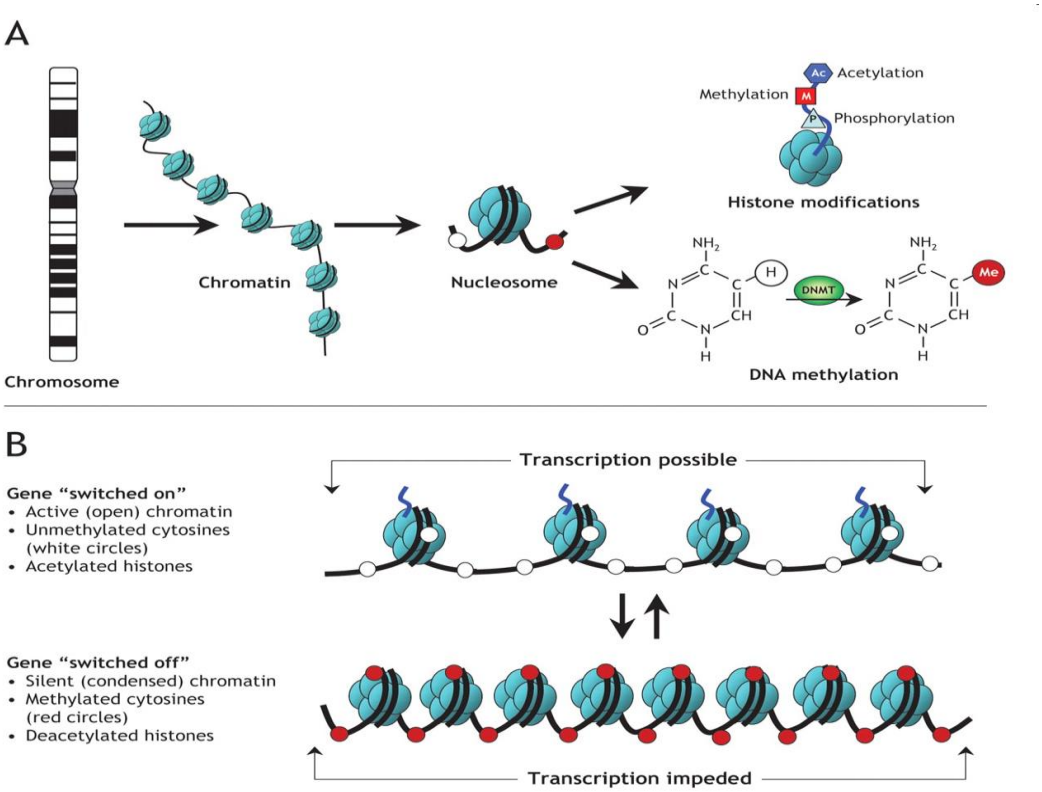


Figure 1. Chromatine organization into nucleosomes (A) and epigenetic modifications (B).

I.B.2. Epigenetic modifications

The histone tail modifications are likely to act in concert with the rather more widely known mediator of chromatin structure and gene expression, namely DNA methylation that happens through the action of DNA methyltransferases (DNMT) **(Figure 1-A)**.

DNA methylation is an important regulatory mechanism of gene expression in eukaryotes. In mammals and humans DNA methylation mostly affects the cytosine (C) base when it is followed by a guanine (G). These CpG sites can be clustered in the so-called “CpG islands” (GCI) but DNA methylation can also be present in non-CG contexts [57]. This modification is known to have a role in the constitutive silencing of chromatin regions, the inactivation of one of the X chromosomes in females, the imprinting of parental alleles, and the silencing of retroviral genes and other individual genes [58]. In addition to DNA methylation, also histone posttranslational modifications act on the chromatin conformation. In fact, methylation and especially trimethylation of histone 3 (H3) at lysine site 27 (H3K27me3)/lysine 9 (H3K9me3) induces gene repression. Conversely, methylation of H3K4 normally indicates active enhancers [59,60]. Hundreds of histone modifications have been found affecting histones into many different residues including lysine (K), arginine (R), serine (S), threonine (T), and glutamate (E) [61].

Recent studies explored in more detail the complexity of information brought from the epigenetic modifications on histone tails. Currently, there are 50 different acetylated isoforms of the histones forming the nucleosome core, with a total of 16 different isoforms for H2B, H3, and H4 and only two for H2A. In addition, these isoforms have been found further modified via methylation on specific lysines and arginines sites (H3 and H4) and via phosphorylation on serine (H3, H4, H2B) [62].

The total combination of the possible histone isoforms carrying the various modifications may give rise to a thousands possible patterns. Recent evidence explored the role of specific proteins which are recruited to particular post translational modifications (PTMs) on histones, thus sheding light to the so called “histone code hypothesis” [63]. Certain PTMs form a docking platform for the recruitment of subsequently acting histone modifiers that associate with the “readers” of these PTMs.

I.B.3. Acetylation and deacetylation

Among the histone modifications, acetylation and deacetylation of specific residues of histone tails have been described having opposing effects on the chromatin activation. The activity of two principal classes of enzymes, the histone acetyltransferases (HATs) and histone deacetylases (HDACs), determines transcriptional activation state of a specific region in the genome. HATs acetylate the ε-groups of the lysine residues of the histone tails, whereas the acetyl groups removal, HDACs-mediated, restores positive charge on these residues. Consistently, active transcriptional regions of the chromatin generally associate with highly acetylated histones H3 and H4 in euchromatic regions of the genome [64]. Histone acetylation plays an important role in chromatin remodelling, affecting on the modulation of transcription [65,66]. In fact, the recruitment of HATs by transcription factors to specific genetic loci determines the grade of local acetylation of histones. Importantly, HATs work as integration hub in many various signaling cascade since they can interact with a large number of transcription factors [67]. Conversely, the histone deacetylation activity, via HDACs, removes acetyl moieties, serving as inactivating signal for chromatinic transcription. HDACs inhibition restores the grade of histone acetylation, chromatin relaxation and gene expression. The imbalance between the activity of HATs and HDACs generally associates with the development of a pathophysiological state. Moreover, HATs and HDACs activity can also target non-histonic substrates such as the tubulin and transcription factors including the tumor suppressor p53, specificity protein 1 (Sp1), Sma-Mothers against decapentaplegic homolog 7 (Smad7), cAMP response element-binding protein (CREB), the pleiotropic transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and signal transducer and activator of transcription 1 (STAT1) [68].

I.B.4. Histone Acetyltransferase (HAT) Families

Histone acetyltransferases (HATs) catalyze the reaction of histones acetylation through addition of an acetyl group of the pseudo-substrate acetyl coenzyme A (acetylCoA) to the lysine residue on the ε-amino group on the N terminal side of histonic core. HATs may act also on non-histone proteic substrates, requiring a new specific nomenclature for these enzymes. For instance, “HATs” that acetylates lysine (K) residues should be referred to as KAT (lysine acetyl transferase) [69]. The family of HATs is found evolutionary well conserved from yeasts to humans.

The most common HATs are classified as cytoplasmic (type A) and nuclear (type B). Nuclear HATs are further grouped into 5 major classes: 1) Gcn5- related N-acetyltransferases (GNAT), 2) p300/cyclic adenosine monophosphate response element-binding protein (CREB) binding protein (CBP), 3) MOZ, yeast YBF2, SAS2, and TIP60 (MYST), 4) transcription factor-related HATs, and 5) nuclear receptor-associated HATs such as TATA-binding protein (TBP)-associated factor (such as TAF130/150) [70]. Most of these histone acetyltransferases show a tipical regulatory function in transcription. The TAF130/250 histone acetylase [71] is a subunit of the Transcription Factor II D (TFIID) complex, a basic component of the Polimerase II (Pol II) transcription machinery in all eukaryotic organisms; it is likely to be associated essentially with all promoters during transcriptional initiation. The p300/CBP histone acetylase [72,73] was described initially as a transcriptional coactivator that functions by interacting with a wide variety of enhancer-binding proteins [74]. However, p300/CBP is tightly associated with the Pol II holoenzyme [75], suggesting the possibility that this histone acetylase could be viewed as a more general component of the transcription machinery. p300/CBP is found in a variety of multicellular organisms from worms to humans, but it does not exist in yeast [76].

I.B.5. Histone Deacetylases (HDACs): Structure–Function Analysis

Two families of histone deacetylases have been described in eukaryotes: the histone deacetylases (HDACs), and the Sir2-like deacetylases (sirtuins).

The HDAC enzymes possess an highly conserved domain, consisting in approximately 390 amino acids, which appears to deacetylate substrates by activation of a water molecule with a divalent zinc cation coupled to a histidine-aspartate exchange system [77]. The HDAC family members can be classified into two classes according to their similarity to yeast histone deacetylases Rpd3 (reduced potassium dependency 3) for the class I, and Hda1 (Histone deacetylase-A) for the class II [78] **(Figure 2)**. HDAC 11 is the unique member of class IV HDAC since it does not share the characteristics of Rpd3 (class I) and Hda1 (class II) histone deacetylases. Four class I (HDAC1, 2, 3, and 8) and five class II (HDAC 4, 5, 6, 7, and 9) HDACs have been identified and partially characterized in humans [79,80], and there are potentially more deacetylases in this family according to the genome sequence [81]. Recently, a second family of histone deacetylases, the sirtuins, was identified. This group of proteins is related to the yeast transcriptional repressor Sir2, and its members can be divided into five classes based on their primary structure [82]. The sirtuin deacetylases contain a conserved 275 amino acid catalytic domain, which is unrelated to that of the HDACs, and the sirtuins operate by a very different mechanism that requires nicotinamide adenine dinucleotide (NAD) as a substrate. Despite these structural and mechanistic differences, proteins from both families have been shown to silence transcription at specific promoters or chromosomal domains by localized histone deacetylation. However, it is likely that members of the two families function in other cellular processes with non-histone substrates [83].

I.B.5.1. HDAC family members

HDAC1 was the first histone deacetylase to be identified and characterized. Sequence analysis of HDAC1 revealed the homology of the human form with yeast protein Rpd3, showing a regulatory transcriptional function. Subsequent fractionation of the histone deacetylase activity in yeast yielded at least two distinct protein complexes, one of which contained Rpd3, and the other of which contained a highly related protein, Hda1. Hda1 contains the conserved HDAC catalytic domain and possesses deacetylase activity, but it is significantly larger in size than Rpd3. Human HDACs group consist in eight cloned proteins classified according their structure and size into the class I, having homology to Rpd3 (HDAC1, 2, 3, and 8), and the class II, with greater similarity to Hda1 (HDAC4, 5, 6, 7, and 9) [84]. All of these proteins share a conserved catalytic domain and *in vitro* histone deacetylase activity. Class II HDACs appears two to three times larger in size than the class I proteins. Class II HDACs protein are further subdivided into two main groups. Class IIa HDACs consist of four members (HDAC4, HDAC5, HDAC7 and HDAC9) with distinct tissue specific patterns of expression, predominantly in muscle and heart [85]. Class IIb HDACs include HDAC6 and HDAC10. The structure of HDAC6 is unusual in that it contains two independently functioning catalytic domains and a carboxy-terminal Zn^{2+}-finger ubiquitin binding domain. HDAC6 functions in the cytoplasm where it deacetylates α-tubulin and alters microtubule stability [86,87]. Its close structural homologue HDAC10 lacks the second functional catalytic domain [88]. HDAC10 has been found in a complex with HDAC3, although the exact functions of this deacetylase are still unknown.

HDAC1 and HDAC2 are the best characterized among the HDAC proteins generally found in stable, multicomponent complexes of proteins, which are then recruited by DNA binding proteins. Three complexes containing HDAC1 and HDAC2 have been characterized thus far: the Sin3, the NuRD

(Nucleosome Remodeling Deacetylase), and CoREST (REST Corepressor 1) complexes [83]. The Sin3 complex includes a central core composed of SAP18 (Sin3A-associated protein of 18 KDa) and SAP30 (Sin3A-associated protein of 30 KDa) proteins, and mSin3A, which stabilizes the complex serving as scaffold structure for various DNA binding proteins [89]. The NuRD complex also contains a core complex of MTA2 (Metastasis-associated protein 2), which is related to protein MTA1 (Metastasis-associated protein 1) and CHD3 (Chromodomain-helicase-DNA-binding protein 3) and CHD4 (Chromodomain-helicase-DNA-binding protein 4) also called Mi-2α and Mi-2β respectively, which possess DNA helicase/ATPase domains found in the SWI/SNF (SWItch/Sucrose Non-Fermentable) family of chromatin remodeling proteins [90].

The CoREST complex includes HDAC1 and HDAC2 but not RbAp46 (Rb-associated protein 46) or RbAp48 (Rb-associated protein 48). Another member of this complex is p110 [78].

HDAC4 and HDAC5 associate with HDAC3 [78] to form a complex with N–CoR (nuclear receptor corepressor) and SMRT(silencing mediator for retinoid and thyroid receptors) [91]. Interaction of HDAC4 or HDAC5 with 14-3-3 proteins has been discovered as sequester mechanism of the protein into the cytoplasm. When they dissociate HDAC-4 and HDAC-5 translocate to the nucleus where they bind HDAC-3 and repress gene expression [92,93].

I.B.5.2. Sirtuin Family Members

The third family of histone deacetylases, sirtuins, are homologues of the yeast Sir2 gene, which function is related to chromatin silencing, cellular metabolism, and aging [94]. In humans have been described seven sirtuins, SIRT1-7, which sequences have a mean of 300-400 amino acids, except that for SIRT1 (747 amino acids). Sirtuins possess several conserved sequence motifs. The catalytic domain of about 275 amino acids consists of two sets of CXXC motifs, which may function as zinc

finger domains [95], and more hydrophobic regions that may function as leucine zippers [96]. Sirtuins deacetylase activity is dependent by the presence in the reaction of NAD+ [97], whereas yeast Sir2 possesses an intrinsic ADP-ribosyltransferase activity [95].

SIRT-1 deacetylates p53, repressing its activity of transcriptional promoter of anti-apoptotic genes in response to DNA damage [98,99-100,101].

I.B.6. HDACs inhibitors

HDACs inhibitors (HDACI) were developed initially like molecules of oncologic interest because of their anticancer potential. Subsequently, the spectrum of therapeutic applications of these drugs was extended also to other human pathologies such as central nervous system diseases, on the basis of promising *in vivo* applications to polyglutamine-repeat diseases. Great efforts have been made to develop other compounds with characteristics similar to already known HDACI, in order to treat brain disorders [102]. HDAC inhibitors such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (vorinostat, also known as SAHA), inhibit all zinc-dependent HDACs and for this reason are called pan-HDACI **(Figure 2)**. These molecules cross the blood–brain barrier (BBB) and share the mechanism of action, committed to their hydroxamate moiety that is able to bind the zinc ion at the HDAC active site to inactivate the enzyme. Sodium butyrate and 4-phenylbutyrate are fatty acid derivatives that inhibit most class I and II HDACs, except HDAC-6. Valproic acid is another HDACI that not only is capable to cross the BBB but possesses also anticonvulsant properties, in particular this molecule inhibits class I and class IIa HDACs, but not class IIb [103].

MC1568 selectively inhibits the HDAC class IIa. MS-275 is a synthetic benzamide derivative that preferentially inhibits HDAC1, compared with HDAC2, 3 and 9, and has little or no activity against HDAC4, 6, 7, and 8. This drug also passes the BBB easily and appears to produce no severe side effects. Apicidin, a cyclic tetrapeptide, inhibits HDAC2 and 3 in the

low nanomolar range and HDAC8 in the high nanomolar range, but does not affect HDAC1 or class II HDAC [104]. Romidepsin (FK-228), another cyclic tetrapeptide, also potently inhibits HDAC1 and 2 [105]. Tubacin is a catalytic domain-targeting small molecule inhibitor showing high selectivity for HDAC6 and for the deacetylation of α-tubulin, a microtubule component [106]. Nicotinamide, also known as niacinamide, is a precursor of NAD+ and a competitive class III HDAC inhibitor that can be given orally [107].

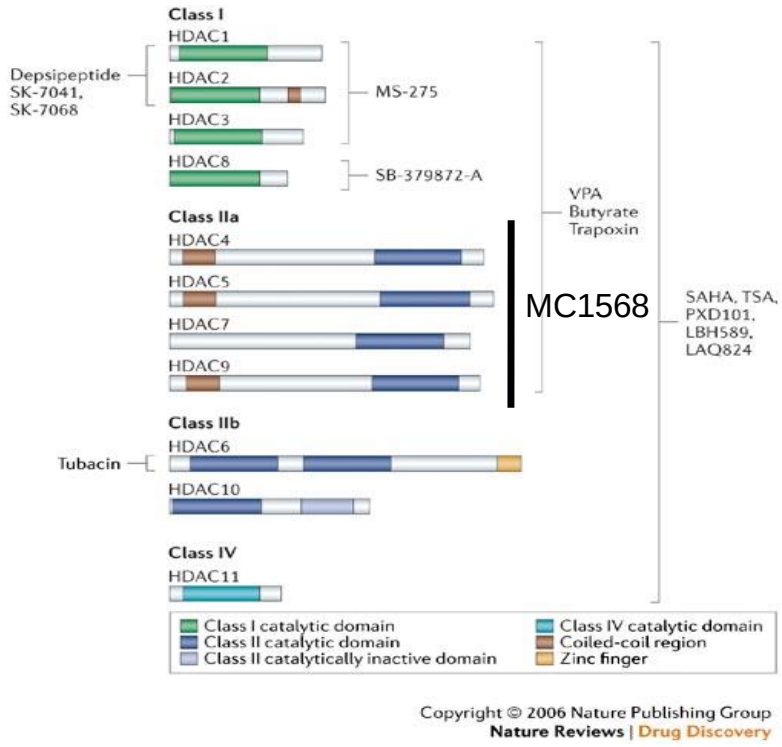


Figure 2. Classification of HDACs and HDACI. Jessica E. Bolden, Melissa J. Peart and Ricky W. Johnstone Nature Reviews Drug Discovery 5, 769-784 (September 2006).

I.B.7. Neuroprotection by HDAC Inhibition in Cellular Models

HDAC inhibition exerts a neuroprotective effect in both *in vivo* and *in vitro* models of brain disorders. Pioneering study support that levels of the HATs CBP/p300 and histone proteins acetylation were decreased during

apoptosis induced by potassium deprivation of cultured primary cerebellar granule cells, and during signaling activation of β-amyloid precursor protein (APP) in cultured primary cerebral cortical neurons from rodents [108]. Moreover, overexpression of CBP/p300 protected these neurons from proapoptotic insults. In cortical neurons, Ryu and colleagues showed that treatment with TSA, sodium butyrate, or vorinostat protected against glutathione depletion-induced oxidative stress; neuroprotection involved the acetylation and subsequent activation of the DNA binding activity of Sp1 [109]. However, it is well known that some HDAC inhibitors, such as TSA, have basal toxicity and prolonged treatment at high doses often induces neuronal death, so compromising their neuroprotective effects [110]. HDAC inhibitor-induced neurotoxicity could be partly due to 'derepression' of genes involved in apoptosis including Bim and B-myb [111]. In fact, Langley and colleagues found that a two-hour pulse treatment with TSA sufficed to rescue cortical neurons from oxidative stress without obvious toxicity; protection was associated with transcriptional activation of the cell-cycle inhibitor $p21^{waf1/cip}$ [112]. Glutamate-induced excitotoxicity has been implicated in the pathophysiology of many neurodegenerative and neuropsychiatric diseases; these include stroke, Huntington's disease, amyotrophic lateral sclerosis, spinal cord and traumatic brain injury, cerebellar degeneration and possibly Alzheimer's disease, Parkinson's disease and mood disorders. Notably, Leng and Chuang demonstrated that valproic acid, 4-phenylbutyrate, or TSA treatment protected against glutamate-induced excitotoxicity in brain, with concomitant transcriptional activation and induction of α-synuclein, a presynaptic protein of unknown function. Recent experiments demonstrate that overexpression of endogenous α-synuclein plays a neuroprotective role; this appears to involve upregulation of the cytoprotective protein B-cell lymphoma 2 (Bcl-2) but downregulation of Ube2n (Ubiquitin conjugating enzyme E2 N), a proapoptotic, ubiquitin-conjugating enzyme. A subsequent study showed that α-synuclein

protected cerebellar granule cells from 6-dihydroxydopamine-induced death. It appears that α-synuclein is neuroprotective in the cytoplasm, but becomes neurotoxic once translocated to the nucleus, where it inhibits HAT activity [113,114, 115]. A more recent study found that valproic acid and other class I and II HDAC inhibitors (e.g. sodium butyrate, 4-phenylbutyrate, and TSA) potentiated these neuroprotective effects against excitotoxicity, when used in conjunction with lithium, another mood stabilizer with a robust neuroprotective profile [116]. Taken together, these findings suggest that HDAC inhibitors induce the expression of multiple downstream targets that might work collectively to elicit neuroprotective effects. Furthermore, HDAC inhibitors increase the expression of neurotrophins, molecules that play prominent roles in neuronal development, synaptic plasticity, and neuronal survival. For instance, Yasuda et al. found that brain-derived neurotrophic factor (BDNF) was induced in rat cortical neurons by treatment with valproic acid, sodium butyrate or TSA [117]. Hong and colleagues found that both BDNF and glial cell line-derived neurotrophic factor (GDNF) were induced by class I and II inhibitors in primary cultures of astrocytes from rat midbrain [118, 119, 120]. Considerable previous studies have reported that HDAC inhibitors exerted neuroprotective effects by preventing microglia activation, and anti-neuroinflammatory effects in some central nervous system (CNS) diseases [121]. The anti-inflammatory effects of HDAC inhibitors were also found in an animal model of cerebral ischemia. Taken together, the *in vitro* studies demonstrate that HDAC inhibitors exert their neuroprotective effects through multiple mechanisms and that, in addition to neurons, glia is also target of HDAC inhibition and neuroprotection [122].

I.B.7.1. Huntington's Disease (HD)

The role of HDACs in neurodegenerative disorders was primarily discovered through experiments showing that HDAC inhibitors were able to improve the cognitive and motor deficits characteristic of Huntington's disease (HD). HD is a neurodegenerative disorder with autosomal-dominant inheritage. The late onset of this disease accompanies with progressive motor, psychiatric and cognitive decline. A typical feature of this disease is a massive death in cortical and striatal neurons which cause is thought to be in the polyglutamine (CAG) expansion in the 5' coding region of the huntingtin (htt) gene [123, 124].

Mutant huntingtin accumulates in the nucleus where its aggregates in polyQ proteins bind many different transcription factors and coactivators such as CBP [123, 124], thus mutant huntingtin is thought be responsible for transcriptional dysregulation. In fact, a lower CBP availability leads to dysregulation in CBP/CREB - mediated gene expression, histone deacetylation and neuronal loss. Early studies demonstrated the ability of HDAC inhibitors to rescue lethality and photoreceptor neurodegeneration in a *Drosophila* model of polyglutamine disease [125]. These findings were extended to the mouse models of HD by several laboratories, who showed that treatment with HDAC inhibitors such as sodium butyrate and phenylbutyrate attenuate neuronal loss, increase motor function and extend survival in R6/2 mice [126, 127].

These findings strengthen the idea that epigenetic dysregulation plays a critical role in the pathogenesis of HD further suggesting the efficacy of therapeutics as cure for the alterations in epigenetic modifications, since they have also beneficial effects after the onset of the disease [67].

I.B.7.2. Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder that affects 1% of the population over 65 [128].

