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IDENTIFICATION OF EPIGENETIC MECHANISMS REGULATING THE ISOFORM 1 OF SODIUM CALCIUM EXCHANGER (NCX1) IN *IN VITRO* AND *IN VIVO* MODELS OF BRAIN ISCHEMIA AND BRAIN ISCHEMIC PRECONDITIONING

COORDINATOR: PROF. LUCIO ANNUNZIATO

TUTOR:
PROF. GIANFRANCO DI RENZO

CANDIDATE:
NATASCIA GUIDA

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Summary

SUMMARY

The Na^{+}-Ca^{2+} exchanger 1 (NCX1) is reduced in stroke by the RE1-silencing transcription factor (REST), whereas it is increased in Ischemic Brain Preconditioning (PC) by Hypoxia-Inducible Factor 1 (HIF-1). Since *ncx1* brain promoter (ncx1-Br) has five putative consensus sequences for the Specificity protein (Sp) family of transcription factors (Sp1, Sp2, Sp3, Sp4), named Sp1 A-E, we investigated the role of this family in regulating *ncx1* transcription in cortical neurons. Here we found that Sp1 is a transcriptional activator, whereas Sp3 is a transcriptional repressor of *ncx1*, and that both bind *ncx1* brain promoter (ncx1-Br) in a sequence-specific manner, modulating *ncx1* transcription through the Sp1 sites C-E. Furthermore, in brain ischemia (tMCAO) the transcriptional repressors Sp3 and REST colocalize with histone-deacetylases (HDACs) HDAC1-2 at the *ncx1* promoter, with a consequent hypoacetylation. By contrast, in PC+tMCAO the transcriptional activators Sp1 and HIF-1 colocalize with Histone-Acetyltransferase p300 (p300) on *ncx1* promoter sequence with a consequent hyperacetylation. Moreover, when brain ischemia-induced REST and Sp3 up-regulation was prevented by intracerebroventricular (icv) injection of siRNAs for REST and Sp3, NCX1 downregulation was reverted. Interestingly, also siRNAs for HDAC1-2 was able to completely revert NCX1 reduction. On the contrary, siRNA for Sp1 and HIF-1, during PC+tMCAO, blocked NCX1 increase, that was almost completely reverted by double siRNA for HIF-1 and Sp1 or by siRNA for p300. In addition, in neurons transfected for siNCX1, and subjected to Oxygen Glucose Deprivation (OGD) (3 hours) plus Reoxygenation (RX) (24 hours), the protective effect of class I HDAC inhibitor MS-275 was counteracted, whereas neurons overexpressing NCX1 and subjected to ischemic preconditioning (PC+OGD\Rx), the detrimental effect of p300 inhibitor C646 was prevented. Collectively, these results demonstrate that NCX1 expression is regulated by Sp3\REST\HDAC1-2 complex in tMCAO and by the Sp1\HIF-1\p300 complex in PC+tMCAO, and that epigenetic therapy modulating the acetylation of *ncx1* gene promoter may be a new strategy to reduce the neurodetrimental effect of stroke.

Introduction

I. INTRODUCTION

I.A. CEREBRAL ISCHEMIA

Ischemic stroke, is the third leading cause of death and a major cause of long-lasting disability in industrially developed countries, only surpassed by heart disease and cancer. It is a pathological condition resulting from occlusion or hemorrhage of blood vessels supplying oxygen and essential nutrients to the brain. In all cases, stroke ultimately induces death and\or dysfunction of brain cells, as well as neurological impairments that reflect the location and size of the ischemic brain area. Even though a large number of compounds have been prove to reduce ischemic injury in experimental animal models, clinical trials have reported disappointing results because of toxic side effects. At present the only FDA (US Food andDrug Administration) approved treatment is to provide tissue plasminogen activator (tPA) to re-open occluded blood vessels, however, due to a narrow time-window of 4,5 hours after the stroke onset (Hacke et al., 2008), this treatment is only appropriate for every small number of patients. Thus, research on the discovery of novel mechanisms and the development of new drugs for treating cerebral ischemia are imperative.