Although the etiology of PD is not well defined, familiar PD cases share a lack of nuclear α-synuclein. Recent studies explored the role of HDAC inhibitors as therapeutics of this progressive neurodegenerative disease. The initial link between PD and epigenetic dysregulation came from studies on *Drosophila*. Using a *Drosophila* model of PD, Feany and colleagues demonstrated that nuclear targeting of α-synuclein promotes its toxicity and that sequestration of α-synuclein to the cytoplasm is protective [113]. It was further shown that α- synuclein binds directly to histones, reduces levels of acetylated histone H3 and inhibits HAT mediated acetyltransferase activity [113]. One study reported that nuclear α-synuclein binds histones to inactivate HATs including CBP, p300 and P/CAF, causing histone hypoacetylation and apoptosis in human neuroblastoma cells. These findings implicate α-synuclein in the degeneration associated with PD. Administration of HDAC inhibitors *in vivo* or *in vitro* rescued α-synuclein- induced toxicity [113]. These findings underline the potential of HDAC inhibitors for therapeutic intervention in the neurodegeneration and cognitive impairments in PD. Future studies are needed to examine the efficacy of HDAC inhibitors in animal models for PD [67].

I.B.7.3. Alzheimer's disease

Alzheimer's disease (AD) is the fourth most common cause of mortality in the USA, affecting approximately the 50% of people over their eighties in industrialized countries. The clinical onset is characterized by progressive memory and mood changes, ultimately leading to dementia. The typical hallmark of the disease is the accumulation of the extracellular β-amyloid (Aβ) in the brain, and hyperphosphorylation of Tau protein. Toxic Aβ peptides derivate by the aberrant double cleavage of the APP (amiloid precursor protein) which normal processing produces physiological and non toxic substrates. Tau protein is an important protein involved in the

stabilization of microtubules structure, whereas its phosphorylation induces disassembling of cytoskeleton filaments.

A follow-up study by Guan et al. elegantly demonstrated that mice overexpressing HDAC2, but not HDAC1, exhibited decreased dendritic spine density, a decreased synaptic number, a reduced synaptic plasticity, and impaired memory formation [129]. Conversely, *Hdac2* knockout mice show memory improvement. Further, HDAC2 is involved in the regulation of hippocampal mouse synaptic formation and in neuronal plasticity by exerting its deacetylase activity on several promoter genes related to neuronal activity, synaptic formation and plasticity.

The memory impairment showed by HDAC2 overexpressing mice was prevented via administration of the HDAC inhibitor vorinostat. These findings shed light on the role of chromatin modifications caused by HDAC2 in neuronal mechanisms of synaptic plasticity and memory formation. Experiments performed on animal models of AD such as Tg2576 mice, consistently demonstrated that in these animals daily injections of 4-phenylbutyrate reversed spatial memory deficits via normalization of hippocampal tau phosphorylation level, without affecting Aβ levels [130]; whereas on APP23 mice a daily administration of low dose of valproic acid (30 mg/kg, i.p.) significantly reduced Aβ plaques formation, and this effect was highly visible by administrating the drug during the early mouse life (seven months as minimum) [131].

Nicotinamide administration induced chronic but slight increase in endogenous p25, leading to improvement in learning and memory skills. Although nicotinamide possesses a class III HDAC inhibitor effect, it has been demonstrated that it has both sirtuin-dependent and -independent functions. These findings suggest a therapeutic beneficial role of nicotinamide via oral administration as medical treatment for this disease.

I.B.7.4. Amyotrophic Lateral Sclerosis (ALS)

The Amyotrophic Lateral Sclerosis (ALS) is a multifactorial disease where the motor neurons are injured. Among the multiple mechanisms that trigger neuronal death, alterations in genes transcription is crucial to perturb the cellular homeostasis, leading to the activation of many deleterious pathophysiological cascades. Recent findings demonstrate that the administration of sodium phenylbutyrate, an HDAC inhibitor, significantly extended survival and improved both clinical and neuropathological phenotypes in the G93A transgenic ALS mice model. Phenylbutyrate administration ameliorated histone hypoacetylation observed in G93A mice and induced expression of Nuclear Factor-kB (NF-kB) p50, the phosphorylated inhibitory subunit of NF-KB (pIKB) and beta cell lymphoma 2 (bcl-2), but reduced cytochrome c and caspase expression. Moreover, curcumin, an NF-KB inhibitor, and mutation of the NF-KB responsive element in the bcl-2 promoter, were found able to block the butyrate-induced bcl-2 promoter activity. Several evidence demonstrated that the pharmacological induction of NF-KB dependent transcription and bcl-2 gene expression is neuroprotective in ALS mice by inhibiting programmed cell death. Phenylbutyrate phosphorylates IKB, allowing the translocation of NF-KB p50 to the nucleus, or acts directly on the acetylation of NF-KB p50. NF-KB p50 mediates the transactivation of bcl-2 gene expression which up-regulation blocks the cytochrome c release and the subsequent caspase activation, slowing motor neuron death. Several studies report that transcriptional and post-translational pathways ultimately promote motor neuron survival and ameliorate disease progression in ALS mice. According with these findings, it has been provided a possible role of phenylbutyrate as therapeutic approach for the treatment of patients with ALS [132].

I.B.8. HDAC Inhibition in Animal Models of cerebral ischemia

Stroke is the third cause of mortality in the United States and the first in terms of disabilities in adults [133]. It displays an annual incidence of 600,000 new victims which 30% remains severely disabled. Several stage III clinical trials failed and their inefficiency is mostly due to a limited time windows for medical first aid. In order to shed light to new neuroprotective strategies to rescue and repair the ischemic injury, new molecular mechanisms need to be discovered.

In this field, modulation of translational mechanisms could be efficient subject of study among molecular strategies. Ischemic stroke could involve the whole brain (global ischemia) or just a limited area (focal ischemia). Global ischemia occurs in case of cerebral blood flow accidents such as cardiac arrest, surgery, hemorrhage and gaseous asphyxia via monoxide poisoning. Focal ischemia, instead, refers to localized cerebrovascular interruptions leading to selective damages. Hippocampal neurons in CA1 region are very sensible to the blood pressure decrease and their injury usually associates with severe cognitive disabilities.

The substantial delay occurring between ischemic injury and neuronal death is consistent with a role for transcriptional changes. The transcriptional repressor RE1 silencing transcription factor REST (also named NRSF), widely expressed during the embryonic development, plays a crucial role in terminal neuronal differentiation [134, 135]. REST represses specific neural genes involved in synaptic plasticity such as vescicular proteins that play a pivotal role in trafficking mechanisms, structural proteins, voltage-gated channels and ligand-operated receptors [134,135]. REST activity is expressed both in neural progenitors that in non neural cells. During neuronal differentiation, REST orchestrates a large set of epigenetic modifications splitting up neuronal cells from non-neuronal cells. Recent studies report that the high vulnerability of hippocampal neurons during a global ischemia strictly associates with anomalous accumulation of nuclear REST that represses some

transcriptional mechanisms needed for the neural function [136]. In fact, REST represses specific target genes via binding of MeCP2 and other corepressor complexes that possesse Histone 3 Lysine 9 (H3K9) deacetylase and methylase functions. Recent findings proved further the role of REST on hippocampal selective neuronal loss via recruitment of CoREST, G9a and MeCP2 to the promoters of specific genes which epigenetic remodeling is cause of deleterious effects of the global ischemia [137]. The abnormal regulation of REST remodelling also affects a widespread number of others disorders such as the pathogenesis of the Down's Syndrome, Alzheimer's disease, Huntington's disease, epilepsy and X-linked mental retardation [134, 135]. Two recent studies demonstrate the therapeutic role of HDAC inhibitors in the field of neuronal loss and cognitive decline in neurons injured by ischemia. In fact, Chiarugi and collegues reported that intraperitoneal SAHA injection (HDAC inhibitor) in mice from 0 to 6 h after ischemic insult obtained through the middle cerebral artery occlusion (MCAO), was able to prevent H3 deacetylation and promote the neuroprotective expression of Bcl-2 and Hsp70. Thus, the resulting reduction in volume infarct suggests a neuroprotective effect for SAHA [138]. Furthermore, Moskowitz, Dirnagl and collegues observed that the abnormal DNA methylation in mice subjected to ischemic MCAO model was prevented by administration of a combination of the demethylating agent 5-aza-2'-deoxycytidine and trichostatin A (HDAC inhibitor), conferring stroke protection. This effect was evident in mice subjected to mild but not to severe ischemic insult [139]. These findings highlight the potential therapeutic role of HDAC inhibitors in case of ischemic stroke as neuroprotective intervention [67].

I.C. SODIUM CALCIUM EXCHANGER

The Na^+/Ca^{2+} exchanger (NCX) is a nine transmembrane protein, distributed throughout the brain and the heart. NCX was primarily discovered and characterized in late 1960s from many independent laboratories: from Baker et al. in the UK [140, 141], also from Reuter and Seitz in Germany and Switzerland [142], and from Martin and De Luca in the United States [143].

These laboratories individually discovered the presence of a countertransport mechanism that exchanged Na^+ and Ca^{2+} ions across the plasma membrane of different excitable but also non excitable cells. In 1988 and in 1990, Philipson and colleagues successfully performed the purification and cloning of the first isoform of NCX, the so-called NCX1, and some years later, the same investigation group cloned NCX2 [144] and NCX3 [145]. Among NCX isoforms, NCX2 and NCX3 are selectively expressed in the brain [146] and in the skeletal muscle [145]. The regulation of intracellular ionic concentrations of the previously mentioned cations, plays important roles in several cellular homeostasis mechanisms in excitable cells. In fact, sodium regulates cellular osmolarity, plays a crucial role in the induction of action potential [147], and also acts in transducing signaling pathways [148].

Importantly, also calcium is involved in several cytosolic intracellular signaling mechanisms as second messenger. The sodium calcium exchanger works in association with other selective ionic channels and ATP-dependent pumps involved in the physiological mechanism of regulation of cytosolic ions concentrations [149]. NCX exchanges one Ca^{2+} ion with three Na^+ ions in a bidirectional working manner (forward and reverse). The *forward mode* operates extruding one Ca^{2+} ion with the influx of three Na^+ ions. This mechanism plays a crucial role in restoring intracellular Ca^{2+} levels as consequence of physiological rises of the cation. By contrast, in the *reverse mode* NCX carries out three Na^+ and

one Ca^{2+} enters the cell. This operating mode is essential to re- equilibrate the Na^{+} concentration after membrane potential depolarization rises, thus reducing the transmembrane Na^{+} electrochemical gradient. The typical accepted stoichiometry 3:1 for NCX has been revisited in the last years. In addition to the major 3:1 stoichiometry, new ionic flux ratios from the 1:1 to a maximum of 4:1 are validated as functional working mode. The exchanging depends on the intracellular concentration of the two cations [150-151].

I.C.1. State of Art of NCX1, NCX2 and NCX3 isoforms

The knocking-out of NCX1 brings to embryonic lethality, whereas the overexpression of this exchanger isoform triggers heart dysfunction. To overcome these limitations and to shed light on neuroprotective role of NCX1, two conditional genetically modified mice have been generated. These animals, under tamoxifen administration, were able to decrease or increase NCX1 expression in neurons belonging to cortex and hippocampus, the two main regions involved in stroke. It has been demonstrated that the conditional overexpression of NCX1 in hippocampal and cortical neurons was able to trigger the phosphorylation of Akt [152], that elicits neuronal survival during brain ischemia. By constrast, the conditional knock-out of NCX1 in the same neuronal populations reduced the amount of phospho-Akt (p-Akt), leading to a worsening ischemic brain damage [153].

The neuroprotection exerted by the ischemic preconditioning is reached through different molecular effectors. It has been found that the isoforms 1 and 3 of the Na^{+}/Ca^{2+} exchanger play a crucial role in this protective mechanism. It has been demonstrated that the reduction of NCX1 expression induced by ischemia was prevented when the animals were exposed preventively to preconditioning stimulus. After preconditioning plus tMCAO, NCX1 and NCX3 expression was markedly increased

compared to ischemic non preconditioned animals. Preconditioning triggers also the increase in the phosphorylation of Akt, further demonstrating the neuroprotective role of this protein related to NCX1 and NCX3 [154]. The neuroprotective preconditioning effects are dependent on several mechanisms, among these the activation of sensor molecules (such as hypoxia inducible factor-1 - HIF-1) is crucial for neuroprotection. In particular, HIF-1 is a nuclear effector, which expression is induced by OGD or ischemic preconditioning and upregulates the transcription of NCX1 determining a neuroprotective effect in ischemic brain [155]. The regulation of NCX1 transcription has been widely investigated and it has been found that during tMCAO in rats Sp3 (specificity protein 3) and Re-1 silencing transcription factor (REST) colocalize with HDAC1 and HDAC2 on ncx1 promoter thus repressing NCX1 expression, whereas after preconditioning stimulus NCX1 transcription is elicited by the transcriptional activators Sp1 and HIF-1 forming a complex with histone acetyltransferase p300 on ncx1 promoter. Changes in NCX3 mRNA and protein expression were not associated with either the REST/Sp3/HDAC1/HDAC2 complex in tMCAO or the Sp1/HIF-1/p300 complex in ischemic brain preconditioning [156]. Nowadays, the regulation of ncx3 promoter activity is not yet investigated during ischemia or after ischemic preconditioning. It has been demonstrated by Jeon et al. that NCX2 has also a neuroprotective role, in fact knockout mice for NCX2 subjected to tMCAO displayed an increased neuronal loss with a consequential enlargement of infarct volume compared to wild-type animals. In a model of ischemia *in vitro* on hippocampal slices from ncx2-/- mice, it has been reported an increase of intracellular calcium level with membrane depolarization and slower recovery in population spike amplitudes compared to wild-type. These results indicate that NCX2 working in forward mode plays a neuroprotective role, removing calcium from the cells during ischemia [157].

I.C.2. Distribution of NCX isoforms in different tissues

NCX1 is the most expressed protein of the SLC8 gene (solute carrier gene) family encoding for the Na^+- Ca^{2+} exchangers. It was chiefly characterized and cloned as cardiac protein, then it has been disclosed in brain and kidney and minimally in other tissues [158–159]. NCX1 gene is alternatively spliced into two well-known sites giving tissue specificity [146, 160].

The first site is located at the 5' untranslated region (5′UTR) *a*nd does not alter the structure of the encoded protein. Otherwise, the presence of three different promoters independently drives the tissue specific expression for NCX, supposedly in response to different physiological requirements. [161,162].

The second site of splicing takes place into the coding region of NCX transcript, whereas two mutually exclusive and four cassette exons encode for a huge number of isoforms that differs just for the cytosolic inner portion of the exchanger [146,160,163].

The two mutually exclusive exons includes the exon A in the transcript of excitable cells, primarily muscular and nervous cells, and the exon B in non-excitable cells. Nowadays, a combinatory pattern for cassette exons in tissues is not still available. Unlike NCX1, the other members of the SLC8 family show a more stringent tissue-specific expression pattern. In fact, NCX2 is present in neurons, but minimally in other sites and NCX3 was found mainly expressed in the brain and skeletal muscle [164]. Recently, NCX3 has been detected also in the immune system and bones. Remarkably, NCX3, but not NCX2, is alternatively spliced and these spicing variants differ for the cytoplasmic region as it has already been demonstrated for NCX1. Since the three Na^+ - Ca^{2+} exchangers, NCX1, NCX2 and NCX3 isoforms display a high homology of sequence, it is widely accepted that structure and the functional role of NCX1 can be generally extended also to NCX2 and NCX3 isoforms [159-165].

I.C.3. Molecular Biology of NCX3

The cloning of the Na^+- Ca^{2+} exchanger isoform 3 (NCX3) was achieved from rat at the Philipson's laboratory in 1996 [145]. NCX3 displays approximately the 80% sequence homology with the other isoforms of the Na^+-Ca^{2+} exchanger family NCX, including NCX1 and NCX2. The NCX structure expresses a different percentage of homology within the isoforms sequence. For istance, the 9 transmembrane domains share more than the 75% of homology within NCX1 and NCX2, but not the cytoplasmic f-loop with just the 60%, thus suggesting that the transport mechanism requires the conservation of the transmembrane regions, whereas the NCX3 capacity is specifically restricted to the cytoplasmic loop. The exact structure of calcium-binding domain (CBD1) of NCX3 remains unknown. Nonetheless, the conservation of some key acidic and basic residues leads to the same Ca^{2+} binding sites and a structure most likely similar to NCX1. Therefore, the regulation of NCX3 capacity of exchange by CBD1 is probably comparable to NCX1 [166]. The comparison of CBD2 has proven to be more difficult because of the alternative splicing of the NCX family causing various possibilities of sequence for CBD2.

The NCX gene is spliced in different variants. It has been described more than 15 splicing variants for the isoform 1 and a exiguous number of variants for NCX3. NCX2 is the unique gene that does not present alternative splicing forms. NCX1 gene displays a tissue specific distribution [160,163;167]. The NCX3 gene is composed of 9 exons (exons from 1 to 9) [163]. Interestingly, exon 2 and 3– also named exon A and B, respectively - are mutually exclusive, whereas exon 4, named exon C, is optional. Thus, in the rat, three splice variants are detected. A variant containing exon A and C is found in skeletal muscle (NCX3-AC), while variants expressing the exon B are expressed in the brain (NCX3-B and NCX3-BC). Furthermore, in humans three truncated forms of NCX3 have been discovered of which two variants are expressed in the fetal brain (exons4- 9 and 6-9) [168]. The third truncated variant is expressed in

skeletal muscle (exons 2 and 6- 9) [169]. These human truncated forms are likely to be under the control of alternative promoters. Recently, the comparison of CBD2 deriving from NCX3-B and NCX1-AD exhibited a similar conformation in structures like the β-sandwich and the α-helix of the F-G loop [170]. The splicing variants of NCX1 display a various orientation in the α-helix which has been found associated with the activation or inhibition during the rise in intracellular calcium $[Ca^{2+}]_I$ [171]. Conversely, the NCX3 helix found subsequently to the splicing region shows a unique orientation among the splicing variants, expressing an $[Ca^{2+}]_i$-dependent activation. NCX1 exons A and B have been found not only individually involved in the signal transmission to the transmembrane domains, but also in the mechanism to relieve the Na^+-mediated inactivation [166]. The other NCX1 exons (C, D, E, and F) may regulate the Ca^{2+}-binding affinity of CBD1 [172]. Overall, NCX3 splicing exons A, B and C are evolutionary well conserved among species, even a minor sequence homology compared with the NCX1 and NCX2 exons, which has been estimated approximatively around 55 and 65 %.Thus, it is quite impossible to apply the effect observed during the alternative splicing of NCX1 to NCX3. In addition, among the splicing variants, NCX3 exhibited different Ca^{2+} binding sites, which were identified three for NCX3-B and two for NCX3-AC. Interestingly, they expressed a different affinity for the Ca^{2+} [173].

According to the NCX structure, we can infer that differences among the axon A and B may have effect both on the Na^+-dependent inactivation [166] and on the sensitivity to Ca^{2+} through changes in conformation of CBD1-CBD2. Finally, we can assume that in the last years important works elegantly investigated the mechanism of translocation in NCX and its regulation though CBD1 and CBD2 domains. Remains to reveal the mechanism that allows to the intracellular loop to influences the transmembrane segments and shed light on the role of the different alternatively splicing form of NCX3 [174].

I.C.4. Identification of NCX3 minimal promoter sequence

In the 250 bp region immediately upstream of the exon 1 it has been identified the human SLC8A3 minimal promoter that displays similarities with SLC8A1 brain promoter. SLC8A3 minimal promoter includes in its sequence Sp1 and AP-2 (Activating Enhancer Binding Protein 2 Alpha) elements that are able to confer constitutive transcription and tissue specific expression, respectively. GC bases enrich both the sequences of SLC8A3 promoter and of exon 1, forming typical cytosine and guanine dinucleotide (CpG) island as it occurs at the 5' end of several housekeeping genes and oncogenes. The sequence of SLC8A3 promoter includes multiple AP-2 sites important for the regulation of physiological functions in nervous system, in fact members of the AP-2 transcription factor family are crucial for the vertebrate development of neuronal tube and neuronal crest derivatives. During mouse development, these transcription factors also differentiate in spatial and temporal expression [175]. The members of AP-2 family display an high tissue specific expression in neuroepithelium and neural crest [176]. Several consensus elements for AP-2 factors are present in promoters of peculiar neuronal genes such as choline acetyltransferase, proenkephalin; human fragile mental retardation promoter [FMR1], [177,178] as well as in other cellular types and genes (i.e., keratin, sodium-phosphate cotransporter [Npt2] of kidney proximal tubules and viral genomes as MMTV [179–180]). A single promoter region is required for the tissue-specific expression of the *SLC8A3* gene, whereas the transcription of SLC8A1 gene depends from multiple tissue-specific promoters. SLC8A3 gene contains elements for the muscle-specific transcription factors MyoD (Myogenic Differentiation) and GATA factors 2/3; in agreement with the fact that the ortholog rodent gene is predominantly expressed in brain and skeletal muscles [145]. The SLC8A3 promoter has also consensus sequences for transcription factors AP1 (activating protein-1) , and Egr-1 (Early growth response protein 1 -

also known as NGF1-A, Krox24, Zif/268, or Tis 8). These are a subgroup of immediate early genes (IEGs) that are activated rapidly and transiently in neuronal cells in response to environmental stimuli, such as neurotransmitters and neurotrophin receptor stimulation, regulating the expression of subset of genes termed delayed-response genes. The dimerization of the Jun proteins (c-Jun; JunB and Jun D) and Fos proteins (c-Fos, FosB, Fra-1, etc.) through their leucine zipper motifs leads to the formation of AP-1 complex that binds to the consensus sequence TGACTCA. This sequence was first named as the element responsive to phorbol ester activation of protein kinase C (PKC). Jun proteins can also homodimerizes or heterodimerizes with Fos or with transcription factors of the ATF/CREB family. The composition of the AP1 complex can affect the affinity for consensus sequences and influence transactivation potential. Many different stimuli can regulate the transcription factors of the Egr family in mammalian brain, since they lack of bZip domain their binding to DNA can be achieved through zinc finger motifs. Egr-1 is induced rapidly after the activation of the NMDA receptor [181]. This increase of Egr-1-mRNA has been demonstrated in primary cultures of cerebellar, corticostriatal, cortical, and hippocampal neurons after stimulation by intraperitoneal administration of NMDA [182]. The MK-801, a blocker of NMDA receptors, downregulates the expression of Egr-1 [183]. The physiological synaptic activity can stimulate NMDA receptor and upregulates the Egr-1 expression, whereas kainate triggers the induction of all four members of the Egr family [184,185]. The link between Egr-1 and SLC8A3 gene transcription is not yet clarified, however it seems that NMDA receptor activation triggers the upregulation of SLC8A3 transcription. The physiological role of this functional link may be due to the role played by NCX3 in extruding calcium after NMDA receptor activation. A functional link between NMDA receptor and NCX3 is also suggested by their high expression in the hippocampus, especially in the CA1 region [182,164]. The SLC8A3 gene promoter contains the sequence

TGACGTGC identified like a CRE element [186, 187]. The CRE element is included in several other cAMP-responsive gene promoters, such as cfos, Zif/268, or Egr-1 and proenkefalin [188]. Calcium influx as well as cAMP triggers the activation of the c-fos gene through the CRE element that, for this reason, is called Ca^{2+} response element (CaRE). The presence of the CRE element has attracted much attention since it could confer rapid inducibility to the SLC8A3 gene, similar to that of IEGs. Several DRE elements are present in the SLC8A3 gene in the region downstream of the TATA box and in the exon 1; these sites are able to bind DREAM (downstream regulatory element antagonist modulator) [189,190,191-192].

Figure 3. Regulation of ncx3 human gene transcription: role of DREAM and CREB. Gabellini N . Molecular Neurobiology, 2004**.**

I.D. PUTATIVE TRANSCRIPTION FACTORS REGULATING NCX3 AND THEIR ROLE IN NEUROPATHOLOGICAL CONDITIONS

I.D.1. CREB

In neurons, a wide range of extracellular stimuli activates signaling pathways like so CREB family members. Gene expression dependent by CREB pathway activation has been implicated in a complex and various series of processes ranging from development to plasticity, and eventually related also to diseases. CREB (cAMP response element binding protein) belongs to the bZIP superfamily of transcription factors. Within this superfamily, CREB and the closely related factors CREM (cAMP response element modulator) and ATF-1 (activating transcription factor 1) include a subcategory referred to as the CREB family. Belonging to the bZIP transcription factors, CREB family members contain a leucine zipper domain that facilitates CREB dimerization, and a C-terminal basic domain which role is binding with DNA. There is a high degree of homology among CREB, CREM, and ATF-1, allowing them to form both homo- and heterodimers, and each of these complexes takes place at the same side of *cis*-regulatory element as reviewed in De Cesare et al. 1999; Mayr and Montminy 2001 and Shaywitz and Greenberg 1999 [193, 194, 195]. The remaining domains of CREB family members mediate the interaction with coactivators and components of the transcriptional machinery to the DNA, facilitating RNA synthesis of genes having an important role in the nervous system. The most common CREB isoforms, $CREB_{\alpha}$ and $CREB_{\Delta}$, display a kinase inducible domain (KID) between two domains enriched of glutamine residues, the so-called Q1 and Q2/CAD (constitutive active domain). The unique difference between these two activators is the presence of an α domain. Following stimulation, the KID domain

phosphorylated at the residue of Serine 133 (Ser-133), can bind the transcriptional coactivator, CREB binding protein (CBP) via its KIX domain [196, 197, 198, 199]. As result of the interaction stimulus-dependent between these two domains, the transcriptional machinery starts the synthesis of the inducible genes. Q2/CAD domain interacts with components of the basal transcriptional machinery allowing the stimulus-independent CRE-driven gene expression [200, 201, 202, 203].

I.D.2. DREAM

DREAM was primarily identified as the transcriptional repressor of the human gene for the opioid peptide dynorphin. Dynorphins are members of the opioid peptide family which are involved in memory formation and chronic pain adaptation. Dynorphin peptide controls the release of neurotransmitters as well as it can block LTP in the hippocampus by inhibiting excitatory neurotransmission from synaptic terminals [204, 205]. The DNA sequence required for the transactivation of prodynorphin transcription via cAMP and Ca^{2+} has been identified in the first exon of the gene (at position +40), and termed dynorphin downstream regulatory element (DRE) [190]. The transcription factor binding to the DRE was subsequently identified and includes four Ca^{2+} binding domains of the EF hand type. Nowadays, it represents the unique transcription factor known to be directly regulated by Ca^{2+}. When loaded with Ca^{2+}, DREAM undergoes conformational changes causing its detachment from DRE sites, thus relieving the transcriptional repression [206,207]. The ability to bind DNA of some members of the basic helix-loop-helix (bHLH) transcription factor family is also regulated by Ca^{2+}, through the interaction with Ca^{2+}-loaded calmodulin [208,209].

The DREAM sequence presumably includes four EF-hands, displaying high homology to Ca^{2+}-binding proteins of the recoverin subfamily. Mutation of two residues within any of the functional EF-hands, the so-called EFmutDREAMs, did not affect the binding to the DRE but

completely prevented the unbinding in the presence of increasing concentrations of Ca^{2+}. It seems like the derepression of DRE-dependent gene expression after PKA activation [190] is related to specific protein–protein interactions between DREAM and nuclear effectors of the transcriptional effects of cAMP. Analysis of the 5' regulatory regions of many Ca^{2+}-regulated genes has shown the presence of one or several functional DRE sites downstream from their TATA boxes [190, 191]. This suggests that DREAM mediated transcriptional derepression is a generally required step prior to transcriptional activation of many genes.

A new search showed identity between DREAM and calsenilin, a protein able to interact with presenilin-2 found in a yeast two-hybrid screen using the carboxy terminal part of presenilin-2 as a bait [210]. Double immunostaining after overexpression of N-tagged calsenilin in Cos cells showed the colocalization with presenilin-2 in the cytoplasm and the endoplasmic reticulum and suggested that the colocalization at the ER, in particular, was increased after calcium stimulation [210]. More recently, also using a yeast two-hybrid screen to identify components of the protein complex responsible for the A-type potassium currents, a protein identical to DREAM called KchIP-3 (potassium channel interacting protein 3) was found to selectively interact with the amino terminal region of Kv4 potassium channels [211].

Interestingly, the interaction with the potassium channel does not depend on the presence of calcium. However, the change in KchIP-3/DREAM conformation that follows binding to Ca^{2+} profoundly affects channel properties [211]. Interestingly, in this study two other proteins related to DREAM, KchIP-1 and -2, were identified. In terms of the interaction with potassium channels, KchIP-1 and -2 are similar to KchIP-3.

However, it is not known whether they are also able to interact with presenilin-2 and especially, whether they are able to specifically bind to DNA. Thus, the possible existence of a DREAM family of nuclear repressors remains to be investigated. Taken together these results

indicate that DREAM/KchIP-3/calsenilin might have pleiotropic functions through the interaction with specific DNA sequences and/or with proteins in different cell compartments [212-213].

DREAM, calsenilin, and KChIP3 are the products of a unique gene [214, 215], although the proteins are localized in different cellular compartments: nucleus, endoplasmic reticulum, or lysosomes and plasma membrane, respectively. Regulation of mRNA translation at alternative start codons may generate proteins with variable N-terminal peptides, which could be important for their localization and function. Furthermore, isoforms lacking the EF-hand domains are generated by alternative splicing in mouse [215,192].

I.D.3. SP transcription family factors

The Sp/XKLF family (Specificity protein/Kruppel-like factor) is a family of transcription factors that share a common structure of cysteine histidine zinc fingers. The so-called Cys2His2zinc are conserved sequences placed in specific pattern and allow to the factors to bind DNA sequences [216]. The Kruppel-like factor name comes from the studies performed on the segmentation gene discovered in Drosophila. In fact, this gene has been discovered to express the same zinc finger motif as well as in the human orthologue. In humans, it is well known as Sp1 transcription factor [217].

Sp1 factors contact the DNA in threes specific regions of their sequence at level of residues KHA, RER and RHK, respectively for the first, second, and third zinc finger.

Sp/XKLF factors bind DNA sequences having the box sequence GC-(GGGGCGGGG) and GT-(GGTGTGGGG), even changes in aminoacidic zinc finger are responsible for their binding affinity. GC and GT boxes play a crucial role in the transcriptional events related to the expression of housekeeping genes as well as tissue specific and viral genes [216]. Interestingly, these motifs have been found to have a role in maintaining

the demethylated state in CpG islands, as well as for the adenine phosphoribosyltransferase (APRT) genes.

Sp family includes two major subcategories: Sp1 and KLFs. Sp1 factors share a similar structure at the N-terminal side but not at level of their zinc finger. KLFs, instead, represent a more heterogeneous group of factors [216]. Nowadays, Sp/XKLF family includes many homologies in the transcription factors members. In fact, among this family has been detected 8 Sp and 15 KLFs factors. Sp factors from 1 to 8 (Sp1-8) sequence takes place near the HOX gene cluster.

Sp1 and Sp7 (Osterix) are placed on the Chromosome 13 at 12q13.13 (HOX C); whereas Sp2 and Sp6 (KLF14) on 17q21.31/32 (HOXB); Sp3 and Sp5 on 2q31.1 (HOX D); Sp4 and Sp8 on 7p21.2 (HOX A). The regulation mechanism for the transcription of these genes through GC and GT boxes is extremely complicated. Moreover, EKLF (Erythroid Kruppel-like Factor), Sp4 and Sp7 has been discovered to be expressed specifically in same tissues as confirmed in *in vivo* experiments with mice [218, 219]. Sp1 and Sp3 are homely expressed albeit expressing specific functions are shown in gene ablation studies [220].

I.D.4. Structural Characteristics of the Sp Factors

Sp1 was primarily identified as trans-activator factor of the SV40 early promoter [221]. In the past, many works considered Sp1 as an essential factor for the regulation of transcription of the genes having GC or GT boxes . Anyway, this thought was abandoned when different transcription factors, called Sp2, 3 and 4, were cloned. Later, they discovered also Sp5, Sp6 or KLF14, Sp7 or Osterix and Sp8 [222]. Sp5-8 share various brief domains with Sp1-4. Among the Sp factors from 1 to 4, (Sp1, Sp2, Sp3 and Sp4) the presence of similar modular structure make possible to include these four factors in a subgroup. Instead, Sp1, Sp3 and Sp4 contain two principal glutamine-rich transactivation domains named A and B involved in the transcription activation. A series of serine/threonine-rich

sequences are placed adjacent to the A and B domain, being a possible target of post-translational modification. Sp2 factor has a unique glutamine-rich domain albeit it presents a highly charged domain C and a serine/threonine-rich region in common with the other factors. Sp2 displays a different consensus-binding site as consequence of a substitution of a residue of histidine with a leucine residue in zinc finger 1 [223]. The Sp factors generally have in their sequence a box following N-terminal to the zinc finger domain named Buttonhead box [224]. The Buttonhead box (Btd element) includes a sequence of 11 conserved amino acids having a possible role in the transactivation potential of the factors as confirmed by the deletion of an overlapping region that induce a reduction in Sp1 activity *in vitro* [225]. Moreover, domain C, and primarily its Btd element [226], are involved in activation SREBP-mediated (sterol-regulatory element-binding proteins) of Sp1 or Sp3. Harrison and collegues in 2000 identified another conserved sequence, named Sp box (SPLALLAATCSR/KI), at level of the N-terminus of the proteins. Closely to the N-terminus, this element contains an endoproteolytic cleavage site that functions as target proteasome-dependent degradation *in vitro* [227].

Even not required for cleavage, the highly conserved sequence of the Sp1 box indicates that could have a role in regulation of Sp factors proteolysis. Another possible role for the Sp box could refer to the control of transactivation potential through interaction with a presumed repressor [228]. Although the functions of the Btd and Sp boxes are not completely clear, their absence in the XKLF subgroup suggest a relationship between the Sp transcription factors. Moreover, the N-terminal regions of Sp5-8 are completely different, except for the Btd and the Sp boxes, from those of Sp1-4 and more closely related to each other.

I.D.5. Early growth response-1 (EGR-1)

Egr-1 is a member of immediate early genes and it has three C_2H_2 zinc fingers DNA-binding domains that can recognize GC- rich sequences of target genes. This transcription factor can be induced rapidly by various stimulants such as growth factors, neurotransmitters, stress and injury. EGR-1 is widely expressed in the brain and regulates the transcription of different target genes, among these BDNF is one of the most important. In particular, several studies suggest a detrimental role for EGR-1 because of its upregulation after ischemic stroke [229]. Interestingly, it has been demonstrated that after ischemic insult, EGR-1 was upregulated and by binding the promoter of BDNF gene decreased its expression. This mechanism leads to a worsening of ischemic injury by attenuating neuroprotective effects of BDNF [230].

I.D.6. Activator protein 2 (AP-2)

Transcription factor AP-2α (TFAP2α) belongs to AP-2 family transcription factors which consists of five members either in humans and in mice. All AP-2 transcription factors are encoded by seven exons except for AP-2δ, and they share, at the carboxyl terminus, a highly conserved domain consisting in a helix-span-helix that is able to determine the dimerization and the binding to DNA in concert with a central basic region. In fact, these transcription factors are capable of forming heterodimers or homodimers. The N-terminal region comprises the transactivation domain, rich in proline and glutamine. AP-2 transcription factors have a nuclear localization because of their particular role in regulating gene transcription. AP-2 proteins are able to establish a cross-talk with several transduction pathways; for example they modulate the pathway of the developmental signaling molecule Wnt. AP-2 factors are susceptible of several posttranslational modifications such as PKA phosphorylation and sumoylation, and they can associate with other various transcription factors like p300, Sp-1, p53. In mice AP-2α, AP-2β and AP-2 γ, are widely

expressed in neural crest cells, in nervous system and throughout the epithelia [231].

I.E. PHARMACOLOGICAL REGULATION OF NCX3 EXCHANGER ACTIVITY

The search for drugs capable of modulating the activity of NCX is evidently more important in function of its possible therapeutic use for those pathological states in which NCX has been proved to be altered.

I.E.1. Inhibitors

Inorganic cations

The Sodium Calcium Exchanger NCX is well known to bind many different cations [232]. Among these, many exert an inhibitory effect due to the direct binding to NCX molecules or via competition with Ca^{2+} thus working as substrate for the antiporter. In fact, Ni^{2+} inhibits the exchanger when it works in reverse mode [232], probably through a competition mechanism that hinders the external transport of Ca^{2+}[232]. The affinity of Ni^{2+} for the three isoforms of NCX is variable. Notably, NCX3 displays a minor sensitivity to Ni^{2+} or Co^{2+} in the order of 10-fold compared to NCX1 or NCX2 [232]. Recently, in NCX1 it was discovered a sequence of three aminoacids involved in the Ni^{2+} specific sensitivity, respectively Asp-130 at level of the alpha1-repeat, and Asp-825 and Glu-837 in the alpha2-repeats [233].

Peptides

A wide number of peptides are mentioned to inhibit the activity of the exchanger.

Endogenous Exchange Inhibitory Peptides

Within the f loop dwells a sequence of 20 amino acids, called XIP, which plays its auto-inhibitory effect via a mechanism dependent by Na^+ [234,235]. The same sequence in a synthetic peptides exerts the same inhibitory function on the exchanger. XIP is reported to block both forward that reverse operation mode of all the three isoforms of NCX, since they share a similar XIP structure sequence. The three specific regions, named XIP1, XIP2, and XIP3 have a very conserved sequences despite some little amino acidic-residue variations [236,237]. When the ligand binds the XIP sequence, a conformational change in C-terminus of the f loop occurs, leading to inhibition of ionic transport [238]. This effect is selectively allowed by the presence of a specific sequence between the aminoacids 5 and 16 of XIP [236,238]. Remarkably, basic and aromatic residues found in this sequence play a crucial role of the XIP-mediated inhibition. It has been synthetized a XIP bearing a molecule of glucose attached to the Tyrosine 6 residue, since the glycosylated peptide more efficiently penetrates the cells. In fact, the link with the glucose allows to the peptide associated to enter the cell via the glucose transporters 1 and 3 [239]. It is interesting to note that male rats infused intracerebroventricularly with the glycosylated form of XIP, express an enormous increase of the infarct volume after Permanent Middle Cerebral Artery Occlusion (pMCAO) [240]. These findings suggest a possible important role for NCX modulation in case of the dramatic effect of neuronal death under ischemia.

Heterocycles

Amiloride Derivatives

Amiloride has been discovered to block NCX activity when used at millimolar concentrations [241]. Its effects are also evident in the inhibition of both epithelial Na^+ channel when used in micromolar concentrations that in Na^+/H^+ exchanger when it is used in the order of millimolar.

Remarkably, this further effect is cause of lack of specificity for the exchanger Na^+/Ca^{2+} NCX. To this aim, in the last year new amiloride analogs have been synthetized. Among the different classes of analogs for amiloride developed, the first class of molecules does not display an inhibitory effect on the epithelial Na^+ channel and plasma membrane NCX, albeit its effect is more evident on Na^+/H^+ exchanger, when it is used in concentrations that may oscillate between 1 to 10 μM [242, 243,244]. Conversely, the second class of amiloride analogs are selective for epithelial Na^+ channel and NCX but not for the Na^+/H^+ exchanger. Notably, three specific compouds belonging to the second group, the dimethylbenzamyloride (DMB), the 3'-4'-dichlorobenzamyl, and the alpha-phenylbenzamil, have been discovered to selectively block the NCX in excitable cells like neurons [242, 243]. Another analogue, the [N-(4-chlorobenzyl)]2,4-dimethylbenzamyl (CB-DMB), is described as the better inhibitor for NCX activity, whereas it does not present an inhibitory effect on Na^+/H^+ antiporter neither for the epithelial Na^+ channels (K_i > 400 μM) [241]. Amiloride analogs efficiency is evident in blocking NCX activity both in forward [243] than in reverse operating mode [245], in fact they compete with Na^+ ion in a reversible manner binding. Notably, it has been recently proposed this category of molecules as Na^+ analogs, indeed they interact with the third Na^+-binding site, acting as inactivators of the exchanger [246].

Diarylaminopropylamine Derivatives: Bepridil

Among the dyarilaminooripylamine derivates, the inhibitory effect of Bepridil is exerterd on various ionic mechanisms, including the Ca^{2+} current mediated through L- [247] and T-Type channels [248], the delayed rectifier K^+ currents, the transient outward current [249], the K^+ current, activated by intracellular Na^+ [250]. Moreover, Bepridil acts also blocking the NCX activity, albeit the effect is dependent by the operation mode of the exchanger [251]. In fact, the Bepridil is more specific in blocking the

NCX activity working in the forward mode than in reverse. Recently, it has been discovered that Bepridil acts on the intracellular portion of the NCX, since its inhibitory effect is lost when cells were treated intracellularly with trypsine [251]. Experimental use of Bepridil, respectively in a model of Chemical Hypoxia *in vitro* and Ischemia *in vivo* (pMCAO), induced a further glial and neuronal damage [252,240].

I.E.2. Activators

Inorganic Cations

Lithium may stimulate, even if with minimum affinity, the Ca^{2+} intake through a mechanism dependent by the intracellular concentrations of Na^{+} for all the NCX isoforms. Its effect is quite low for NCX1 rather than in NCX2 or NCX3 [232]. NCX1 chimeras generated by substitution with the α-2 repeat of NCX3, present a greater sensitivity to Lithium similar to that one described for the isoform 3 of the exchanger NCX. By contrast, the chimeras containing the α-2 repeat of NCX1 expressed in NCX3 presented a loss on lithium sensitivity in NCX3 with values similar to the native NCX1. These findings suggest that the the α-2 repeat is required for both stimulation of NCX isoforms, either in NCX1 that in NCX3 [232,253].

Redox agents

NCX activity is highly sensible to the redox state, since the presence of reducing compounds and oxidating agents induces the activation of the Sodium Calcium Exchanger. Among molecules that influence the redox state and consequently the functional activity of NCX we remember reducing substrates in the form of glutathione (GSH), 1,4-Dithiothreitol (DTT), Fe^{2+}, O_2^{-} superoxide and oxidating agents like Fe^{3+}, H_2O_2, glutathione disulfide (GSSG) and O_2 [254]. It was proposed that these agents could activate the exchanger activity by promoting thiol-disulfide interchange in the protein carrier [254]. Recent studies suggest that the

activation of NCX activity may be due to the reduction of disulfide bond with a consequently formation of new link [254]. Cystein residues associated with the formation of disulfide bonds are presumably the Cys-14, Cys-20, and Cys-780 [255]. The analysis of mutated exchangers has indicated that cysteines are not responsible for the stimulation of the exchange activity induced by a mixture of redox agents [255]. It has been suggested that the stimulation of wild-type exchanger by Fe-DTT is mainly due to the removal of the Na^+-dependent inactivation process [255]. Since redox changes in NCX activity have been implicated in several aspects of cell physiology and pathophysiology, it is possible to speculate that agents capable of stimulating NCX might constitute a possible therapeutic strategy in those pathological conditions in which oxidative stress is involved [256].

Organic compounds

Agonists of G-Protein-Coupled Receptors

It has been reported that the agonists of G-protein-coupled receptors, such as α- and β-receptors, histamine, $5HT_{2c}$, and endothelin-1 and angiotensin-II receptors, are capable of stimulating NCX activity by a pathway which involves either PKA and/or PKC [257, 258, 259, 260, 261, 262].

Peptides

Among the peptides capable of stimulating NCX activity, only insulin and concanavalin A have been proven to exert such effect. In fact, both peptides stimulate Na^+-dependent Ca^{2+} uptake [263, 264].

I.F. ROLE OF NCX3 EXCHANGER IN PATHOPHYSIOLOGICAL CONDITIONS

NCX3 by controlling intracellular homeostasis of calcium and sodium, may play a crucial role in ischemic event. NCX3 assumes two different behaviours in the ischemic core region and in the penumbral region. In the first case, ischemic insult brings to an accumulation of intracellular Na^+ ions because of Na^+/K^+ pump failure, forcing NCX3 to operate in reverse mode. In the penumbral region, NCX3, sustained by ATPase activity, operates in forward mode extruding calcium ions and decreasing cell injury. It has been demonstrated that permanent middle cerebral artery occlusion (pMCAO) is able to cause changes in NCX protein expression in ischemic core and in penumbral region. In particular, 6 to 24 hours after pMCAO, NCX3 decreased in the ischemic core; this event was probably due to the cleavage of NCX3 protein, aroused by the activation of caspases and calpain during ischemia suggesting that the decrease of NCX3 can be involved in the process leading to cell death. By the injection of specific antisense oligodeoxynucleotides (AS-ODNs), NCX1, NCX2 and NCX3 gene products were knocking down. The rats knocking down for NCX3 displayed enlargement of the infarct volume with a worsening of neurological deficits [265]. It has been demonstrated that BHK (Baby hamster kidney) cells stably transfected with the isoform 3 of sodium calcium exchanger, unlike those transfected with NCX1, NCX2 or wild-type cells, displayed a major resistance to chemical hypoxia plus reoxygenation by virtue of their Ca^{2+} buffering properties in conditions of ATP depletion. NCX3 is the unique isoform able to operate in absence of ATP, whereas NCX1 and NCX2 are strictly dependent by this nucleotide for their action [266]. These data suggest a relevant role for NCX3 in counteracting ischemic damage.

A further demonstration of the neuroprotective role of NCX3 in brain ischemia is provided by knockout mice (ncx3-/-) for this isoform of the exchanger. In primary cortical neurons and in organotypic hippocampal cultures obtained from NCX 3 -/- mice and subjected to OGD plus reoxygenation, neuronal death was increased after ischemic insult compared to wild-type mice. Furthermore, ncx3 gene suppression leads to a worsening of brain damage after tMCAO [267].

It has been demonstrated that NCX3 is potentially neuroprotective in a model of Alzheimer's disease. In neurons, the exposure to amyloid-β_{1-42} ($A\beta_{1-42}$) determines an early increase in intracellular calcium concentration that triggers the activation of calpain. Calpain generates NCX3 hyperfunctional proteolytic fragment with a consequential increase of NCX3 currents in reverse mode of operation. In particular, the enhanced NCX3 activity contributes to increase calcium content in endoplasmic reticulum (ER), thus delaying ER stress and cell death. In the late phase, when the NCX3 proteolytic cleavage cesead, a reduction in ER calcium content happens, thus triggering ER stress and consequential neuronal death. These results suggest that neurons activate via NCX3, an early survival strategy against the deleterious stimulus represented by $A\beta\text{-}_{1-42}$ in the early phase of their exposure to this peptide [268]. NCX3 is highly expressed in CA1 and CA3 regions, the hippocampal subfields involved in long term potentiation (LTP), suggesting a role for this exchanger isoform in learning and memory [269, 256]. Because of NCX3 plays a crucial role in removing calcium at the synaptic site of hippocampal neurons [270], it has been investigated its role in LTP modulation. Ncx3 -/- neurons showed an alteration of LTP caused by the reduction of NCX currents in the forward mode of operation. Indeed, the ablation of NCX3 gene, delaying intracellular calcium removal after neuronal activation and increasing the intracellular concentration of this ion in resting conditions, decreases the magnitude of LTP at hippocampal Schaffer collateral-CA-1 synapse level. NCX3 knockout mice displayed a significant reduction in spatial-learning

and memory performances compared to wild-type mice, demonstrating a key role of this exchanger isoform in learning phenomena [271].