I.A.1. Pathophysiology of Cerebral Ischemia

Cerebral ischemic event triggers a set of complex pathological mechanisms eventually leading to death and\or dysfunction of brain cells. Excitotoxicity and ionic imbalance, oxidative\nitrosative stress, inflammation and apoptosis are some of the ischemia-induced pathophysiological processes, each of which has a distinct time frame, some occurring over minutes, others over hours and days, causing injury to neurons, glia and endothelial cells. Within the core of the ischemic area, where blood flow is most severely restricted, excitotoxic and necrotic cell death occurs within minutes. In the periphery of the ischemic area, where collateral blood flow can buffer the full effects of the stroke, the degree of ischemia and the timing of reperfusion determine the outcome for individual cells. In this ischemic penumbra cell death occurs less

rapidly via mechanisms such as apoptosis and inflammation (Dirnagl et al., 1999).

I.A.1.1. Excitotoxicity and Ionic imbalance

After ischemia, $Na^{+}\backslash K^{+}$-ATPase and Ca^{2+}-ATPase, which are important ion pumps found on the plasma membrane of neurons, can no longer function due to energy depletion. Their impaired functions cause neuronal plasma membrane depolarization, release of potassium into the extracellular space and entry of sodium and calcium into the cells (Caplan, 2000). Consequently, calcium dependent proteases, lipases and DNases become active, eventually leading to catabolism and death of many cells in the ischemic core. Furthermore, membrane depolarization results in neurotransmitter release, most prominently the release of the excitatory neurotransmitter glutamate, which plays a central role in the pathology of cerebral ischemia (Simon et al., 1984). Increase in synaptic glutamate concentration overactivates its receptors, N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and metabotropic glutamate (mGlu) receptors, which in return cause further membrane depolarization and greater calcium influx, exacerbating excitotoxicity (Bruno et al., 2001; Peng et al., 2006).

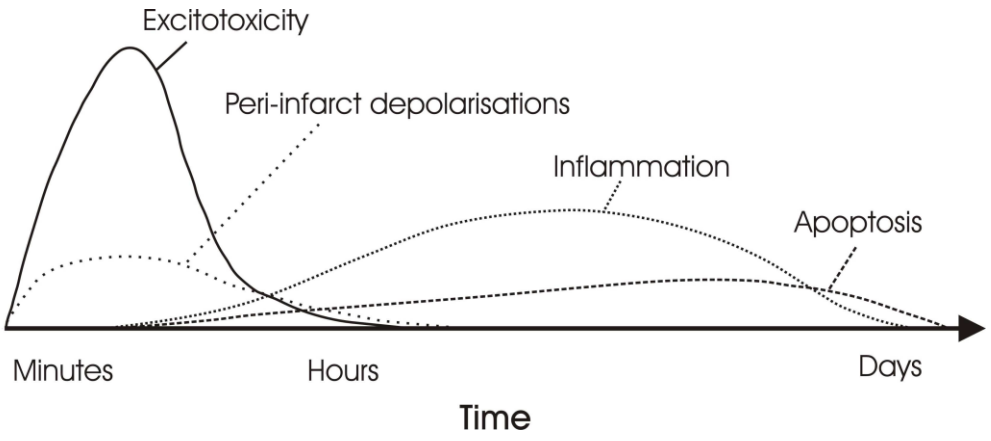

Figure 1. Damaging cascades of events after cerebral ischemia (Dirnagl et al., 1999).

I.A.1.2. Oxidative and nitrosative stress

Unlike other organs, the brain is especially vulnerable to reactive oxygen species due to neurons having relatively low levels of endogenous antioxidants (Coyle and Puttfarcken, 1993). High levels of intracellular calcium, sodium and ADP induce mitochondria to produce deleterious levels of reactive oxygen species, and consequently overly abundant oxygen radicals cause the destruction of cellular macromolecules and participate in signaling mechanisms that result in apoptotic cell death (Sugawara and Chan, 2003). Moreover, there is a surge in production of superoxide, NO and peroxynitrate, following reperfusion. Thrombolytic therapy has a 4,5 hour time window of efficacy. The reason for this limited time window is that the surge in production of free radicals associated with delayed reperfusion brings a second wave of oxidative and nitrosative stress that increases the risk of brain hemorrhage and edema.