I.G. TRANSCRIPTIONAL REGULATION OF NCX3 GENE IN NEURONS AND NEURONAL CELLS

I.G.1. Regulation of NCX3 promoter activity by DREAM in cerebellar neurons

The human NCX3 proximal promoter contains specific enhancers such as cAMP response element (CRE) [169]. Gomez- Villafuertes and colleagues have demonstrated a specific role for DREAM in regulating Ca^{2+} homeostasis and viability in cerebellar neurons. NCX3 is the most abundant Na^{+}/Ca^{2+} exchanger in cerebellar granules, and excitotoxins through the action of calpains trigger the rapid degradation of this exchanger isoform. NCX3 plays a crucial role in the maintenance of Ca^{2+} homeostasis in these neurons, underlying the importance of sheding light on the mechanism regulating NCX3 expression in neurons. DREAM through a mechanism Ca^{2+} dependent can modulate the expression of several genes and it has been found to bind to specific DRE sites to repress transcription [272]. Binding of Ca^{2+} to DREAM via EF-hand motifs reduces its affinity for DNA, leading to its detachment from the DRE sites and to derepression of target genes. DREAM – dependent transcriptional derepression is also observed after protein kinase A (PKA) activation through a mechanism that involves the interaction of DREAM with phosphorylated α-CRE modulator (α-CREM). DREAM interacts with CREB in Ca^{2+}- dependent manner and represses CRE- dependent transcription, preventing the recruitment of CBP [191]. In the kinase-inducible domain (KID) of CREB and in leucine zipper of αCREM there are two leucine-charged residue-rich domains (LCD) that are able to interact with

other two LCDs in DREAM, this physical interaction led to the loss of DREAM binding to DRE sites and derepression.

Since the LCD motif located within the KID in CREM is also present in CREB, and maps in a region critical for the recruitment of CBP, in the absence of Ca^{2+} DREAM binds to the LCD in the KID of CREB [191]. As a result, DREAM impairs recruitment of CBP by phospho CREB and blocks CBP-mediated transactivation at CRE sites in a Ca^{2+}-dependent manner. Gomez-Villafuertes and colleagues have demonstrated that DREAM mediates ncx3 gene repression in the cerebellum of transgenic mice overexpressing the Ca^{2+}- insensitive EF-hand mutant EF-m-DREAM. So, transgenic cerebellar granules have increased levels of cytosolic Ca^{2+} and are less viable when cultured under mild membrane-depolarizing conditions. Importantly, their viability is normalized when their Ca^{2+} extruding ability is restored by lentiviral-mediated overexpression of NCX3. Thus, the Ca^{2+}-modulated transcriptional repressor DREAM controls the expression of the NCX3 protein, which is fundamental for the maintenance of the Ca^{2+} homeostasis and viability of the neurons [272]. A cAMP-dependent regulation of the NCX3 promoter has been associated with the presence of a CRE site [169].The EFmDREAM mutant has an intact leucine-charged residue rich domain, which is responsible of the interaction with CREB but does not respond to Ca^{2+} stimulation, so it should heterodimerize with CREB and function as a dominant mutant to also block the CRE-dependent transcription of the NCX3 gene [191]. Thus, the synergistic stimulation of the NCX3 gene by Ca^{2+} and cAMP signaling would be blocked by the dominant EFmDREAM. The regulation by DREAM is specific for the NCX3 gene, because the DREAM mutant does not affect the transcription of NCX1 and NCX2 [191, 272].

I.G.2. Regulation of NCX3 promoter activity by CREB in SH-SY5Y cell line

In proliferating and differentiated SH-SY5Y cells, it has been investigated the regulation of the transcription of SLC8A3 promoter following the elevation of cAMP and the intracellular calcium increase obtained by partial depolarization of the plasma membrane with KCl. In proliferating and differentiated cells, the SLC8A3- reporter activity was increased about two fold following treatment with the membrane permeable cAMP derivative N^6, 2′-O-Dibutyryladenosine-3′:5′- cyclic monophosphate (Bt_2cAMP). The induction triggered by cAMP was abolished owing to the reporter construct lacking the CRE site in both proliferating and differentiated cells, demonstrating a pivotal role of CRE site in the increase of the SLC8A3- reporter activity cAMP-dependent. In proliferating cells, a partial depolarization of the plasma membrane is achieved through KCl and allows calcium entry. This depolarization that affects the plasma membrane downregulated the transcription of the SLC8A3 promoter via calcium of about 40% compared to untreated control cells. The activity of the reporter construct lacking the CRE site decreased by the Ca^{2+}, was similar to that displayed by the wild-type promoter, indicating that the CRE was not involved in this downregulation. The downregulation that occurs in basal transcription activity could be produced by CaMKII (Ca^{2+} /calmodulin-dependent protein kinase II) phosphorylation of CREB at Ser-142 [169,192]. The negative effect of Ca^{2+} on basal SLC8A3 transcription could depend on the downregulation of AP2 consequent to the activation of PLC (phospholipase C) and PKC (protein kinase C) after depolarization with KCl, shown to occur in SH-SY5Y cells [273,274]. When cAMP and Ca^{2+} levels were raised simultaneously, it is possible to appreciate the inhibitory effect of the Ca^{2+} elevation. In this case, the CaMKII inhibitor KN-93 reversed the downregulation of the cAMP-stimulated activity by Ca^{2+} [275].

Furthermore, to support the involvement of CaMKII activity in this downregulation, it has been evaluated the immunoreactivity of anti-phospho Ser-133 following the cAMP and Ca^{2+} elevation. CREB was strongly phosphorylated following the cAMP increase, contrary to what happens after the depolarizing treatment. However, when cAMP and Ca^{2+} were elevated simultaneously, phospho-CREB was reduced, raising the possibility that phosphorylation of the Ser-142 by the CaMKII could decrease the affinity of the antibody to anti-phospho Ser- 133. However, data were not conclusive because the possibility that KN-93 inhibitor could instead block Ca^{2+} entry through VGCC was not ruled out [276,277]. Furthermore, the cAMP-stimulated activity of the promoter lacking the CRE site was strongly downregulated by Ca^{2+} in proliferating cells.

By contrast, calcium induced SLC8A3 promoter in fully differentiated neurons exposed to BDNF for 4 days via ATF/CREB family, in fact the absence of CRE/CaRE sequence disrupted this induction. BDNF is one of the main regulators of CREB-induced gene transcription and it is an activator of SLC8A3 gene expression in stably transfected cells. The deletion of the CRE element in the SLC8A3 promoter causes the loss of stimulation by BDNF [275]. The phosphorylation of CREB obtained via CaMKII and CaMKIV leads to an increase of promoter activity, in fact CaMKs activation could be triggered during the differentiation promoted by BDNF in neuroblastoma cell line. It was also observed a different phosphorylation pattern of ATF/CREB polypeptides in response to the cAMP elevation in differentiating neurons. BDNF, during the first day of exposure, induces cAMP elevation and apparently causes the phosphorylation of transcription factor ATF1 instead of CREB [275]. It is possible that specific isoforms of CaMKs could be upregulated in differentiated neurons. CaMKII can phosphorylate Ser-63 of ATF-1, corresponding to Ser-133 of CREB, but is unable to phosphorylate Ser-72 of ATF-1, corresponding to the inhibitory Ser-142 of CREB. Therefore transcription factor ATF-1 can be only positively regulated by CaMKII,

suggesting that transcriptional activation of cAMP-responsive genes by CaMKII may be mediated by ATF-1 [278], in fact the Ca^{2+} elevation does not change the extent of anti-phospho Ser-133 immunoreactivity; however it is also possible that the activation of CaMKII and CaMKIV by BDNF induces the phosphorylation of CREB at Ser-142 and Ser-143. The triple phosphorylation of CREB at Ser-133, Ser- 142, and Ser-143 [279] is induced by calcium influx, thus these phosphorylations that occur in these peculiar serines disrupt the interaction of the KIX domain of CREB with CBP, forming a transcription complex different from that induced by cAMP. For this reason, calcium pathway may trigger a specific program of gene expression that is different from that induced by cAMP. The triple phosphorylation of CREB, responsible of the increased transcription status of the SLC8A3 gene via calcium, occurred in embryonic cortical neurons (E18) cultured for 6–8 d but was absent in the earlier stages. In conclusion, the upregulation of SLC8A3 transcription by Ca^{2+} influx is greater in fully differentiated SH-SY5Y exposed to BDNF for 4 d than in cells exposed to BDNF for 1 d [169-192].

I.H. MODULATION OF NCX3 EXPRESSION IN *IN VIVO* AND *IN VITRO* MODELS OF STROKE

I.H.1. NCX3 gene is downregulated in brain ischemia and upregulated in postconditioned ischemic brain

Recent studies explored a new neuroprotective mechanism as potential therapeutic treatment in case of an ischemic stroke episode. A short but sublethal ischemic event, the so-called ischemic post-conditioning, has been found to prevent the harmful effects of primary prolonged ischemia [280]. It has been investigated the role played by the different isoforms of the Na^{+}-Ca^{2+} exchanger NCX (NCX1, NCX2 and NCX3) during the post-conditioning. To this aim, it has been evaluated the expression of the NCX

isoforms in the ipsilateraltemporoparietal cortex of rats exposed to ischemic post-conditioning event. The expression levels of NCX isoforms in postconditioned rats were compared with those of rats which received the tMCAO alone [281].

The reduction in NCX3 expression occurring in cerebral ischemia alone was completely prevented by ischemic post-conditioning. Interestingly, the NCX3 protein expression showed an increase 24 hours after post-conditioning.

In order to evaluate whether this NCX3 protein increase was due to a stimulation of transcriptional events, the levels of mRNA coding for NCX3 were analyzed. Notably, mRNA levels from the ipsilateraltemporoparietal cortex of rats subjected to ischemic post-conditioning at different reperfusion time intervals were analyzed through Real-Time Polymerase chain reaction (RT-PCR) and were compared with those of the same brain region of rats subjected to ischemia alone [281].

After ischemia, the NCX3 mRNA expression was found reduced approximately of 50% at 24 hours after reperfusion, whereas in post-conditioned animals from 5 to 24 hours after reperfusion NCX3 mRNA expression progressively increased compared with sham-operated animals [281].

I.H.2. NCX3 sumoylation participates in SUMO1 protective role during ischemic preconditioning

NCX3 is susceptible also for new post-transcriptional modifications such as sumoylation. The Small Ubiquitin-like Modifier (SUMO) conjugation has been described as enzymatic modification of target proteins via covalent binding of SUMO substrate. As the sumoylation protects the target proteins from degradation, it allows the modulation of intracellular protein localization, activity and stability [282]. Among the SUMO proteins, four isoforms have been identified (SUMO 1 to 4). SUMO 1, SUMO 2 and SUMO 3 are expressed in the brain, whereas the fourth isoform (SUMO 4)

is mainly localized in the kidney [283]. The sumoylation modification is promoted by different stress conditions. In case of focal cerebral ischemia, a different cerebral sumoylation pattern was described [284], and it may play a neuroprotective role. In fact, SUMO-1 knock down mice showed decreased cell survival to the Oxygen-Glucose Deprivation (OGD) model, whereas overexpression of SUMO 1 has been found to protect neurons from OGD-induced injury. Interestingly, among the several neuroprotective molecules supposed to play a crucial role in the ischemic preconditioning, some of these have been described also as possible substrates for sumoylation [285]. Emerging evidences support the role for sumoylation in ionic homeostasis modulation. In fact, recent studies confirmed the presence of sumoylation enzymes and substrates at the plasma membrane, thus sumoylation might regulate stability and expression of the transmembrane proteins interested in the brain ischemia event, specifically NCX3. SUMO1 is involved in the neuroprotective mechanisms elicited by *in vivo* ischemic preconditioning. NCX3, a downstream key player of neuroprotection in stroke [265,154,286] has also been proposed as a new possible target of SUMO1. In rats subjected to tMCAO, the silencing of SUMO1 significantly worsens the ischemic injurious outcome and partially reverted the preconditioning-mediated neuroprotective effects.

Double immunofluorescence analysis for NCX3 and SUMO1 revealed a neuronal signal increase of the two proteins in both preconditioning and preconditioning followed by tMCAO, suggesting a possible spatial and temporal interaction between NCX3 and SUMO1. Bioinformatic analysis of NCX3 sequence displayed the presence of nine putative sumoylation sites that might be recognized by SUMO1. In fact, deletion mutagenesis experiments demonstrated that the removal of either the whole f-loop (252-750 aa), or its subregion (528-676 aa), prevented the co-immunoprecipitation of NCX3 with SUMO1. This suggests that NCX3 sumoylation site was located in the 528-676 aa region of the f-loop, a

region involved in the regulation of NCX stability and activity [34,35]. Interestingly, the SUMO1 silencing produced a downregulation of NCX3 protein levels during preconditioning plus tMCAO ischemic model, suggesting a role for SUMO1 in mediating a protection related to NCX3. Consequently, all these findings present a possible neuroprotective role in which the NCX3 sumoylation mediated by SUMO1 may prevent degradation of NCX3 occurring during ischemic conditions. Furthermore, NCX3 might be one of the SUMO1-mediated neuroprotective mechanisms involved in ischemic preconditioning [287].

I.H.3. OGD/Reoxygenation - induced NCX3 downregulation at post-transcriptional level

Cortical neurons deprived for three hours of oxygen and glucose (OGD *in vitro* model) expressed lower amount of NCX3 protein compared to control neurons. Interestingly, this effect was not found for NCX1 which protein expression levels remained unaffected [288]. The downregulation of NCX3 did not affect the relative transcript decrease in turn, suggesting a possible effect exerted by anoxic and scarce energetic conditions on protein expression through post-transcriptional modifications [288]. In fact, the downregulation of NCX3 in cortical neurons exposed to OGD conditions was prevented by using the inhibitor for the proteasomal system, the MG-132, safeguarding the basal protein expression, whereas three hours of OGD has been found to downregulate NCX3 protein expession via proteasomal system activation [288].

Aim of Study

II. AIM OF STUDY

The human gene for member 3 of solute carrier family 8 (SLC8A3), encoding the Na^+/Ca^{2+} exchanger isoform 3 (NCX3), was identified on chromosome 14q24 and consists of nine exons. Three different splicing isoforms have been found for NCX3, and specifically in the brain the exons 3 and 5 are spliced to form an mRNA that contains only seven exons [275]. NCX3 minimal promoter sequence has been described and it is located at 250 bp region upstream of exon-1 [275]. The consensus sequences for specific transcription factors have been identified in this minimal promoter via bioinformatic analysis. Among the transcription factors able to bind this promoter sequence, it has been found that the transcriptional repressor DREAM and the transcriptional activator CREB decrease and increase NCX3 promoter activity, respectively [275,272]. NCX3 plays an important role in neuronal survival in *in vitro* and *in vivo* models of cerebral ischemia [267]. In fact, the ischemic brain damage is significantly increased by NCX3 knocking - down [267], whereas during ischemic preconditioning (a well-known mechanism of neuroprotection) there is an increase of NCX3 [154]. About transcriptional regulation of NCX3 it has been found that after ischemia NCX3 mRNA expression was reduced approximately of 50% at 24 hours [281] while in ischemic postconditionig ncx3 mRNA is increased of 40% [154,281]. Regarding the transcription factors and the epigenetic mechanisms regulating NCXs, it has been found that NCX1 isoform is reduced in brain ischemia by Sp3/REST/HDAC1/HDAC2 complex [156] whereas it is increased in ischemic brain preconditioning by Sp1/HIF- 1/p300 complex [155], and these changes in NCX1 expression are achieved trought modifications of histone acetylation status of ncx1 promoter. The epigenetic mechanisms such as histone acetylation of NCX3 promoter and consequent changes in NCX3 expression in neurons have never been investigated. The first aim of this study was to evaluate the role of HDACs inhibitors (acting on class I

and II of HDACs) on the modulation of NCX3 gene and protein in primary cortical neurons (DIV 7). We found that pan HDAC inhibitor TSA and HDAC class IIa inhibitor MC1568 both increased ncx3 promoter activity. Importantly, MC1568 increased NCX3 mRNA at 24h and 48h. Furthermore, the other aims of this thesis were to identify the specific isoform or isoforms of HDACs belonging to class II involved in the regulation of ncx3 expression and the correlation between the expression of specific HDACs isoforms and ncx3 gene activity in *in vivo* models of ischemic stroke.

Material and Methods

III. MATERIALS AND METHODS

III.1. Materials

Luciferase reporter kits and luciferase vectors were from Promega (Milan, IT). Synthetic oligonucleotides were from Primm (Milan, IT). siRNAs for HDAC4 (siHDAC4) (SI01836492), HDAC5 (siHDAC5) (SI01521002), HDAC7 (siHDAC7) (SI05620702), HDAC9 (siHDAC9) (SI02024995) were from Qiagen (Milan, IT). The HDAC inhibitor MS-275 (EPS002), MC1568 (M1824) and trichostatin A (TSA) were obtained from Sigma (Milan, IT). These three compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted before application to a final DMSO concentration lower than 0.2%. All common reagents were of the highest quality and were purchased from Sigma (Milan, IT).

III.2. Primary Cortical Neurons

Primary cortical neurons were prepared from 17-day-old Wistar rat embryos (Charles River, Calco, IT) and used after 7 days. Cytosine arabinoside (2.5 μM) was added the second day to reduce glial contamination. The experiments on primary cortical neurons were performed according the procedures described in experimental protocols approved by Ethical Committee of the “Federico II” University of Naples. Briefly, dissection and dissociation were performed in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) containing glucose (30 mM/l). Tissues were incubated with papain for 10 minutes at 37°C and dissociated by trituration in Earl’s Balanced Salt Solution (EBSS) containing DNAse (0,16 U/ml), bovine serum albumin (10 mg/ml), and ovomucoid (10 mg/ml). Neurons, were plated in plastic Petri dishes (Falcon™ Becton-Dickinson, Buccinasco, IT) pre-coated with poly-D-lysine (20 μg/ml), were grown in MEM/F12 containing glucose, 5% of deactivated fetal bovine serum (FBS) and 5% of horse serum (HS), glutamine (2 mM/l), penicillin (50 Units/ml), and streptomycin (50 μg/ml) (Invitrogen, Milan, IT). Cell density was $2x10^6$ cells/well for 12-well plate for luciferase assay, $5x10^6$ cells/well for 60 mm

for qRT-PCR and $15x10^6$ cells/well for 100 mm for Western Blot analysis and ChIP analysis.

III.3. Transfection with Expression Plasmids or Small Interfering RNA (siRNA) and Luciferase Reporter Assay, in Cortical Neurons

Cortical neurons were transfected with 50nM of siCTL, siHDAC4, siHDAC5, siHDAC7 or siHDAC9. For RNA interference, 50nM of specific siHDAC4, siHDAC5, siHDAC7 or siHDAC9 were used. To overexpress HDAC4, HDAC5, and HDAC9 neurons were transfected with above mentioned constructs in the following amounts: 1.3 µg for 12-well plates, 7 µg for 60 mm plates and 15 µg for 100 mm plates. Each transfection was performed at 7 DIV in Optimem with Lipofectamine LTX (15338-100, Invitrogen, Milan, IT), as suggested by the producer. After 2 hours it was replaced with fresh medium. For luciferase assay experiments, cortical neurons at 7 DIV were transfected in 12-well plates. Cells were co-transfected with 1.2 µg of total DNA vectors; the reporters (800 ng each) were the following: (1) the pGL3 construct, (2) the pGL3-ncx3 (*short ncx3 promoter*), (3) the pGL3-ncx3-CREmut (GACGT), (4) the pGL3-ncx3-DREmut (GTCAGTCA). Mutagenesis of the CRE and DRE sites in the promoter was performed using the QuickChange site-directed mutagenesis kit from Stratagene. Each transfection mix also contained 140 ng of the pRL-TK control vector expressing renilla luciferase gene. After a 2h incubation period, the medium was replaced with a fresh one and analyzed after 24h with Dual-Luciferase Reporter Assay System kit (E1910) (Promega, Milan, IT), as already reported [289].

III.4. Quantitative Real-Time PCR (qRT-PCR) Analysis

The first-strand cDNA and quantitative real-time PCR was carried out as previously described [289, 290]. Using 1/10 of the cDNAs as a template, the quantitative real-time PCR was carried out in a 7500 fast real-time PCR system (Applied Biosystems, Monza, IT) by Fast SYBR Green

Master Mix (cod. 4385610; Applied Biosystems, Monza, IT). Samples were amplified simultaneously in triplicate in one assay as follows: heating 2 min @ 50º C, denaturation 10 min @ 95º C, amplification and quantification 35 cycles of 15 sec @ 95º C; 1 min @ 60º C with a single fluorescence measurement. PCR data was collected using ABI Prism 7000 SDS software (Applied Biosystems). After PCR, products were electrophoretically separated on 1,5% agarose gels and bands were visualized with ethidium bromide and documented using a Gel Doc Imaging System (Bio-Rad, Hercules, CA). Normalization of the data was performed by HPRT as an internal control. Differences in mRNA content between groups were calculated as normalized values by using $2^{-\Delta\Delta ct}$ formula and results were tested for significance using Relative Expression Software Tool (REST ©) [290]. The oligonucleotide sequences for NCX3 and Hypoxanthine phosphoribosyltransferase (HPRT) were already published.

III.5. Western Blotting

For Western blot analysis, cells (or tissues) were collected in ice-cold lysis buffer [289] containing anti-protease cocktail (P8340 Sigma, Milan, IT). For HDAC4, HDAC5 (50 µg) and NCX3 (30 µg), proteins were separated on 8% SDS polyacrylamide gels, whereas for DREAM expression, proteins (30 µg) were separated on 12% SDS-polyacrylamide gels. Both were transferred onto Hybond ECL nitrocellulose membranes (Amersham, Milan, IT). Membranes were blocked with 5% non fat dry milk in 0.1% Tween 20 (Sigma, Milan, IT) (2 mM Tris-HCl and 50 mM NaCl pH 7.5) for 2 hours at room temperature, and then they were incubated overnight at 4°C in the blocking buffer with the 1:1000 monoclonal antibodies against HDAC4 (sc-11418), HDAC5 (sc- 11419), DREAM (sc-9142) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and 1:2000 β-actin (A 4700) (Sigma, Milan, IT), 1:5000 α-tubulin (Sigma, Milan, IT), and NCX-3 (Swant, Bellinzona, Switzerland). Finally, after the incubation with primary

antibodies, membranes were washed with 0.1% Tween 20, followed by incubation with secondary antibodies for 1 h at room temperature. Immunoreactive bands were detected with the ECL reagent (Amersham). The optical density of the bands, normalized to β-actin, was determined by Chemi-Doc Imaging System (Bio-Rad, Hercules, CA).

III.6. Chromatin Immunoprecipitation (ChIP)

Brain tissue and cortical neurons were processed into chromatin by use of published protocols [289, 290] with some modifications. Cells and tissues were cross-linked with 1% formaldehyde, and then reaction was stopped by adding glycine to a final concentration of 0.125 M. Brain tissue and cells were washed three times in cold PBS containing proteinase inhibitors and then collected in a buffer containing: 50mM Tris pH 8.1, 1% SDS, 10 mM EDTA, and anti-protease cocktail. For cell and tissue samples, chromatin was fragmented by sonication into 200–500 bp fragments (6 rounds for cells and 15 rounds for brain tissue of 15 1-s pulses at 50% of maximum potency) by a Bandelin Sonopuls HD 2070 ultrasonic homogenizer (Bandelin, Berlin, Germany). Equal amounts of chromatin lysates (50 µg for cells and 70 µg for tissues) were incubated overnight with 5µg of antibody for HDAC4, HDAC5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), acetyl-Histone H4, RNA Polymerase II (Millipore, Milan, IT), and normal rabbit or mouse IgG were used as negative controls. After immunoprecipitation, the DNA-histone complex was collected with 40 µl of salmon sperm DNA/protein A or G -agarose beads for 2 hours (16-157, 16-201) (Millipore, Milan, IT). After rotating for 2 hours at 4°C on a spinning wheel, the beads were washed once with each of the following buffers in the order shown: high-salt buffer (0.1% SDS, 1% Triton, 2mM EDTA, 20mM Tris HCl pH 8.1, 500 mM NaCl); low-salt buffer (0.1% SDS, 1% Triton, 2mM EDTA, 20mM Tris HCl pH 8.1, 150mM NaCl); LiCl buffer (0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris HCl pH 8.1), and two times at room temperature with TE buffer (10mM

Tris pH 8.1 and 1mM EDTA). The precipitated fragments were eluted with a buffer containing 1% SDS and 0.1M NaHCO3. DNA was analyzed by qRT-PCR using Fast SYBR Green Master Mix (cod 4385610; Applied Biosystems, Milan, IT). Heating 2 min @ 50ºC, denaturation 10 min @ 95ºC, amplification and quantification 35 cycles of 30 sec @ 95ºC; 1 min @ 60°C with a single fluorescence measurement. The binding activity was graphically represented as the percentage of total input of chromatin and the results were analyzed using a previously described formula [291].

The following oligonucleotides were used for the amplification of immunoprecipitated DNA: A) Forward 5'-CGAGGAGCGTTCTGAGAGTCTCC -3' and Reverse 5'-GCTTAGCGGTGACTGGAATCTACG -3'. For each amplification, melting curves and gel electrophoresis of the PCR product were used to verify their identities. Samples were amplified simultaneously in triplicate in one assay run.

III.7. Experimental groups

Male Sprague-Dawley rats (Charles River) weighing 250 to 300 g were housed under diurnal lighting conditions (12 h darkness/light). Experiments were performed according to the international guidelines for animal research. All experiments were approved by the Institutional Animal Care and Use Committee of the "Federico II" University of Naples, IT.

III.8. Transient Focal Ischemia

Transient focal ischemia was induced as previously described [155], by suture occlusion of the middle cerebral artery (MCA) in male rats anesthetized using 1.5% sevoflurane, 70% N_2O, and 28.5% O_2. Achievement of ischemia was confirmed by monitoring regional cerebral blood flow through laser Doppler (PF5001; Perimed). Animals not showing a cerebral blood flow reduction of at least 70% were excluded from the

study. Animals that did not show a cerebral blood flow reduction of at least 70% were excluded from the experimental group, as well as animals that died after ischemia induction. Rats were divided into 2 experimental groups: (1) sham-operated (CTL); (2) ischemic, subjected to transient MCA occlusion (tMCAO). The sham-operated animals underwent the same experimental conditions except that the filament was not introduced; in the ischemic group, the MCA was occluded for 100 minutes. All animals were euthanized 24 h after the 100 min tMCAO. Rectal temperature was maintained at 37±0.5°C with a thermostatically controlled heating pad and a catheter was inserted into the femoral artery to measure arterial blood gases before and after ischemia (Rapid Laboratory 860, Chiron Diagnostic). All surgical procedures were performed under an operating stereomicroscope.

III.9. Statistical analysis

The data were evaluated as means ± SEM. Statistically significant differences among means were determined by ANOVA followed by Student-Newman-Keuls test. The threshold for statistical significance data was set at $p<0.05$.

Results

IV RESULTS

IV.1. HDAC class II A inhibitor MC1568 increases ncx3 promoter activity

To evaluate whether a specific class of HDACs could regulate NCX3 expression, experiments of luciferase assays were performed in neurons pre-treated for 2 hours with the pan-HDAC inhibitor Trichostatin A (TSA) at 100 nM [292] or with class I HDAC inhibitor MS-275 at 5 µM [156], and with the class IIA HDAC inhibitor MC1568 at 5 µM [293]. For these experiments we used a pGL3 construct containing the human NCX3 proximal promoter region named (pGL3-ncx3) already used in a previously paper [272]. At 24 hours TSA and MC1568 increased ncx3 promoter activity compared to cells transfected with pGL3-ncx3 alone. By contrast, no modification of NCX3 promoter activity was observed after MS-275 pretreatment **(Figure 4)**.

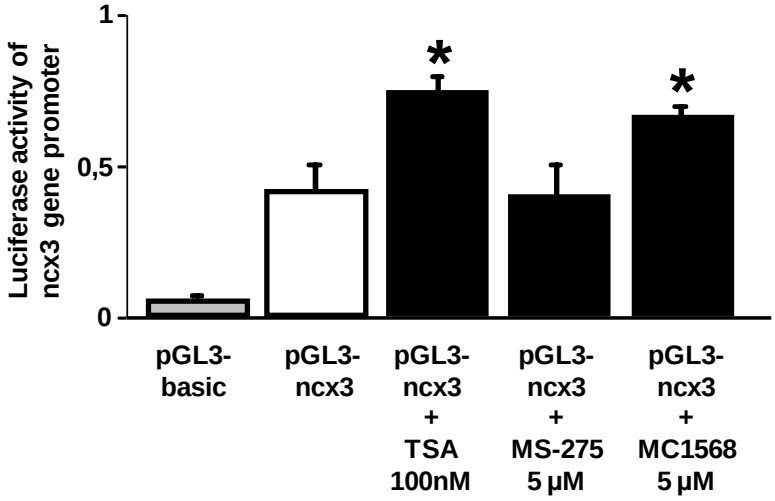


Figure 4. Effect of HDACI on ncx3 promoter activity. Cortical neurons were transfected with pGL3-ncx3 promoter and then treated with the pan-HDACI (TSA), with MS-275 an inhibitor for HDACs of class I and with MC1568 an HDACI specific for HDACs of class IIA. 24 hours post-treatment, neurons were lysed in 1x passive lysis buffer. Lysates were analyzed for luciferase

activity. The luciferase activity was expressed as firefly-to-renilla ratio. Each column represents the mean ± s.e.m. of n=3 independent experiments (*P<0.05 vs pGL3-ncx3).

IV.2. MC1568 increases ncx3 luciferase activity in time - dependent manner

NCX3 lucifarase activity was significantly increased by MC1568 at 24 hours compared to the cells transfected with pGL3-ncx3 alone. This increase in NCX3 luciferase activity reached a maximum after 48 h of incubation **(Figure 5)**.

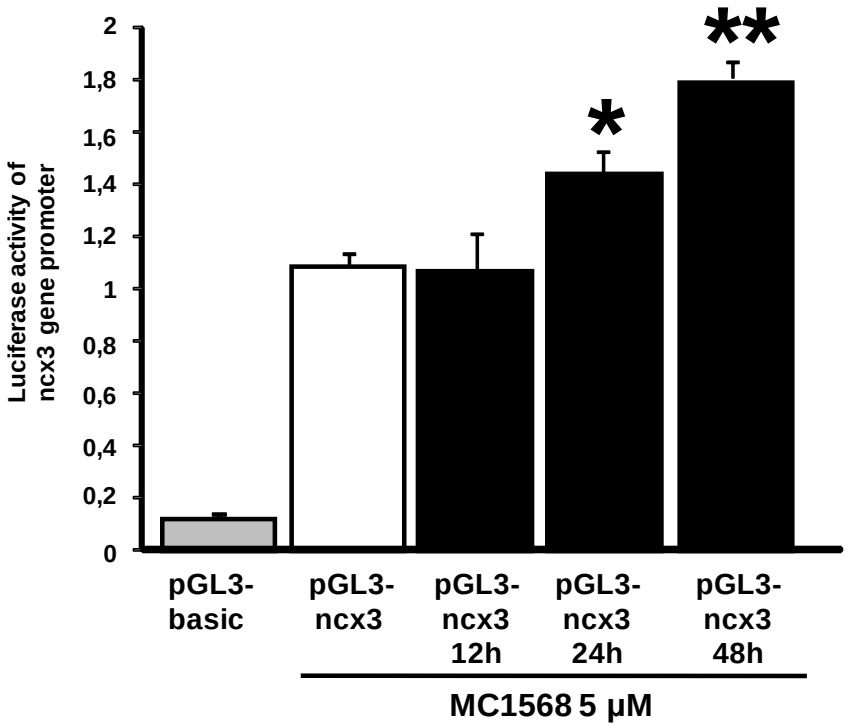
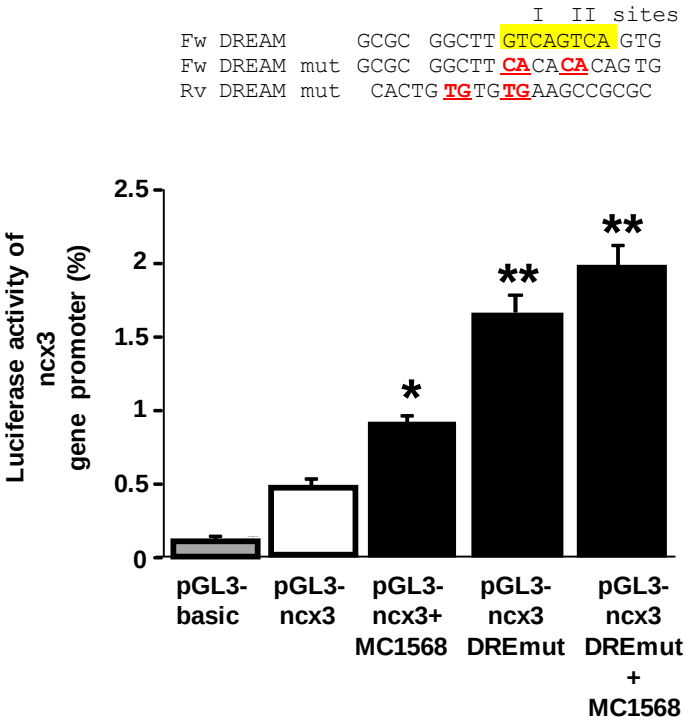


Figure 5. Effect of MC1568 in time dependent manner on ncx3 promoter activity. Cortical neurons were transfected with pGL3-ncx3 promoter and then treated with MC1568 (5 µM) at different time. 24 hours post-treatment, neurons were lysed in 1x passive lysis buffer. Lysates were analyzed for luciferase activity. The luciferase activity was expressed as firefly-to-renilla ratio. Each column represents the mean ± s.e.m. of n=3 independent experiments (*P<0.05 vs pGL3-ncx3, **P<0.05 vs all).

IV.3. MC1568 regulates ncx3 promoter activity via DREAM sequence

Luciferase assays were conducted to evaluate whether the DRE site (GTCAGTCA) and CRE site (GACGT) on ncx3 promoter were responsible of the MC1568-induced NCX3 increase. In particular, DREAM and CREB binding sites were mutated in the pGL3-ncx3 construct by site-directed mutagenesis to generate the pGL3-ncx3-DREmut (CACACACA) and pGL3-ncx3-CREmut (ACGGT) constructs. These two constructs were transfected in cortical neurons treated or not with MC1568 (5µM). The transfection of pGL3-ncx3-DREmut construct caused a significant increase of promoter activity compared to neurons transfected with pGL3-ncx3 alone or transfected with pGL3-ncx3 and subsequently treated with MC1568. In neurons transfected with pGL3-ncx3 carrying mutations in DRE sites and treated with MC1568, ncx3 promoter activity was not increased significantly compared to cells transfected with pGL3-ncx3-DREmut alone. The transfection of pGL3-ncx3-CREmut construct caused a significant decrease of promoter activity **(Figure 6)**.

a



b

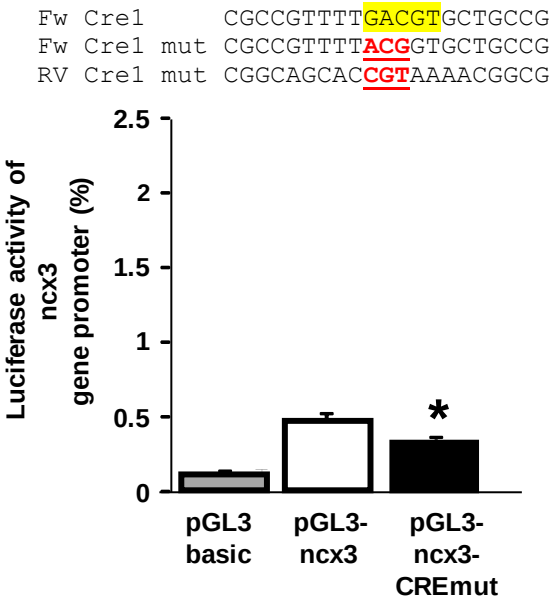


Figure 6. Effect of MC1568 on the transcriptional activity of the pGL3-ncx3 after site-directed mutagenesis of DREAM and CREB binding sites. Mutated nucleotides of ncx3 promoter are

underlined; replaced nucleotides are highlighted in yellow on the wild-type sequences. **(a)** Cortical neurons were transfected with pGL3-ncx3 wildtype or pGL3-ncx3-DREmut and then treated or not with MC1568. **(b)** Cortical neurons were transfected with pGL3-ncx3 wildtype or pGL3-ncx3-CREmut. 24 hours post-transfection, neurons were lysed in 1x passive lysis buffer. Lysates were analyzed for luciferase activity. The luciferase activity was expressed as firefly-to-renilla ratio. Each column represents the mean ± s.e.m. of n=3 independent experiments. (*P<0.05 vs pGL3-ncx3, **P<0.05 vs all).

IV.4. HDAC class IIA isoforms 4 and 5 reduce NCX3 mRNA and protein expression

HDAC Class IIa is composed of HDAC 4, 5, 7 and 9 [122]. Thus, to detect which of these isoforms was possibly involved in the MC1568-dependent increase of NCX3 mRNA and protein, neurons were silenced by specific siRNAs against HDAC 4, 5, 7 and 9 named siHDAC4, siHDAC5, siHDAC7 and siHDAC9.

Real Time PCR (qRT–PCR) at 24 hours and western blot analysis at 48 hours revealed that in neurons, NCX3 mRNA and protein increased with siHDAC4 and with siHDAC5 whereas any changing was detected with siHDAC7 and siHDAC9 all compared to Control (CTL).

To further confirm the role of HDAC4 and HDAC5 in regulating NCX3 expression, neurons were transfected with constructs overexpressing HDAC 4, 5, 7 and 9. In neurons overexpressing HDAC4 and HDAC5, NCX3 mRNA and protein were both reduced whereas cells overexpressing the other two HDAC isoforms belonging to class IIA did note determine any modification of NCX3 **(Figure 7)**.

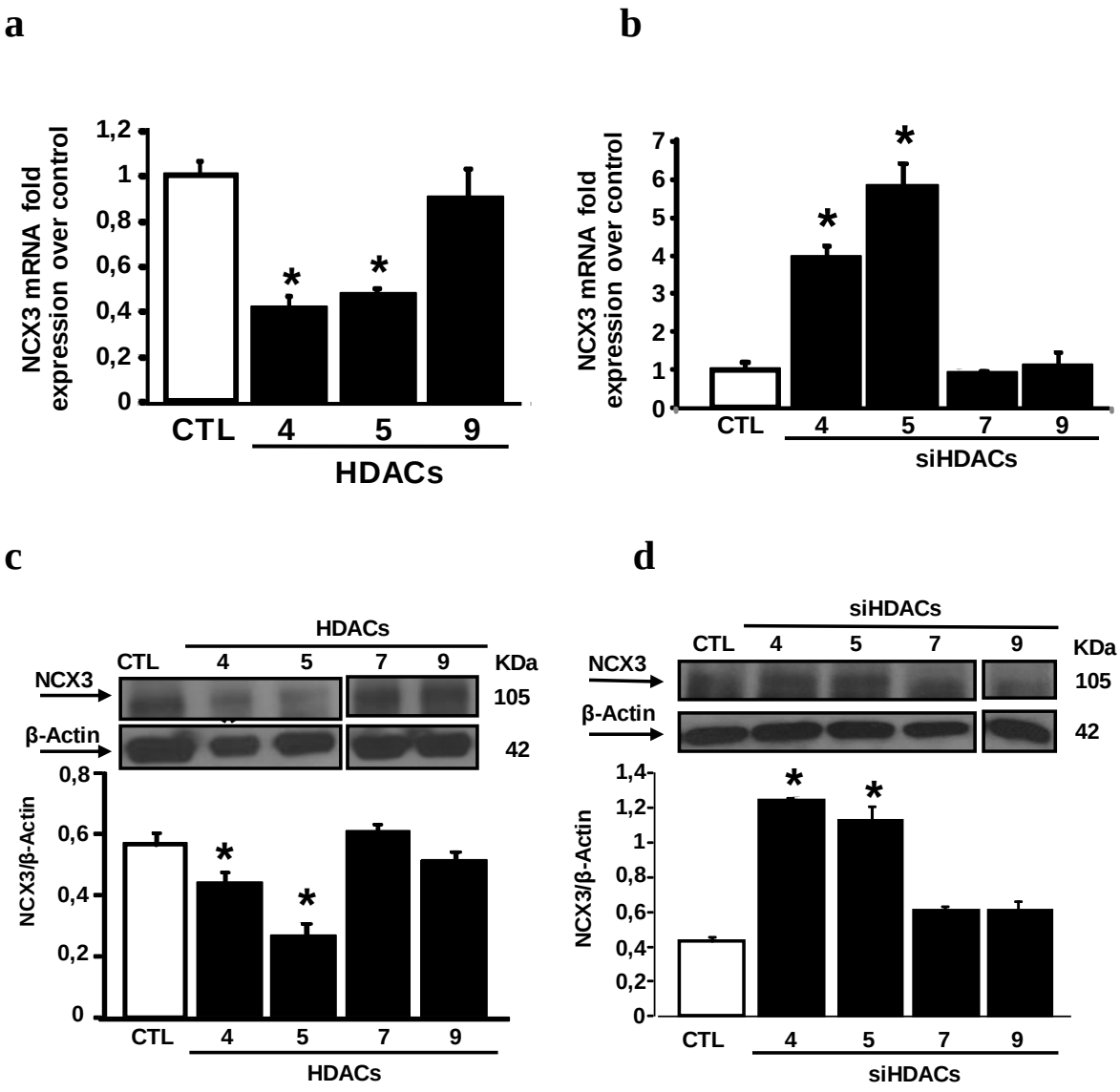


Figure 7. Effects of HDACs class IIA on ncx3 gene and protein. (a,c). qRT–PCR and representative WB with quantification of NCX3 in cortical neurons transfected with constructs for HDAC4, HDAC5, HDAC7 and HDAC9. Each column represents the mean ± s.e.m. of n=3 independent experiments (*P<0.05 vs CTL). **(b,d).** qRT–PCR and representative WB with quantification of NCX3 in cortical neurons transfected with siHDAC4, siHDAC5, siHDAC7 and

siHDAC9. Each column represents the mean ± s.e.m. of n=3 independent experiments (*P<0.05 vs CTL).

IV.5. DREAM, HDAC4 and HDAC5 protein expression is increased in brain ischemia

NCX3 isoform has been deeply investigated in the pathophysiology of stroke. In particular it has been found that its knock-down worsens the ischemic damage or reduces preconditioning-induced neuroprotection, respectively [265,281]. Western blot analysis, performed in the cortex of rats at 24 and 48 h after tMCAO, revealed a significative increase of DREAM, HDAC4 and HDAC5 in parallel with a reduction of NCX3 protein **(Figure 8)**.

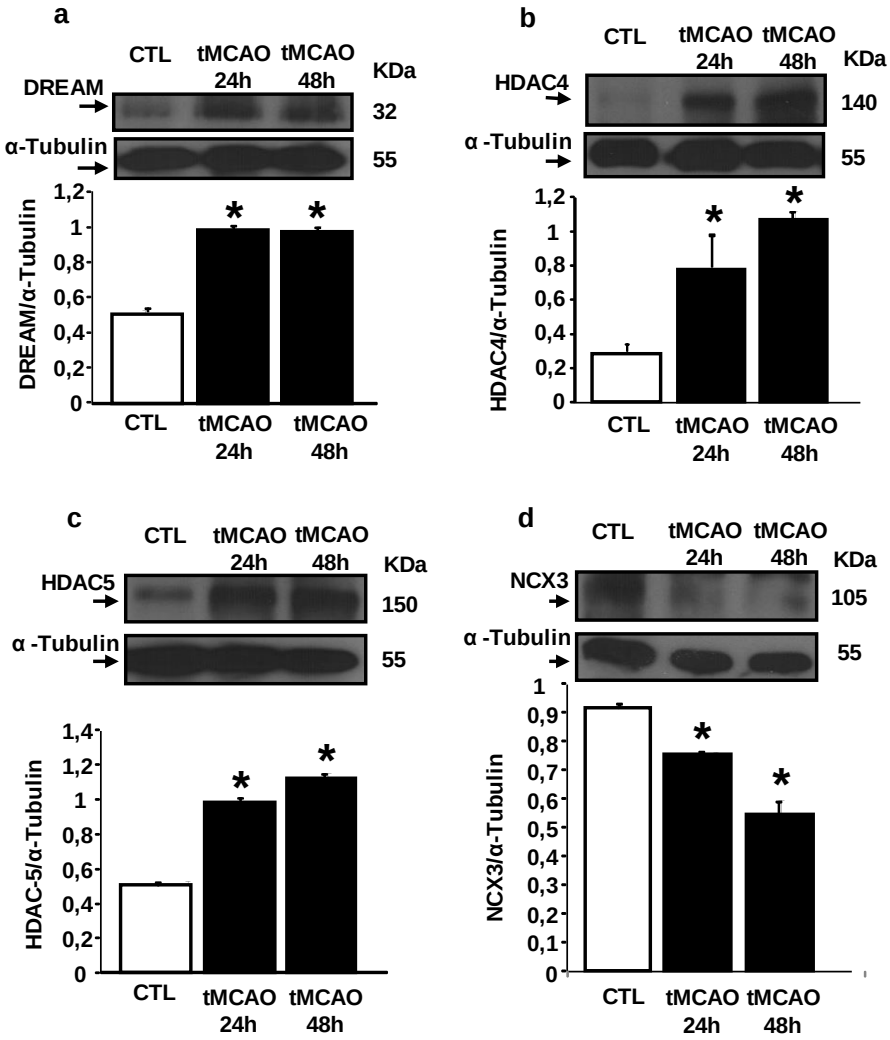


Figure 8. Effect of tMCAO on DREAM, HDAC4, HDAC5 and NCX3 expression. (a, b, c, d) Representative WB with quantification of DREAM, HDAC4, HDAC5 and NCX3 protein expression in: (1) control group (CTL), (2) tMCAO at 24h and (3) tMCAO at 48h. Each column represents the mean ± s.e.m. (n= 5 animals for each column) (*P<0.05 vs CTL).

IV.6. Stroke induces an increase of HDAC4 and HDAC5 binding on ncx3 promoter

The deacetylation of histones is a modification associated generally to transcriptional repression that is achieved through the reduction of access of the transcription machinery to the genes [67]. By ChIP analysis we

found that tMCAO determined at level of the ncx3 promoter sequence an increase of the binding of DREAM, HDAC4 and HDAC5 and a reduction of the amount of the ncx3 promoter associated with acetylated histone H4 protein and RNA-Pol II **(Figure 9)**.