I.A.1.3. Apoptosis

Ischemic injury preferentially induces cell death via an apoptotic-like mechanism rather than necrosis. Because the ischemic penumbra sustains milder injury and preserves ATP, apoptosis predominates in this region (González, 2006). Triggers of apoptosis include ionic imbalance, oxygen free radicals, death receptor ligation, DNA damage and protease activation. Cytochrome c release from the outer mitochondrial membrane, an event which is promoted or prevented by Bcl-2 family of proteins, initiates the intrinsic apoptotic cascade. In addition, extrinsic apoptosis pathway, which could be activated by inflammatory signals such as TNF family of ligands, is also operative after ischemia (del Zoppo et al., 2000). Eventually, downstream effector caspases are activated targeting the substrates that dismantle the cell by cleaving homeostatic, cytoskeletal, repair, metabolic and cell signaling proteins (Namura et al., 1998)

I.A.1.4. Inflammation

Inflammation contributes to cerebral ischemic injury. Effects of individual components of the inflammatory cascade, however, can be beneficial depending on the stage of tissue injury, the magnitude of the response and whether the inflammatory component also activates neuroprotective pathways (Bruce et al., 1996; Nawashiro et al., 2000). After stroke immune cells can gain access to brain parenchymal tissue. Infiltration of bone marrow-derived cells into the ischemic brain persists for weeks following stroke, and while the initial infiltration leads to worsening of tissue damage and exacerbation of neurological deficits, subsequent aspects of the infiltration such as the phagocytosis of debris and the release of cytokines that promote glial scar formation could be crucial for effective wound healing. On the other hand, numerous cytokines and chemokines are produced by activated endothelial cells, microglia, neurons, platelets, leukocytes, and fibroblasts and contribute to ischemic brain injury (Gong et al., 1998). In particular, IL-1, TNF-α and toll-like receptors (TLRs) are important inflammatory factors with detrimental effects for stroke outcome (Zaremba and Losy, 2001; Cao et al., 2007; Lehnardt et al., 2007). In contrast, TGF-β was reported to play a neuroprotective role in the pathogenesis of stroke (Wiessner et al., 1993).

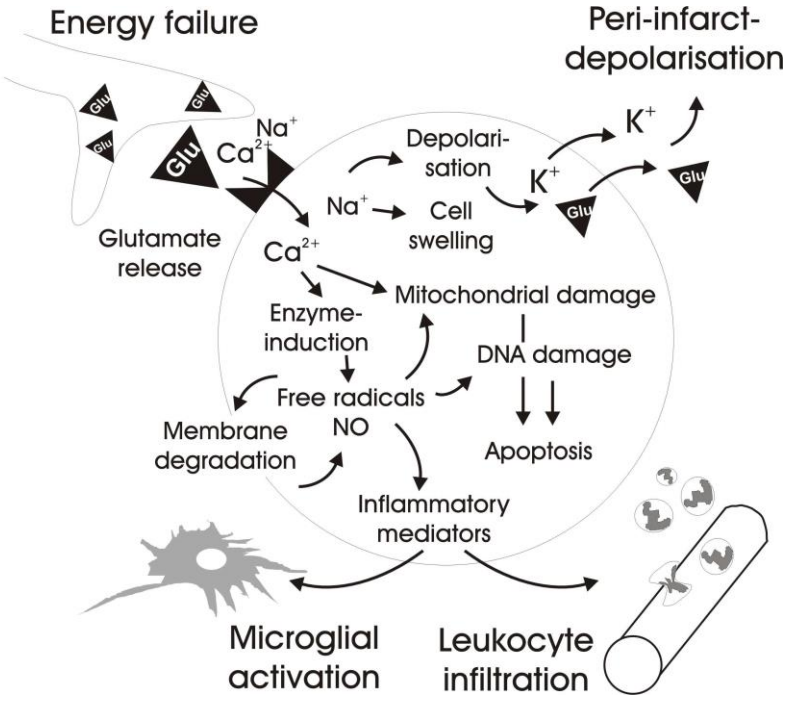

Figure 2. Depiction of the major pathophysiological events evoked by cerebral ischemic injury. Glutamate excitotoxicity, peri-infarct depolarisations, oxidative\nitrosative stress, apoptosis and inflammation contribute to injury following brain ischemia (Dirnagl et al., 1999).