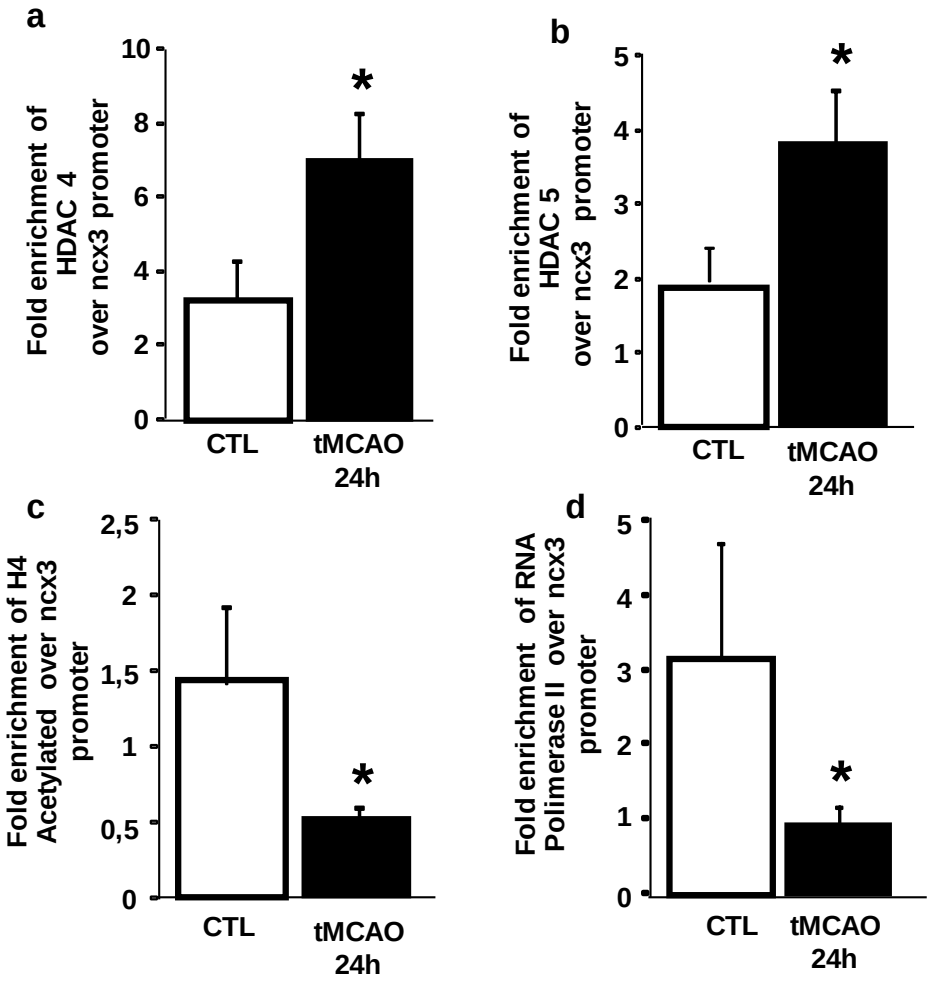


Figure 9. Stroke induces transcriptional modifications on ncx3 promoter. ChIP analysis with anti-HDAC4, HDAC5, anti- H4acetyl and anti- RNA polymerase II of ncx3 promoter in CTL and in tMCAO. IgG was used as negative control. Each column represents the mean ± s.e.m. (n= 5 animals for each column) (*P<0.05 vs CTL).

Discussion

V DISCUSSION

The results of this thesis indicate that in rat cortical neurons ncx3 luciferase activity is increased by the class IIa HDAC inhibitor MC1568, but not by the class I inhibitor MS-275. Furthermore, the effect of MC1568 on NCX3 promoter activity occurs via DREAM sequence. Among the HDAC class IIA only HDAC4 and HDAC5 isoforms are able to regulate ncx3 expression. In addition, during brain ischemia HDAC4 and 5 were increased in parallel with a reduction of NCX3.

Many studies demonstrated that the ODNs, not only for NCX1 but also for NCX3 gene product, caused a worsening of ischemic damage in animals subjected to pMCAO [265] and that NCX3 may play a great protective role in chemical hypoxia since it seems to be the unique isoform of the exchanger able to function in spite of ATP depletion [266].

NCX3 is neuroprotective in brain ischemia, in fact knockout mice ncx3-/- displayed a worsening of neuronal death after an ischemic insult [267]. About transcriptional regulation of NCX3 it has been found that after ischemia NCX3 mRNA expression was reduced approximately of 50% at 24 hours [281] while in ischemic postconditionig ncx3 mRNA is increased of about 40%[154,281]. Recently it has been found that in preconditioned rats NCX3 protein reduction is blocked by the binding of SUMO-1 at 72 hours after ischemia [287]. Previous studies demonstrated that MC1568 by regulating HDAC4 reverts DEHP-induced neuronal cell death [294] and protects neural processes against neurotoxic insult [295] .

A crucial finding of this work is that only HDAC4 and HDAC5 isoforms are able to regulate the transcription and the expression of NCX3. Interestingly, the other two isoforms belonging to the HDAC class IIA HDAC7 and 9, did not change the expression of NCX3. NCX3 promoter has putative consensus sequences for the CRE binding protein (CREB) and for the Ca^{2+}-binding protein downstream regulatory element (DRE)

antagonist modulator (DREAM) [275]. CRE sequence is involved in the stimulation of ncx3 promoter [275], whereas DRE sequence for its repression [272]. We found that mutation of DRE and CRE sequences causes an increase or a reduction of ncx3 promoter activity, respectively. Specifically, MC1568 by inhibiting HDAC class IIA increases NCX3 promoter activity via DREAM sequence, indeed mutation of DRE sequence on ncx3 promoter (pGL3-ncx3-DREmut) together with HDAC class IIA inhibition due to MC1568 treatment did not cause any significant effect compared to pGL3-ncx3-DREmut.

About DREAM and HDAC4, it has been reported that the former is a repressor of NCX3 transcription [272], whereas the latter is a mediator of neuronal cell death. Indeed, the exposure of cultured cerebellar granule neurons (CGNs) to low potassium medium, reported as apoptotic stimulus, triggers rapidly the translocation of HDAC4 into the nucleus with subsequent neuronal death [296]. Interestingly we found that DREAM, HDAC4 and HDAC5 protein expression is increased at 24h and 48h after brain ischemia. NCX3 isoform has been deeply investigated in the pathophysiology of stroke. In particular, we found that the increase in the expression of HDAC4, HDAC5 and DREAM occurred in parallel with the NCX3 reduction. The deacetylation of histones is an epigenetic modification determining a transcriptional repression due to restricted access of the transcriptional machinery to the promoter regions of genes [67]. In particular, by ChIP analysis we found that in brain ischemia HDAC4 and HDAC5 are recruited to ncx3 promoter decreasing H4 acetylation and RNA-Pol II binding. By these experiments it could be hypothesized that HDAC4 and HDAC5 by binding ncx3 promoter could lead to ncx3 reduction and consequent neuronal cell death. Collectively, these data demonstrate that MC1568 increases ncx3 promoter activity through DRE site and that the transcription factor DREAM by recruiting HDAC4 and 5 could reduce ncx3 mRNA and protein expression at basal level and in brain ischemia. Intriguingly, the development of new HDACs

inhibitors specific for each isoform can be considered a new step for the improvement of therapeutic strategies against cerebral ischemia and all neurodegenerative diseases where HDACs-increased activity or expression are involved.

References

REFERENCES

[1] Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 22:391-397.

[2] Seok Joon Won, Doo Yeon Kim and Byoung Joo Gwag (2002) Cellular and Molecular Pathways of Ischemic Neuronal Death Journal of Biochemistry and Molecular Biology, 35: 67-86

[3] Gill, D. L., Ghosh, T. K. and Mullaney, J. M. (1989) Calcium signaling mechanisms in endoplasmic reticulum activated by inositol 1,4,5-trisphosphate and GTP. Cell Calcium 10, 363- 374.

[4] Gunter, T. E. and Pfeiffer, D. R. (1990) Mechanisms by which mitochondria transport calcium. Am. J. Physiol. 258, C755- C786.

[5] Carafoli, E. (1991) Calcium pump of the plasma membrane. Physiol. Rev. 71, 129-153.

[6] Caplan LR (2000) Acute stroke: seeing the full picture. Hosp Pract (1995) 35:65-71; discussion 71-62; quiz 113.

[7] Benveniste, H., Drejer, J., Schousboe, A. and Diemer, N. H. (1984) Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral mecrodialysis. *J. Neurochem.* 43, 1369-1374.

[8] Takagi, K., Ginsberg, M. D., Globus, M. Y., Dietrich, W. D., Martinez, E., Kraydieh, S. and Busto, R. (1993) Changes in amino acid neurotransmitters and cerebral blood flow in the ischemic penumbral region following middle cerebral artery occlusion in the rat: correlation with histopathology. *J. Cereb. Blood Flow Metab.* 13, 575-585.

[9] Choi, D. W. (1987) Ionic dependence of glutamate neurotoxicity. *J. Neurosci.* 7, 369-379.

[10] Goldberg, M. P., Weiss, J. H., Pham, P. C. and Choi, D. W. (1987) N-methyl-D-aspartate receptors mediate hypoxic neuronal injury in cortical culture. *J. Pharmac. Exp. Ther.* 243, 784-791.

[11] Greenberg, J. H., Uematsu, D., Araki, N., Hickey, W. F. and Reivich, M. (1990) Cytosolic free calcium during focal cerebral ischemia and the effects of nimodipine on calcium and histologic damage. *Stroke* 21, IV72-IV77.

[12] Simon, R. P., Swan, J. H., Griffiths, T. and Meldrum, B. S. (1984) Blockade of N-methyl-D-aspartate receptors may protect against ischemic damage in the brain. *Science* 226, 850-852.

[13] K.P. Doyle, R.P. Simon, M.P. Stenzel-Poore, (2008) Mechanisms of ischemic brain damage, Neuropharmacology 55 310–318.

[14] S.L. Mehta, N. Manhas, R. Rahubir, (2007) Molecular targets in cerebral ischemia for developing novel therapeutics, Brain Res. Rev. 54 34–66.

[15] Neil R. Sims, Hakan Muyderman, (2010) Mitochondria, oxidative metabolism and cell death in stroke Biochimica et Biophysica Acta 1802 80–91

[16] Sugawara T, Chan PH (2003) Reactive oxygen radicals and pathogenesis of neuronal death after cerebral ischemia. Antioxid Redox Signal 5:597-607.

[17] Seth Love (2003) Apoptosis and brain ischaemia Progress in Neuro-Psychopharmacology & Biological Psychiatry 27 267– 282

[18] Cohen, G.M., (1997) Caspases: the executioners of apoptosis. Biochem. J. 326, 1 –16.

[19] Earnshaw, W.C., Martins, L.M., Kaufmann, S.H., (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu. Rev. Biochem. 68, 383– 424.

[20] Hengartner, M.O., (2000) The biochemistry of apoptosis. Nature 407, 770– 776.

[21] Kaufmann, S.H., Hengartner, M.O., (2001) Programmed cell death: alive and well in the new millennium. Trends Cell Biol. 11, 526– 534.

[22] Strasser, A., O'Connor, L., Dixit, V.M., (2000) Apoptosis signaling. Annu.Rev. Biochem. 69, 217– 245.

[23] Cao, G., Pei, W., Lan, J., Stetler, R.A., Luo, Y., Nagayama, T., Graham,S.H., Yin, X.M., Simon, R.P., Chen, J., (2001) Caspase-activated DNase/DNA fragmentation factor 40 mediates apoptotic DNA fragmentation in transient cerebral ischemia and in neuronal cultures. J. Neurosci. 21, 4678–4690.

[24] Luo, Y., Cao, G., Pei, W., O'Horo, C., Graham, S.H., Chen, J., (2002) Induction of caspase-activated deoxyribonuclease activity after focal cerebral ischemia and reperfusion. J. Cereb. Blood Flow Metab. 22, 15–20.

[25] Harrison, D.C., Davis, R.P., Bond, B.C., Campbell, C.A., James, M.F., Parsons, A.A., Philpott, K.L., (2001) Caspase mRNA expression in a rat model of focal cerebral ischemia. Mol. Brain Res. 89, 133–146.

[26] Krupinski, J., Lopez, E., Marti, E., Ferrer, I., (2000) Expression of caspases and their substrates in the rat model of focal cerebral ischemia. Neurobiol. Dis. 7, 332– 342.

[27] Sasaki, C., Kitagawa, H., Wen Ri, Z., Warita, H., Sakai, K., Abe, K., (2000) Temporal profile of cytochrome c and caspase-3 immunoreactivities and TUNEL staining after permanent middle cerebral artery occlusion in rats. Neurol. Res. 22, 223– 228.

[28] Velier, J.J., Ellison, J.A., Kikly, K.K., Spera, P.A., Barone, F.C., Feuerstein, G.Z., (1999) Caspase-8 and caspase-3 are expressed by different populations of cortical neurons undergoing delayed cell death after focal stroke in the rat. J. Neurosci. 19, 5932– 5941.

[29] Botchkina, G.I., Geimonen, E., Bilof, M.L., Villarreal, O., Tracey, K.J., (1999) Loss of NF-kappaB activity during cerebral ischemia and TNF cytotoxicity. Mol. Med. 5, 372– 381.

[30] Rupalla, K., Allegrini, P.R., Sauer, D., Wiessner, C., (1998) Time course of microglia activation and apoptosis in various brain regions after permanent focal cerebral ischemia in mice. Acta Neuropathol. 96, 172–178.

[31] Sairanen, T., Carpen, O., Karjalainen-Lindsberg, M.L., Paetau, A., Turpeinen, U., Kaste, M., Lindsberg, P.J., (2001) Evolution of cerebral tumor necrosis factor-[alpha] production during human ischemic stroke. Stroke 32, 1750– 1757.

[32] Antonsson, B., (2001) Bax and other pro-apoptotic Bcl-2 family ‘‘killerproteins’’ and their victim the mitochondrion. Cell Tissue Res. 306, 347– 361.

[33] Wang, G.X., Li, G.R., Wang, Y.D., Yang, T.S., Ouyang, Y.B., (2001) Characterization of neuronal cell death in normal and diabetic rats following experimental focal cerebral ischemia. Life Sci. 69, 2801– 2810.

[34] Antonawich, F.J., Krajewski, S., Reed, J.C., Davis, J.N., (1998) Bcl-xl Bax interaction after transient global ischemia. J. Cereb. Blood Flow Metab.18, 882– 886.

[35] Chen, J., Zhu, R.L., Nakayama, M., Kawaguchi, K., Jin, K., Stetler, R.A., Simon, R.P., Graham, S.H., (1996) Expression of the apoptosis-effector gene, Bax, is up-regulated in vulnerable hippocampal CA1 neurons following global ischemia. J. Neurochem. 67, 64– 71.

[36] Gillardon, F., Lenz, C., Waschke, K.F., Krajewski, S., Reed, J.C., Zimmermann, M., Kuschinsky, W., (1996) Altered expression of Bcl-2, Bcl-X, Bax, and c-Fos colocalizes with DNA fragmentation and ischemic cell damage following middle cerebral artery occlusion in rats. Brain Res. Mol. Brain Res. 40, 254–260.

[37] MacGibbon, G.A., Lawlor, P.A., Sirimanne, E.S., Walton, M.R., Connor, B., Young, D., Williams, C., Gluckman, P., Faull, R.L.M., Hughes, P., Dragunow, M., (1997) Bax expression in mammalian neurons undergoing apoptosis, and in Alzheimer’s disease hippocampus. Brain Res. 750, 223–234.

[38] Matsushita, K., Matsuyama, T., Kitagawa, K., Matsumoto, M., Yanagihara, T., Sugita, M., (1998) Alterations of BCL-2 family proteins precede cytoskeletal proteolysis in the penumbra, but not in infarct centres following focal cerebral ischemia in mice. Neuroscience 83, 439–448.

[39] Niwa, M., Hara, A., Iwai, T., Sassa, T., Mori, H., Uematsu, T., (1997) Expression of Bax and Bcl-2 protein in the gerbil hippocampus following transient forebrain ischemia and its modification by phencyclidine.Neurol. Res. 19, 629– 633.

[40] Rong Jin & Lin Liu & Shihao Zhang & Anil Nanda & Guohong Li (2013) Role of Inflammation and Its Mediators in Acute Ischemic Stroke J. of Cardiovasc. Trans. Res. 6:834–851

[41] Iadecola, C., & Anrather, J. (2011). The immunology of stroke: from mechanisms to translation. Nature Medicine, 17, 796–808.

[42] Lai, A. Y., & Todd, K. G. (2006). Microglia in cerebral ischemia: molecular actions and interactions. Canadian Journal of Physiology and Pharmacology, 84, 49–59.

[43] Denes, A., Vidyasagar, R., Feng, J., Narvainen, J., McColl, B. W., Kauppinen, R. A., et al. (2007). Proliferating resident microglia after focal cerebral ischaemia in mice. Journal of Cerebral Blood Flow and Metabolism, 27, 1941–1953.

[44] Thomas,W. E. (1992). Brain macrophages: evaluation of microglia and their functions. Brain Research, 17, 61–74.

[45] Benveniste, E. N. (1998). Cytokine actions in the central nervous system. Cytokine & Growth Factor Reviews, 9, 259–275.

[46] Che, X., Ye, W., Panga, L., Wu, D. C., & Yang, G. Y. (2001). Monocyte chemoattractant protein-1 expressed in neurons and astrocytes during focal ischemia in mice. Brain Research, 902, 171–177.

[47] Liu, T., Clark, R. K.,McDonnell, P. C.,Young, P. R.,White, R. F., Barone, F. C. (1994). Tumor necrosis factor-α expression in ischemic neurons. Stroke, 25, 1481–1488

[48] Wang, X., Yue, T. L., Barone, F. C.,White, R. F., Gagnon, R. C., Feuerstein, G. Z. (1994). Concomitant cortical expression of TNF-α and IL-1 α mRNAs follows early response gene expression in transient focal ischemia. Molecular and Chemical Neuropathology, 23, 103–114.

[49] Rosenberg, G. A., Navratil, M., Barone, F., & Feuerstein, G. (1996). Proteolytic cascade enzymes increase in focal cerebral ischemia in rat. Journal of Cerebral Blood Flow and Metabolism, 16, 360–366.

[50] Clark, A. W., Krekoski, C. A., Bou, S. S., Chapman, K. R., Edwards, D. R. (1997). Increased gelatinase A (MMP-2) and gelatinase B (MMP-9) activities in human brain after focal ischemia. Neuroscience Letters, 238, 53–56.

[51] Castellanos, M., Leira, R., Serena, J., Pumar, J. M., Lizasoain, I., Castillo, J. (2003). Plasma metalloproteinase-9 concentration predicts hemorrhagic transformation in acute ischemic stroke. Stroke, 34, 40–46.

[52] Montaner, J., Alvarez-Sabin, J., Molina, C., Angles, A., Abilleira, S., & Arenillas, J. (2001).Matrix metalloproteinase expression after human cardioembolic stroke: temporal profile and relation to neurological impairment. Stroke, 32, 1759–1766.

[53] M. F. Mehler, (2008) Epigenetics and the nervous system, *Annals of Neurology*, vol. 64, no. 6, pp. 602–617.

[54] Tessarz P., Kouzarides T. (2014) Histone core modifications regulating nucleosome structure and dynamics. Nat Rev Mol Cell Biol.703-8.

[55] Thiagalingam S, Cheng KH, Lee HJ, Mineva N, Thiagalingam A, Ponte JF. (2003) Histone deacetylases: unique players in shaping the epigenetic histone code. Ann N Y Acad Sci. 983:84-100.

[56] Jenuwein T, Allis CD. (2001)Translating the histone code. Science. 293(5532):1074-80. Review.

[57] R. Fernández-Santiago, M. Ezquerra. (2016)Epigenetic Research of Neurodegenerative Disorders Using Patient iPSC-Based Models. Stem Cells International Volume 2016.

[58] Ng, H. H. ,and Bird, A.(1999).DNA methylation and chromatin modification. Curr. Opin.Genet.Dev. 9, 158–163.

[59] B. Guillemette, P. Drogaris, H., H. S. Lin et al., (2011) “H3 lysine 4 is acetylated at active gene promoters and is regulated by H3 lysine 4 methylation,” *PLoS Genetics*, vol. 7, no. 3.

[60] Z.Wang, C. Zang, J.A.Rosenfeld et al., (2008) “Combinatorial patterns of histone acetylations and methylations in the human genome,” *Nature Genetics*, vol. 40, no. 7, pp. 897–903.

[61] M. F. Mehler, (2008) “Epigenetics and the nervous system,” *Annals of Neurology*, vol. 64, no. 6 pp. 602–617.

[62] Hansen JC, Tse C, Wolffe AP (1998) Structure and function of the core histone N-termini: more than meets the eye. Biochemistry 37:17637-17641.

[63] Strahl BD, Allis CD (2000) The language of covalent histone modifications. Nature 403:41-45.

[64] Thiagalingam S, Cheng KH, Lee HJ, Mineva N, Thiagalingam A, Ponte JF (2003) Histone deacetylases: unique players in shaping the epigenetic histone code. Ann N Y Acad Sci.;983:84-100.

[65] Kouzarides T. (2007) Chromatin modifications and their function. Cell;128:693–705.

[66] Lee KK, Workman JL. (2007) Histone acetyltransferase complexes: one size doesn't fit all. Nat Rev Mol Cell Biol;8:284–295.

[67] Abel T, Zukin RS. (2008) Epigenetic targets of HDAC inhibition in neurodegenerative and psychiatric disorders. Curr Opin Pharmacol.;8(1):57-64.

[68] De-Maw Chuang, Yan Leng, Zoya Marinova, Hyeon-Ju Kim, and Chi-Tso Chiu (2009) Multiple roles of HDAC inhibition in neurodegenerative conditions**.** Trends Neurosci.; 32(11): 591–601.2009 Sep

[69] Allis CD, Berger SL, Cote J, et al. (2007) New nomenclature for chromatin-modifying enzymes. Cell.;131:633–636.

[70] Schneider A, S Chatterjee, O Bousiges, B. R Selvi, A Swaminathan, R Cassel, F Blanc, T K. Kundu, A-L Boutillier (2013) Acetyltransferases (HATs) as Targets for Neurological Therapeutics Neurotherapeutics.; 10(4): 568–588.

[71]Mizzen C.A., Yang X.Y., Kokubo T., Brownell J.E., Bannister A.J., Owe nHughes T., Workman J., Wang L., Berger S.L., Kouzarides T., Nakatani Y., Allis C.D. (1996) The $TAF_{II}250$ subunit of TFIID has histone acetyltransferase activity. Cell 87:1261–1270.

[72] Bannister A.J., Kouzarides T. (1996) The CBP co-activator is a histone acetyltransferase. Nature 384:641–643.

[73] Ogryzko V.V., Schiltz R.L., Russanova V., Howard B.H., Nakatani Y (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 87:953–959.

[74] Janknecht R., Hunter T. (1996) A growing coactivator network. Nature 383:22–23.

[75] Nakajima T., Uchida C., Anderson S., Parvin J., Montminy M. (1997) Analysis of a cAMP-responsive activator reveals a two-component mechanism for transcriptional induction via signal-dependent factors. Genes & Dev. 11:738–747.

[76] Struhl K. (1998) Histone acetylation and transcriptional regulatory mechanisms. Genes Dev.; 12 (5): 599-606.

[77] Finnin, M.S., Donigian, J.R., Cohen, A., Richon, V.M., Rifkind, R.A., Marks, P.A., Breslow, R., and Pavletich, N.P. (1999). Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature *401*, 188–193.

[78] Grozinger, C.M., Hassig, C.A., and Schreiber, S.L. (1999) Three proteins define a class of human histone deacetylases related to yeast Hda1p. Proc. Natl. Acad. Sci. USA *96*, 4868–4873.

[79] Gray, S.G., and Ekstrom, T.J. (2001) The human histone deacetylase family. Exp. Cell Res. *262*, 75–83.

[80] Zhou, X., Marks, P.A., Rifkind, R.A., and Richon, V.M. (2001) Cloning and characterization of a histone deacetylase, HDAC9. Proc. Natl. Acad. Sci. USA *98*, 10572–10577.

[81] Venter, J.C., Adams, M.D., Myers, E.W., Li, P.W., Mural, R.J., Sutton, G.G., Smith, H.O., Yandell, M., Evans, C.A., Holt, R.A., et al.. (2001) The sequence of the human genome. Science *291*,1304–1351.

[82] Frye, R.A. (2000) Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochem. Biophys. Res. Commun. *273*, 793–798.

[83] Grozinger CM (2002) Deacetylase enzymes: biological functions and the use of small-molecule inhibitors. Chem Biol.;9(1):3-16.

[84] Gray, S.G., and Ekstrom, T.J. (2001) The human histone deacetylase family. Exp. Cell Res. *262*, 75–83.

[85] Majdzadeh N, Wang L, Morrison B, Bassel-Duby R, Olson E, D'Mello S (2008) HDAC4 inhibits cell-cycle progression and protects neurons from cell death. Dev Neurobiol 68:1076-1092.

[86] Hubbert C, Guardiola A, Shao R, Kawaguchi Y, Ito A, Nixon A, Yoshida M, Wang XF, Yao TP (2002) HDAC6 is a microtubule-associated deacetylase. Nature 417:455-458.

[87] Matsuyama A, Shimazu T, Sumida Y, Saito A, Yoshimatsu Y, Seigneurin-Berny D, Osada H, Komatsu Y, Nishino N, Khochbin S, Horinouchi S, Yoshida M (2002) In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation. EMBO J 21:6820-6831

[88] Tong JJ, Liu J, Bertos NR, Yang XJ (2002) Identification of HDAC10, a novel class II human histone deacetylase containing a leucine-rich domain. Nucleic Acids Res 30:1114-1123.

[89] Ayer, D.E. (1999) Histone deacetylases: transcriptional repression with SINers and NuRDs. Trends Cell Biol. *9*, 193–198.

[90] Knoepfler, P.S., and Eisenman, R.N. (1999) Sin meets NuRD and other tails of repression..Cell *99*, 447–450.

[91] Huang EY, Zhang J, Miska EA, Guenther MG, Kouzarides T, Lazar MA. (2000) Nuclear receptor corepressors partner with class II histone deacetylases in a Sin3-independent repression pathway. Genes Dev 14: 45-54

[92] Grozinger CM, Schreiber SL. (2000) Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization. Proc Natl Acad Sci U S A.;97(14):7835-40.

[93] Thiagalingam S, Cheng KH, Lee HJ, Mineva N, Thiagalingam A, Ponte JF. (2003) Histone deacetylases: unique players in shaping the epigenetic histone code. Ann N Y Acad Sci.;983:84-100.

[94] Leonard Guarente (2000) Sir2 links chromatin silencing, metabolism, and aging Genes Dev 14: 1021-1026

[95] Frye RA. (1999) Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity. Biochem Biophys Res Commun.;260(1):273-9.

[96] Brachmann CB, Sherman JM, Devine SE, Cameron EE, Pillus L, Boeke JD. (1995) The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability. Genes Dev. ;9(23):2888-902.

[97] Imai S, Armstrong CM, Kaeberlein M, Guarente L. (2000) Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature. 403(6771):795-800.

[98] Luo J, Su F, Chen D, Shiloh A, Gu W. (2000) Deacetylation of p53 modulates its effect on cell growth and apoptosis. Nature;408(6810):377-81.

[99] Luo J, Nikolaev AY, Imai S, Chen D, Su F, Shiloh A, Guarente L, Gu W. (2001) Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell.;107(2):137-48.

[100] Kuzmichev A, Zhang Y, Erdjument-Bromage H, Tempst P, Reinberg D. (2002) Role of the Sin3-histone deacetylase complex in growth regulation by the candidate tumor suppressor p33(ING1). Mol Cell Biol. ;22(3):835-48.

[101] Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, Guarente L, Weinberg RA. (2001) hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell.;107(2):149-59.

[102] Kazantsev A, Thompson L (2008) Therapeutic application of histone deacetylase inhibitors for central nervous system disorders. Nat Rev Drug Discov 7:854-868.

[103] Gurvich N, Tsygankova OM, Meinkoth JL, Klein PS (2004) Histone deacetylase is a target of valproic acid-mediated cellular differentiation. Cancer Res 64:1079-1086.

[104] Khan N, Jeffers M, Kumar S, Hackett C, Boldog F, Khramtsov N, Qian X, Mills E, Berghs SC, Carey N, Finn PW, Collins LS, Tumber A, Ritchie JW, Jensen PB, Lichenstein HS, Sehested M (2008) Determination of the class and isoform selectivity of small-molecule histone deacetylase inhibitors. Biochem J 409:581-589.

[105] Itoh Y, Suzuki T, Miyata N (2008) Isoform-selective histone deacetylase inhibitors. Curr Pharm Des 14:529-544.

[106] Haggarty SJ, Koeller KM, Wong JC, Grozinger CM, Schreiber SL (2003) Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation. Proc Natl Acad Sci U S A 100:4389-4394

[107] Green KN, Steffan JS, Martinez-Coria H, Sun X, Schreiber SS, Thompson LM, LaFerla FM (2008) Nicotinamide restores cognition in Alzheimer's disease transgenic mice via a mechanism involving sirtuin inhibition and selective reduction of Thr231-phosphotau. J Neurosci 28:11500-11510.

[108] Rouaux C, Jokic N, Mbebi C, Boutillier S, Loeffler JP, Boutillier AL (2003) Critical loss of CBP/p300 histone acetylase activity by caspase-6 during neurodegeneration. EMBO J 22:6537-6549.

[109] Ryu H, Lee J, Olofsson BA, Mwidau A, Dedeoglu A, Escudero M, Flemington E, Azizkhan-Clifford J, Ferrante RJ, Ratan RR, Deodoglu A (2003) Histone deacetylase inhibitors prevent oxidative neuronal death independent of expanded polyglutamine repeats via an Sp1-dependent pathway. Proc Natl Acad Sci U S A 100:4281-4286.

[110] Jeong MR, Hashimoto R, Senatorov VV, Fujimaki K, Ren M, Lee MS, Chuang DM (2003) Valproic acid, a mood stabilizer and anticonvulsant, protects rat cerebral cortical neurons from spontaneous cell death: a role of histone deacetylase inhibition. FEBS Lett 542:74-78.

[111] Biswas SC, Liu DX, Greene LA (2005) Bim is a direct target of a neuronal E2F-dependent apoptotic pathway. J Neurosci 25:8349-8358.

[112] Langley B, D'Annibale MA, Suh K, Ayoub I, Tolhurst A, Bastan B, Yang L, Ko B, Fisher M, Cho S, Beal MF, Ratan RR (2008) Pulse inhibition of histone deacetylases induces complete resistance to oxidative death in cortical neurons without toxicity and reveals a role for cytoplasmic p21(waf1/cip1) in cell cycle-independent neuroprotection. J Neurosci 28:163-176.

[113] Kontopoulos E, Parvin JD, Feany MB (2006) Alpha-synuclein acts in the nucleus to inhibit histone acetylation and promote neurotoxicity. Hum Mol Genet 15:3012-3023.

[114] Leng Y, Chuang DM (2006) Endogenous alpha-synuclein is induced by valproic acid through histone deacetylase inhibition and participates in neuroprotection against glutamate-induced excitotoxicity. J Neurosci 26:7502-7512.

[115] Monti B, Polazzi E, Batti L, Crochemore C, Virgili M, Contestabile A (2007) Alpha-synuclein protects cerebellar granule neurons against 6-hydroxydopamine-induced death. J Neurochem 103:518-530.

[116] Feng HL, Leng Y, Ma CH, Zhang J, Ren M, Chuang DM (2008) Combined lithium and valproate treatment delays disease onset, reduces neurological deficits and prolongs survival in an amyotrophic lateral sclerosis mouse model. Neuroscience 155:567-572.

[117] Yasuda S, Liang MH, Marinova Z, Yahyavi A, Chuang DM (2009) The mood stabilizers lithium and valproate selectively activate the promoter IV of brain-derived neurotrophic factor in neurons. Mol Psychiatry 14:51-59.

[118] Chen PS, Peng GS, Li G, Yang S, Wu X, Wang CC, Wilson B, Lu RB, Gean PW, Chuang DM, Hong JS (2006) Valproate protects dopaminergic neurons in midbrain neuron/glia cultures by stimulating the release of neurotrophic factors from astrocytes. Mol Psychiatry 11:1116-1125.

[119] Wu X, Chen PS, Dallas S, Wilson B, Block ML, Wang CC, Kinyamu H, Lu N, Gao X, Leng Y, Chuang DM, Zhang W, Lu RB, Hong JS (2008) Histone deacetylase inhibitors up-regulate astrocyte GDNF and BDNF gene transcription and protect dopaminergic neurons. Int J Neuropsychopharmacol 11:1123-1134.

[120] Lin CH, Chen TJ, Chen SS, Hsiao PC, Yang RC (2011) Activation of Trim17 by PPARγ is involved in di(2-ethylhexyl) phthalate (DEHP)-induced apoptosis on Neuro-2a cells. Toxicol Lett 206:245-251.

[121] Cao CX, Yang QW, Lv FL, Cui J, Fu HB, Wang JZ (2007) Reduced cerebral ischemia-reperfusion injury in Toll-like receptor 4 deficient mice. Biochem Biophys Res Commun 353:509-514.

[122] Chuang DM, Leng Y, Marinova Z, Kim HJ, Chiu CT (2009) Multiple roles of HDAC inhibition in neurodegenerative conditions. Trends Neurosci 32:591-601.

[123] Bates GP. (2001) Huntington's disease. Exploiting expression. Nature;413:691, 693–691, 694.

[124] Sugars KL, Rubinsztein DC. (2003) Transcriptional abnormalities in Huntington disease. Trends Genet 19:233–238.

[125] Steffan JS, Bodai L, Pallos J, Poelman M, McCampbell A, Apostol BL, Kazantsev A, Schmidt E, Zhu YZ, Greenwald M, Kurokawa R, Housman DE, Jackson GR, Marsh JL, Thompson LM. (2001) Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in Drosophila. Nature; 413:739–743.

[126] Ferrante RJ, Kubilus JK, Lee J, Ryu H, Beesen A, Zucker B, Smith K, Kowall NW, Ratan RR, Luthi-Carter R, Hersch SM. (2003) Histone deacetylase inhibition by sodium butyrate chemotherapy ameliorates the neurodegenerative phenotype in Huntington's disease mice. J Neurosci;23:9418–9427.

[127] Hockly E, Richon VM, Woodman B, Smith DL, Zhou X, Rosa E, Sathasivam K, Ghazi-Noori S, Mahal A, Lowden PA, Steffan JS, Marsh JL, Thompson LM, Lewis CM, Marks PA, Bates GP.(2003) Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, ameliorates motor deficits in a mouse model of Huntington's disease. Proc Natl Acad Sci U S A;100:2041–2046.

[128] Gasser T. (2001) Genetics of Parkinson's disease. J Neurol;248:833–840.

[129] Guan JS, Haggarty SJ, Giacometti E, Dannenberg JH, Joseph N, Gao J, Nieland TJ, Zhou Y, Wang X, Mazitschek R, Bradner JE, DePinho RA, Jaenisch R, Tsai LH (2009) HDAC2 negatively regulates memory formation and synaptic plasticity. Nature 459:55-60.

[130] Barrett RM, Wood MA (2008) Beyond transcription factors: the role of chromatin modifying enzymes in regulating transcription required for memory. Learn Mem 15:460-467.

[131] Qing H, He G, Ly PT, Fox CJ, Staufenbiel M, Cai F, Zhang Z, Wei S, Sun X, Chen CH, Zhou W, Wang K, Song W (2008) Valproic acid inhibits Abeta production, neuritic plaque formation, and behavioral deficits in Alzheimer's disease mouse models. J Exp Med 205:2781-2789.

[132] Ryu H, Smith K, Camelo SI, Carreras I, Lee J, Iglesias AH, Dangond F, Cormier KA, Cudkowicz ME, Brown RH Jr, Ferrante RJ. (2005) Sodium phenylbutyrate prolongs survival and regulates expression of anti-apoptotic genes in transgenic amyotrophic lateral sclerosis mice. ; J Neurochem.93(5):1087-98.

[133] Lo EH, Dalkara T, Moskowitz MA. (2003) Mechanisms, challenges and opportunities in stroke. Nat Rev Neurosci;4:399–415.

[134] Ballas N, Mandel G. (2005) The many faces of REST oversee epigenetic programming of neuronal genes. Curr Opin Neurobiol;15:500–506.

[135] Roopra A, Huang Y, Dingledine R. (2001) Neurological Disease: Listening to Gene Silencers. Mol Interv;1:219–228.

[136] Calderone A, Jover T, Noh K-M, Tanaka H, Yokota H, Lin Y, Grooms S, Regis R, Bennett MV, Zukin RS. (2003) Ischemic insults de-repress the gene silencer rest in neurons destined to die. J Neurosci;23:2112–2121.

[137] Formisano L, Noh KM, Miyawaki T, Mashiko T, Bennett MV, Zukin RS. (2007) Ischemic insults promote epigenetic reprogramming of mu opioid receptor expression in hippocampal neurons. Proc Natl Acad Sci USA;104:4170–4175.

[138] Faraco G, Pancani T, Formentini L, Mascagni P, Fossati G, Leoni F, Moroni F, Chiarugi A. (2006) Pharmacological inhibition of histone deacetylases by suberoylanilide hydroxamic acid specifically alters gene expression and reduces ischemic injury in the mouse brain. Mol Pharmacol;70:1876–1884.

[139] Endres M, Meisel A, Biniszkiewicz D, Namura S, Prass K, Ruscher K, Lipski A, Jaenisch R, Moskowitz MA, Dirnagl U. (2000) DNA methyltransferase contributes to delayed ischemic brain injury. J Neurosci;20:3175–3181.

[140] Baker PF and Blaustein MP (1968) Sodium-dependent uptake of calcium by crab nerve. *Biochim Biophys Acta* 150:167–170.

[141] Baker PF, Blaustein MP, Hodgkin AL, and Steinhardt RA (1969) The influence of calcium on sodium efflux in squid axons. *J Physiol* 200:431–458.

[142] Reuter H and Seitz N (1968) The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. *J Physiol* 195:451–470.

[143] Martin DL and De Luca HF (1969) Influence of sodium on calcium transport by the rat small intestine. *Am J Physiol* 216:1351–1359.

[144] Li Z, Matsuoka S, Hryshko LV, Nicoll DA, Bersohn MM, and Burke EP (1994) Cloning of the NCX2 isoform of the plasma membrane Na^+-Ca^{2+} exchanger. *J Biol Chem* 269:17434–17439.

[145] Nicoll DA, Quednau BD, Qui Z, Xia YR, Lusis AJ, and Philipson KD (1996b) Cloning of a third mammalian Na^+-Ca^{2+} exchanger, NCX3. *J Biol Chem* 271:24914– 24921.

[146] Lee SL, AS Yu, and Lytton J (1994) Tissue-specific expression of Na^+-Ca^{2+} exchanger isoforms. *J Biol Chem* 269:14849–14852.

[147] Lipton P (1999) Ischemic cell death in brain neuron *Physiol Rev* 79:1431–1568.

[148] Yu L and Colvin RA (1997) Regional differences in expression of transcripts for Na^+/Ca^{2+} exchanger isoforms in rat brain. *Mol Brain Res* 50:285–292.

[149] Blaustein MP and Lederer WJ (1999) Sodium/calcium exchange: its physiological implications. *Physiol Rev* 79:763–854.

[150]Fujioka Y, Fujioka Y, Hiroe K, and Matsuoka S (2000) Regulation kinetics of Na^+-Ca^{2+} exchange current in guinea-pig ventricular myocytes. *J Physiol* 529:611–623.

[151] Hang TM and Hilgemann DW (2004) Multiple transport modes of the cardiac Na^+/Ca^{2+} exchanger. *Nature (Lond)* 427:544–548.

[152] Secondo A et al, (2015) Involvement of the Na+/Ca2+ exchanger isoform 1 (NCX1) in neuronal growth factor (NGF)-induced neuronal differentiation through Ca^{2+}-dependent Akt phosphorylation. J Biol Chem.;290(3):1319-31.

[153] Molinaro P et al, (2015) Neuronal NCX1 overexpression induces stroke resistance while knockout induces vulnerability via Akt. J Cereb Blood Flow Metab. J Cereb Blood Flow Metab (in press).

[154] Pignataro G et al, (2011) NCX1 and NCX3: two new effectors of delayed preconditioning in brain ischemia. Neurobiol Dis.;45 (1):616-23.

[155] Valsecchi V. et al, (2011) NCX1 is a novel target gene for hypoxia-inducible factor-1 in ischemic brain preconditioning. Stroke. 42(3):754-63.

[156] Formisano L. et al, (2015) Sp3/REST/HDAC1/HDAC2 Complex Represses and Sp1/HIF-1/p300 Complex Activates ncx1 Gene Transcription, in Brain Ischemia and in Ischemic Brain Preconditioning, by Epigenetic Mechanism. J Neurosci.;35(19):7332-48.

[157] Jeon D et al, (2008) Na^{+}/Ca^{2+} exchanger 2 is neuroprotective by exporting Ca^{2+} during a transient focal cerebral ischemia in the mouse. Cell Calcium;43(5):482-91.

[158] Kofuji, P., Hadley, R. W., Kieval, R. S., Lederer, W. J. and Schulze, D. H. (1992) Expression of the Na^{+}–Ca^{2+} exchanger in diverse tissues: a study using the cloned human cardiac Na^{+}–Ca^{2+} exchanger. Am. J. Physiol. Cell Physiol. 263, C1241–C1249

[159] Quednau, B. D., Nicoll, D. A. and Philipson, K. D. (2004) The sodium/calcium exchanger family-SLC8. Pflugers Arch. ¨ 447, 543–548

[160] Kofuji, P., Lederer, W. J. and Schulze, D. H. (1994) Mutually exclusive and cassette exons underlie alternatively spliced isoforms of the Na/Ca exchanger. J. Biol. Chem. 269, 5145–5149

[161] Barnes, K. V., Cheng, G., Dawson, M. M. and Menick, D. R. (1997) Cloning of cardiac, kidney, and brain promoters of the feline ncx1 gene. J. Biol. Chem. 272, 11510–11517

[162] Nicholas, S. B., Yang, W., Lee, S. L., Zhu, H., Philipson, K. D. and Lytton, J. (1998) Alternative promoters and cardiac muscle cell-specific expression of the Na+/Ca2+ exchanger gene. Am. J. Physiol. Heart Circ. Physiol. 274, H217–H232

[163] Quednau, B. D., Nicoll, D. A. and Philipson, K. D. (1997) Tissue specificity and alternative splicing of the Na+/Ca2+ exchanger isoforms NCX1, NCX2, and NCX3 in rat. Am. J. Physiol. Cell Physiol. 272, C1250–C1261

[164] Papa, M., Canitano, A., Boscia, F., Castaldo, P., Sellitti, S., Porzig, H., Taglialatela, M. and Annunziato, L. (2003) Differential expression of the Na+–Ca2+ exchanger transcripts and proteins in rat brain regions. J. Comp. Neurol. 461, 31–48

[165] Lytton J (2007) Na^+/Ca^{2+} exchangers: three mammalian gene families control Ca^{2+} transport. Biochem J.;406(3):365-82.

[166] Hilge M, Aelen J, Foarce A, Perrakis A, Vuister GW (2009) Ca^{2+} regulation in the Na^+ /Ca^{2+} exchanger features a dual electrostatic switch mechanism. Proc Natl Acad Sci U S A 106: 14333–14338.

[167] Dyck C, Omelchenko A, Elias CL, Quednau BD, Philipson KD, Hnatowich M, Hryshko LV (1999) Ionic regulatory properties of brain and kidney splice variants of the NCX1 Na^+-Ca^{2+} exchanger. J Gen Physiol 114:701–711

[168] Lindgren RM, Zhao J, Heller S, Berglind H, Nister M (2005) Molecular cloning and characterization of two novel truncated isoforms of human Na^+ /Ca^{2+} exchanger 3, expressed in fetal brain. Gene 348:143–155.

[169] Gabellini N, Bortoluzzi S, Danieli GA, Carafoli E (2002) The human SLC8A3 gene and the tissuespecific Na^+ /Ca^{2+} exchanger 3 isoforms. Gene 298:1–7

[170] Breukels V, Touw WG, Vuister GW (2012a) NMR structure note: solution structure of Ca^{2+} binding domain 2B of the third isoform of the Na^+/Ca^{2+} exchanger. J Biomol NMR 54: 115–121.

[171] Giladi M, Lee SY, Hiller R, Chung KY, Khananshvili D (2015) Structure-dynamic determinants governing a mode of regulatory response and propagation of allosteric signal in splice variants of Na^+ /Ca^{2+} exchange (NCX) proteins. Biochem J 465:489–501

[172] Giladi M, Bohbot H, Buki T, Schulze DH, Hiller R, Khananshvili D (2012) Dynamic features of allosteric Ca^{2+} sensor in tissue-specific NCX variants. Cell Calcium 51:478–485.

[173] Breukels V, Touw WG, Vuister GW (2012b) Structural and dynamic aspects of Ca^{2+} and Mg^{2+} binding of the regulatory domains of the Na^+ /Ca^{2+} exchanger. Biochem Soc Trans 40:409–414.

[174] Lauriane Y.M. Michel, Joost G.J. Hoenderop, and Rene´ J.M. Bindels Rev Physiol Biochem Pharmacol (2015) Towards Understanding the Role of the Na^+ - Ca^{2+} Exchanger Isoform 3.

[175] Moser M., Ruschoff J., Buettner R. (1997) Comparative analysis of AP-2 alpha and AP-2 beta gene expression during murine embryogenesis. *Dev. Dyn.* 208, 115–124.

[176] Mitchell P.J., Timmons P.M., Hebert J.M., Rigby P.W., Tjian R. (1991) Transcription factor AP-2 is expressed in neural crest cell lineages during mouse embryogenesis. *Genes Dev.* 5, 105–119.

[177] Quirin-Stricker C., Mauvais C., Schmitt M. (1997) Transcriptional activation of human choline acetyltransferase by AP2- and NGFinduced factors. *Brain Res. Mol. Brain Res.* 49, 165–167.

[178] Imagawa M., Chiu R., Karin M. (1987) Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. *Cell* 51, 251–260.

[179] Mitchell P.J., Wang C., Tjian R. (1987) Positive and negative regulation of transcription in vitro: enhancer-binding protein AP-2 is inhibited by SV40. *Cell* 50, 847–861.

[180] Williams T., Tjian R. (1991) Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. *Genes Dev.* 5, 670–682.

[181] Platenik J., Kuramoto N., Yoneda Y. (2000) Molecular mechanisms associated with longterm consolidation of the NMDA signals. *Life Sci.* 67, 335–364.

[182] Beckmann A.M., Davidson M.S., Goodenough S., Wilce P.A. (1997) Differential expression of Egr-1-like DNA-binding activities in the naive rat brain and after excitatory stimulation. *J. Neurochem.* 69, 2227–2237.

[183] Worley P.F., Christy B.A., Nakabeppu Y., Bhat R.V., Cole A.J., Baraban J.M. (1991) Constitutive expression of zif268 in neocortex is regulated by synaptic activity. *Proc. Natl. Acad. Sci. USA* 88, 5106–5110.

[184] Honkaniemi J., Sharp F.R. (1999) Prolonged expression of zinc finger immediate-early gene mRNAs and decreased protein synthesis following intracerebral administration of 3-nitropropionic acid. *Eur. J. Neurosci.* 11, 10–17.

[185] Gass P., Herdegen T., Bravo R., Kiessling M. (1994) High induction threshold for transcription factor KROX-20 in the rat brain: partial coexpression with heat shock protein 70 following limbic seizures. *Brain Res. Mol. Brain Res.* 23, 292–298.

[186] Sassone-Corsi P., Visvander J., Ferland L., Mellon P.L., Verma I.M. (1988) Induction of protooncogene fos transcription though the adenylate cyclase pathway: characterization of a cAMP-responsive element. *Genes & Devel.* 2, 1529–1538.

[187] Kobierski L.A., Chu H.M., Tan Y., Comb M.J. (1991) cAMP-dependent regulation of proenkephalin by JunD and JunB: positive and negative effects of AP-1 proteins. *Proc. Natl. Acad. Sci. USA* 88, 10222–10226.

[188] Sheng M., McFadden G., Greenberg M.E. (1990) Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. *Neuron* 4, 571–582.

[189] Carrion A.M., Link W.A., Ledo F., Mellstrom B., Naranjo J.R. (1999) DREAM is a Ca^{2+}-regulated transcriptional repressor. *Nature* 398, 80–84.

[190] Carrion A.M., Mellstrom B., Naranjo J.R. (1998) Protein kinase A-dependent derepression of the human prodynorphin gene via differential binding to an intragenic silencer. *Mol. Cell. Biol.* 18, 6921–6929.

[191] Ledo F., Link W.A., Carrion A.M., Echeverria V., Mellstrom B., Naranjo J.R. (2000) The DREAM-DRE interaction: key nucleotides and dominant negative mutants. *Biochim. Biophys.Acta.* 1498, 162–168.

[192] Gabellini N. (2004), Transcriptional regulation by cAMP and Ca^{2+} links the Na^{+}/Ca^{2+} exchanger 3 to memory and sensory pathways. Mol Neurobiol.;30(1):91-116.

[193] De Cesare, D., Fimia, G.M., and Sassone-Corsi, P. (1999). Signaling routes to CREM and CREB: plasticity in transcriptional activation. Trends Biochem. Sci. 24, 281–285

[194] Mayr, B., and Montminy, M. (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat. Rev. Mol. Cell Biol 2, 599–609.

[195] Shaywitz, A.J., and Greenberg, M.E. (1999). CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu. Rev. Biochem. 68, 821–861.

[196] Chrivia, J.C., Kwok, R.P.S., Lamb, N., Hagiwara, M., Montminy, M.R, and Goodman, R. (1993). Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature 365, 855–859.

[197] Kwok, R.P.S., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bachinger, H.P., Brennan, R.G., Roberts, S.G.E., Green, M.R., and Goodman, R.H. (1994). Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature 370, 223–226.

[198] Parker, D., Ferreri, K., Nakajima, T., LaMorte, V.J., Evans R, Koerber, S.C., Hoeger, C., and Montminy, M.R. (1996). Phosphorylation of

CREB at Ser-133 induces complex formation with CREB binding protein via a direct mechanism. Mol. Cell. Biol. 16, 694–703.

[199] Radhakrishnan, I., Perez-Alvarado, G.C., Parker, D., Dyson, H.J., Montminy, M.R., and Wright, P.E. (1997). Solution structure of KIX domain of CBP bound to the transactivation domain of CREB: a model for activator:coactivator interactions. Cell 91, 741–752.

[200] Ferreri, K., Gill, G., and Montminy, M. (1994). The cAMP-regulated transcription factor CREB interacts with a component of the TFIID complex. Proc. Natl. Acad. Sci. USA 91, 1210–1213.

[201] Quinn, P.G. (1993). Distinct domains of CREB mediate basal and cAMP-regulated gene transcription. J. Biol. Chem. 268, 16999–17009.

[202] Xing, L., Gopal, V.K., and Quinn, P.G. (1995). cAMP response element-binding protein (CREB) interacts with transcription factors IIB and IID. J. Biol. Chem. 270, 17488–17493.

[203] Lonze BE, Ginty DD. (2002) Function and regulation of CREB family transcription factors in the nervous system. Neuron.;35(4):605-23.

[204] Wagner J.J., Terman G.W., Chavkin C. (1993) Endogenous dynorphins inhibit excitatory neurotransmission and block LTP induction in the hippocampus. *Nature* 363, 451–454.

[205] Weisskopf M.G., Zalutsky R.A., Nicoll R.A.(1993) The opioid peptide dynorphin mediates heterosynaptic depression of hippocampal mossy fibre synapses and modulates longterm potentiation. *Nature* 365, 188.

[206] Herdegen T., Leah J.D. (1998) Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. *Brain Res. Brain Res. Rev.*28, 370–490.

[207] Osawa M., Tong K.I., Lilliehook C., et al. (2001) Calcium-regulated DNA binding and oligomerization of the neuronal calcium-sensing protein, calsenilin/DREAM/KChIP3. *J. Biol. Chem.* 276, 41005–41013.

[208] Corneliussen B., Holm M., Waltersson Y., et al. (1994) Calcium/calmodulin inhibition of basic- helix-loop-helix transcription factor domains. *Nature* 368, 760–764.

[209] Onions J., Hermann S., Grundstrom T. (1997) Basic helix-loop-helix protein sequences determining differential inhibition by calmodulin and S-100 proteins. *J. Biol. Chem.* 272, 23930–23937

[210] Buxbaum JD, Choi EK, Luo Y, Lilliemook C, Crowley AC, Merriam DE, Wasco W (1998) Calsenilin: a calcium-binding protein that interacts with the presenilins and regulates the levels of a presenilin fragment. Nature Medicine 4:1177–1181

[211] An WF *et al.* (2000) Modulation of A-type potassium channels by a family of calcium sensors. Nature 403:553–556

[212] Agell N, Aligue R, Alemany V, Castro A, Jaime M, Pujol MJ, Rius E, Serratosa J, Taules M, Bachs O (1998) New nuclear functions for calmodulin. Cell Calcium 23:115–121

[213] Britt Mellström and Jose R. Naranjo (2001) Ca^{2+}-dependent transcriptional repression and derepression: DREAM, a direct effector. CELL & DEVELOPMENTAL BIOLOGY, Vol. 12: pp. 59–63

[214] Buxbaum J.D., Lilliehook C., Chan J.Y., et al. (2000) Genomic structure, expression pattern, and chromosomal localization of the human calsenilin gene: no association between an exonic polymorphism and Alzheimer's disease. *Neurosci. Lett.* 294, 135–138.

[215] Spreafico F., Barski J.J., Farina C., Meyer M. (2001) Mouse DREAM/calsenilin/KChIP3: gene structure, coding potential, and expression. *Mol. Cell. Neurosci.* 17, 1–16.

[216] Philipsen S, Suske G (1999) A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. Nucleic Acids Res 27:2991-3000.

[217] Kadonaga JT, Carner KR, Masiarz FR, Tjian R (1987) Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell 51:1079-1090.

[218] Göllner H, Bouwman P, Mangold M, Karis A, Braun H, Rohner I, Del Rey A, Besedovsky HO, Meinhardt A, van den Broek M, Cutforth T, Grosveld F, Philipsen S, Suske G (2001b) Complex phenotype of mice homozygous for a null mutation in the Sp4 transcription factor gene. Genes Cells 6:689-697.

[219] Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108:17-29.

[220] Göllner H, Dani C, Phillips B, Philipsen S, Suske G (2001a) Impaired ossification in mice lacking the transcription factor Sp3. Mech Dev 106:77-83.

[221] Dynan WS, Tjian R (1983) Isolation of transcription factors that discriminate between different promoters recognized by RNA polymerase II. Cell 32:669-680.

[222] Bouwman P, Göllner H, Elsässer HP, Eckhoff G, Karis A, Grosveld F, Philipsen S, Suske G (2000) Transcription factor Sp3 is essential for post-natal survival and late tooth development. EMBO J 19:655-661.

[223] Kingsley C, Winoto A (1992) Cloning of GT box-binding proteins: a novel Sp1 multigene family regulating T-cell receptor gene expression. Mol Cell Biol 12:4251-4261.

[224] Harrison SM, Houzelstein D, Dunwoodie SL, Beddington RS (2000) Sp5, a new member of the Sp1 family, is dynamically expressed during development and genetically interacts with Brachyury. Dev Biol 227:358-372

[225] Courey AJ, Tjian R (1988) Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell 55:887-898.

[226] Athanikar JN, Sanchez HB, Osborne TF (1997) Promoter selective transcriptional synergy mediated by sterol regulatory element binding

protein and Sp1: a critical role for the Btd domain of Sp1. Mol Cell Biol 17:5193-5200.

[227] Su K, Roos MD, Yang X, Han I, Paterson AJ, Kudlow JE (1999) An N-terminal region of Sp1 targets its proteasome-dependent degradation in vitro. J Biol Chem 274:15194-15202.

[228] Murata Y, Kim HG, Rogers KT, Udvadia AJ, Horowitz JM (1994) Negative regulation of Sp1 trans-activation is correlated with the binding of cellular proteins to the amino terminus of the Sp1 trans-activation domain. J Biol Chem 269:20674-20681.

[229] Milbrandt J (1987) A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. Science 238:797–799.

[230] Yang L et al, (2015) Over-expressed EGR1 may exaggerate ischemic injury after experimental stroke by decreasing BDNFexpression. Neuroscience;290:509-17.

[231] Eckert D et al, (2005) The AP-2 family of transcription factors. Genome Biol.;6(13):246.

[232] Iwamoto T and Shigekawa M (1998b) Differential inhibition of Na^+/Ca^{2+} exchanger isoforms by divalent cations and isothiourea derivative. *Am J Physiol* 275:C423– C430.

[233] Shigekawa M, Iwamoto T, Uehara A, and Kita S (2002) Probing ion binding sites in the Na^+-Ca^{2+} exchanger. *Ann N Y Acad Sci* 976:19–30.

[234] Nicoll DA, Longoni S, and Philipson KD (1990) Molecular cloning and functional expression of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger. *Science (Wash DC)* 250:562–565.

[235] Matsuoka S, Nicoll DA, He Z, and Philipson KD (1997) Regulation of cardiac Na^+/Ca^{2+} exchanger by the endogenous XIP region. *J Gen Physiol* 109:273–286.

[236] Li Z, Nicoll DA, Collins A, Hilgemann DW, Filoteo AG, Penniston JT, Weiss JN, Tomich JM, and Philipson KD (1991) Identification of a peptide

inhibitor of the cardiac sarcolemmal Na^+/Ca^{2+} exchanger. *J Biol Chem* 266:1014–1020.

[237] DiPolo R and Beauge´ LA (1994) Cardiac sarcolemmal Na^+-Ca^{2+} inhibiting peptides XIP and FMRF-amide also inhibit Na^+-Ca^{2+} exchange in squid axons. *Am J Physiol* 267:C307–C311.

[238] He Z, Petesch N, Voges K, Roben W, and Philipson KD (1997) Identification of important amino acid residues of the Na^+-Ca^{2+} exchanger inhibitory peptide, XIP. *J Membr Biol* 156:149–156.

[239] Namane A, Gouyette C, Fillion MP, Fillion G, and Huynh-Dinh T (1992) Improved brain delivery of AZT using a glycosyl phosphotriester prodrug. *J Med Chem* 35:3039–3044.

[240] Pignataro G, Tortiglione A, Scorziello A, Giaccio L, Secondo A, Severino B, Santagada V, Caliendo G, Amoroso S, Di Renzo GF, et al. (2004b) Evidence for a protective role played by the Na^+/Ca^{2+} exchanger in cerebral ischemia induced by middle cerebral artery occlusion in male rats. *Neuropharmacology* 46:439–448.

[241] Sharikabad MN, Cragoe EJ Jr, and Brors O (1997) Inhibition by 5-*N*-(4-chlorobenzyl)-2',4'-dimethylbenzamil of Na^+/Ca^{2+} exchange and L-type Ca^{2+} channels in isolated cardiomyocytes. *Pharmacol Toxicol* 80:57–61.

[242] Taglialatela M, Amoroso S, Di Renzo GF, and Annunziato L (1988b) Membrane events and ionic processes involved in dopamine release from tuberoinfundibular neurons. I. Effect of the inhibition of the Na^+-$Ca2^+$ exchange by amiloride. *J Pharmacol Exp Ther* 246:689–694.

[243] Taglialatela M, Canzoniero LM, Cragoe EJ, Di Renzo, and Annunziato L (1990a) Na^+-$Ca2^+$ exchange activity in central nerve endings. II. Relationship between pharmacological blockade by amiloride analogues and dopamine release from tuberoinfundibular hypothalamic neurons. *Mol Pharmacol* 38:393–400.

[244] Amoroso S, Taglialatela M, Canzoniero LM, Cragoe EJ Jr, di Renzo G, and Annunziato L (1990) Possible involvement of Ca__ ions, protein kinase C and Na^+/H^+ antiporter in insulin-induced endogenous dopamine release from tuberoinfundibular neurons. *Life Sci* 46:885–894.

[245] Amoroso S, De Maio M, Russo GM, Catalano A, Bassi A, Montagnani S, Di Renzo GF, and Annunziato L (1997) Pharmacological evidence that the activation of the Na^+-Ca^{2+} exchanger protects C6 glioma cells during chemical hypoxia. *Br J Pharmacol* 121:303–309.

[246] Kaczorowski GJ, Barros F, Dethmers JK, Trumble MJ, and Cragoe EJ Jr (1985) Inhibition of Na^+/Ca^{2+} exchange in pituitary plasma membrane vesicles by analogues of amiloride. *Biochemistry* 24:1394–1403.

[247] Yatani A, Brown AM, and Schwartz A (1986) Bepridil block of cardiac calcium and sodium channels. *J Pharmacol Exp Ther* 237:9–17.

[248] Cohen CJ, Spires S, and Van Skiver D (1992) Block of T-type Ca channels in guinea pig atrial cells by antiarrhythmic agents and Ca channel antagonists. *J Gen Physiol* 100:703–728.

[249] Berger F, Borchard U, and Hafner D (1989) Effects of the calcium entry blocker bepridil on repolarizing and pacemaker currents in sheep cardiac Purkinje fibres. *Naunyn-Schmiedeberg Arch Pharmacol* 339:638–646.

[250] Mori K, Kobayashi S, Saito T, Masuda Y, and Nakaya H (1998) Inhibitory effects of class I and IV antiarrhythmic drugs on the Na^+-activated K^+ channel current in guinea pig ventricular cells. *Naunyn-Schmiedeberg Arch Pharmacol* 358:641–648.

[251] Watanabe Y and Kimura J (2001) Blocking effect of bepridil on Na^+/Ca^{2+} exchange current in guinea pig cardiac ventricular myocytes. *Jpn J Pharmacol* 85:370–375.

[252] Amoroso S, Tortiglione A, Secondo A, Catalano A, Montagnani S, Di Renzo G, and Annunziato L (2000) Sodium nitroprusside prevents chemical hypoxia-induced cell death through iron ions stimulating the activity of the Na^+-Ca^{2+} exchanger in C6 glioma cells. *J Neurochem* 744:1505–1513.

[253] Iwamoto T, Uehara A, Nakamura TY, Imanaga I, and Shigekawa M (1999) Chimeric analysis of Na^+/Ca^{2+} exchangers NCX1 and NCX3

reveals structural domains important for differential sensitivity to external Ni^{2+} or Li^{+}. *Am J Physiol* 275: C423–C430.

[254] Reeves JP, Bailey CA, and Hale CC (1986) Redox modification of sodium-calcium exchange activity in cardiac sarcolemmal vesicles. *J Biol Chem* 261:4948–4955.

[255] Santacruz-Toloza L, Ottolia M, Nicoll DA, and Philipson KD (2000) Functional analysis of a disulfide bond in the cardiac Na^{+}/Ca^{2+} exchanger. *J Biol Chem* 275:182–188.

[256] L. Annunziato et al, (2004) Pharmacology of Brain Na^{+}/Ca^{2+} Exchanger: From Molecular Biology to Therapeutic Perspectives, *Pharmacol Rev* 56:633–654.

[257] Ballard C and Schaffer S (1996) Stimulation of the Na^{+}/Ca^{2+} exchanger by phenylephrine, angiotensin II and endothelin 1. *J Mol Cell Cardiol* 28:11–17.

[258] Smith BN and Armstrong WE (1996) The ionic dependence of the histamine-induced depolarization of vasopressin neurones in the rat supraoptic nucleus. *J Physiol* 495:465–478.

[259] Stengl M, Mubagwa K, Carmeliet E, and Flameng W (1998) Phenylephrine-induced stimulation of Na^{+}/Ca^{2+} exchange in rat ventricular myocytes. *Cardiovasc Res* 38:703–710.

[260] Eriksson KS, Sergeeva O, Brown RE, and Haas HL (2001) Orexin/hypocretin excites the histaminergic neurons of the tuberomammillary nucleus. *J Neurosci* 21:9273–9279.

[261] Eriksson KS, Stevens DR, and Haas HL (2001) Serotonin excites tuberomammillary neurons by activation of Na^{+}/Ca^{2+} exchange. *Neuropharmacology* 40:345–351.

[262] Woo SH and Morad M (2001) Bimodal regulation of Na^{+}/Ca^{2+} exchanger by betaadrenergic signaling pathway in shark ventricular myocytes. *Proc Natl Acad Sci USA* 98:2023–2028.

[263] Gupta MP, Makino N, Khatter K, and Dhalla NS (1986) Stimulation of Na^+-Ca^{2+} exchange in heart sarcolemma by insulin. *Life Sci* 39:1077–1083.

[264] Makino N, Zhao D, and Dhalla NS (1988) Stimulation of heart sarcolemmal Na^+-Ca^{2+} exchange by concanavalin A. *Biochem Biophys Res Commun* 154:245–251.

[265] Pignataro G et al. (2004)Two sodium/calcium exchanger gene products, NCX1 and NCX3, play a major role in the development of permanent focal cerebral ischemia Stroke, 35(11):2566-70.

[266] Secondo A et al (2007), The Na^+/Ca^{2+} exchanger isoform 3 (NCX3) but not isoform 2 (NCX2) and 1 (NCX1) singly transfected in BHK cells plays a protective role in a model of in vitro hypoxia. Ann N Y Acad Sci. ;1099:481-5.

[267] Molinaro P et al, (2008) Targeted disruption of Na^+/Ca^{2+} exchanger 3 (NCX3) gene leads to a worsening of ischemic brain damage. J Neurosci.;28(5):1179-84.

[268] Pannaccione A et al.(2012), A new concept: Aβ1-42 generates a hyperfunctional proteolytic NCX3 fragment that delays caspase-12 activation and neuronal death.J Neurosci;32(31):10609-17.

[269] Papa M, Canitano A, Boscia F, Castaldo P, Sellitti S, Porzig H, Taglialatela M, and Annunziato L (2003) Differential expression of the Na^+-Ca^{2+}exchanger transcripts and proteins in rat brain regions. *J Comp Neurol* 461:31–48.

[270] Reuter H, Porzig H., (1995) Localization and functional significance of the Na^+/Ca^{2+} exchanger in presynaptic boutons of hippocampal cells in culture. Neuron. ;15(5):1077-84.

[271] Molinaro P et al, (2011) Na^+ -Ca^{2+} exchanger (NCX3) knock-out mice display an impairment in hippocampal long-term potentiation and spatial learning and memory. J Neurosci.;31(20):7312-21.

[272] Gomez-Villafuertes R et al, (2005) Downstream regulatory element antagonist modulator regulates Ca^{2+} homeostasis and viability in cerebellar neurons. J Neurosci.;25(47):10822-30.

[273] Smart D., Smith G., Lambert D.G. (1995) Muopioids activate phospholipase C in SH-SY5Y human neuroblastoma cells via calcium-channel opening. *Biochem. J.* 305, 577–578.

[274] Lüsher, B., Mitchell, P.J., Williams, T., Tjian, R., (1989) Regulation of transcription factor AP-2 by the morphogen retinoic acid and by second messengers. *Genes Dev.* 3, 1507–1517.

[275] Gabellini N., Bortoluzzi S., Danieli G.A., Carafoli E. (2003) Control of the Na^{+}/Ca^{2+} exchanger 3 promoter by cyclic adenosine monophosphate and Ca^{2+} in differentiating neurons. *J. Neurochem.* 84, 282–293.

[276] Anderson M.E., Braun A.P., Wu Y., et al. (1998) KN-93, an inhibitor of multifunctional Ca^{2+}/calmodulin-dependent protein kinase, decreases early after depolarizations in rabbit heart. *J. Pharmacol. Exp. Ther.* 287, 996–1006.

[277] Bhatt H.S., Conner B.P., Prasanna G., Yorio T., Easom R.A. (2000) Dependence of insulin secretion from permeabilized pancreatic beta cells on the activation of Ca^{2+}/calmodulin dependent protein kinase II. A re-evaluation of inhibitor studies. *Biochem. Pharmacol.* 60, 1655–1663.

[278] Shimomura A., Ogawa Y., Kitani T., Fujisawa H., Hagiwara M. (1996) Calmodulin-dependent protein kinase II potentiates transcriptional activation through activating transcription factor 1 but not cAMP response element-binding protein. *J. Biol. Chem.* 271, 17957–17960.

[279] Kornhauser J.M., Cowan C.W., Shaywitz A.J., et al. (2002) CREB transcriptional activity in neurons is regulated by multiple, calcium-specific phosphorylation events. *Neuron* 34, 221–233.

[280] Burda J et al (2006) , Delayed postconditionig initiates additive mechanism necessary for survival of selectively vulnerable neurons after transient ischemia in rat brain. Cell Mol Neurobiol.;26(7-8):1141-51.

[281] Pignataro G et al, (2011) The NCX3 isoform of the Na^+/Ca^{2+} exchanger contributes to neuroprotection elicited by ischemic postconditioning. J Cereb Blood Flow Metab. ;31(1):362-70.

[282] Gill G. (2003) Post-translational modification by the small ubiquitin-related modifier sumo has big effects on transcription factor activity. *Curr Opin Genet Dev*.;13:108-113

[283] Bohren KM, Nadkarni V, Song JH, Gabbay KH, Owerbach D.(2004) A m55v polymorphism in a novel sumo gene (sumo-4) differentially activates heat shock transcription factors and is associated with susceptibility to type i diabetes mellitus. *J Biol Chem*.;279:27233-27238

[284] Cimarosti H, Henley JM. (2008) Investigating the mechanisms underlying neuronal death in ischemia using in vitro oxygen-glucose deprivation: Potential involvement of protein sumoylation. *Neuroscientist*.;14:626-636

[285] Rajan S, Plant LD, Rabin ML, Butler MH, Goldstein SA. (2005) Sumoylation silences the plasma membrane leak k+ channel k2p1. *Cell*.;121:37-47

[286] Bederson JB, Pitts LH, Germano SM, Nishimura MC, Davis RL, Bartkowski HM. (1986) Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. *Stroke*.;17:1304-1308

[287] Cuomo O et al, (2016)Sumoylation of LYS590 of NCX3 f-Loop by SUMO1 Participates in Brain Neuroprotection Induced By Ischemic Preconditioning. Stroke. (in press)

[288] Sirabella R et al, (2009) Anoxia-induced NF-kappaB-dependent upregulation of NCX1 contributes to Ca^{2+} refilling into endoplasmic reticulum in cortical neurons. Stroke.;40(3):922-9.

[289] Formisano L, Guida N, Valsecchi V, Pignataro G, Vinciguerra A, Pannaccione A, Secondo A, Boscia F, Molinaro P, Sisalli MJ, Sirabella R, Casamassa A, Canzoniero LM, Di Renzo G, Annunziato L (2012) NCX1 is a new rest target gene: Role in cerebral ischemia. Neurobiol Dis.

[290] Formisano L, Noh K, Miyawaki T, Mashiko T, Bennett M, Zukin R (2007) Ischemic insults promote epigenetic reprogramming of mu opioid receptor expression in hippocampal neurons. Proc Natl Acad Sci U S A 104:4170-4175

[291] Renthal W, Maze I, Krishnan V, Covington HE, Xiao G, Kumar A, Russo SJ, Graham A, Tsankova N, Kippin TE, Kerstetter KA, Neve RL, Haggarty SJ, McKinsey TA, Bassel-Duby R, Olson EN, Nestler EJ (2007) Histone deacetylase 5 epigenetically controls behavioral adaptations to chronic emotional stimuli. Neuron 56:517-529.

[292] Yildirim F et al (2008) Inhibition of histone deacetylation protects wildtype but not gelsolin-deficient mice from ischemic brain injury. Exp Neurol.;210 (2):531-42.

[293] Koppel I et al, (2013) Differential regulation of Bdnf expression in cortical neurons by class-selective histone deacetylase inhibitors. Neuropharmacology ;75:106-15.

[294] Guida N et al, (2014) Histone deacetylase 4 promotes ubiquitin-dependent proteasomal degradation of Sp3 in SH-SY5Y cells treated with di(2-ethylhexyl)phthalate (DEHP), determining neuronal death. Toxicol Appl Pharmacol.1;280(1):190-8

[295] Collins LM et al, (2015) Class-IIa Histone Deacetylase Inhibition Promotes the Growth of Neural Processes and Protects Them Against Neurotoxic Insult. Mol Neurobiol.;51(3):1432-42.

[296] Bolger TA et al, (2005) Intracellular trafficking of histone deacetylase 4 regulates neuronal cell death. J Neurosci.;25(41):9544-53.