I.A.2. Ischemic preconditioning in brain

Ischemic preconditioning or ischemic tolerance is described as a brief episode of sub-lethal ischemia which renders the brain resistant to subsequent, longer and severe ischemic insults. The terms 'tolerance' and 'preconditioning' were introduced for the first time by Janoff in 1964 (JANOFF, 1964), and ever since, the phenomenon of ischemic preconditioning has been observed in numerous organs such as brain (Kitagawa et al., 1991) and heart (Meldrum et al., 1997), as well as in a wide range of species like the gerbil (Kirino et al., 1991), the rat (Simon et al., 1993) and the mouse (Wu et al., 2001). Amongst various brain regions, hippocampus, cerebral cortex, basal ganglia and thalamus were often reported to acquire ischemic tolerance (Kitagawa et al., 1991). Measurement of cerebral blood flow showed that such tolerance was not accompanied by an improvement of regional tissue perfusion during or after the ischemic episode that induced tolerance (Matsushima and Hakim, 1995; Barone et al.,

1998). Therefore, the state of ischemic tolerance seems to be based on the alteration of neurons themselves at the cellular level.

I.A.2.1. Mechanisms of Ischemic Preconditioning

Mechanisms of ischemic preconditioning could be grouped into three components according to the temporal profile of their development. The first component is the stress sensor\signalling component that can detect various stressful conditions and convert the information into intracellular signals. Hypoxia Inducible Factor (HIF), oxygen sensitive ion channels, potassium channels, calcium and sodium channel families control cellular responses for hypoxia in neurons as well as in many other cell types and belong to the sensor\signaling component of tolerance development (Kemp and Peers, 2007). Once the danger is detected by the cell, an appropriate cellular response is coordinated by the transducer component, which includes various kinases such as p38 mitogen activated protein kinase (MAPK) (Nishimura et al., 2003) and extracellular regulated kinases (ERK) (Jones and Bergeron, 2004), as well as transcription factors like signal transducer and activator of transcription (STATs), cyclic AMP response-element binding-protein (CREB) (Meller et al., 2005) and nuclear factor kappa B (NF-κB) (Digicaylioglu and Lipton, 2001). Lastly, the effector component represents the executive mediators of protection: Reactive oxygen species (ROS) scavenger superoxide dismutase (SOD) (Danielisová et al., 2005), anti-apoptotic protein Bcl-2 (Liu et al., 2002), vascular endothelial growth factor (VEGF) (Bernaudin et al., 2002), nitricoxide (NO) and hexokinase-2 are some of the most reported mediators of neuroprotection conferred by ischemic preconditioning (Kirino, 2002). Ischemic preconditioning-induced neuroprotection may be the result of a combination of different cellular and molecular pathways, with a net outcome of counteracting pathophysiological cascades triggered by lethal ischemic insult. Ischemic tolerance thus reflects a fundamental change in the cellular response to injury that shifts the outcome from cell death to cell survival (Dirnagl et al., 2003). In fact, Stenzel-Poore, with a substantial series of genome-wide gene expression analysis study, suggested that preconditioning maylead to a fundamental

reprogramming of the transcriptional response to ischemic injury, ultimately conferring neuroprotection (Stenzel-Poore et al., 2003). Although transcription factors, such as HIF-1, CREB and NF-κB are already known to be driving neuroprotective gene expression upon an ischemic preconditioning stimulus, we are today more aware that apart from the transcription factors and DNA sequence, regulation of such transcriptional activity requires the cooperation of a third party, namely epigenetic alterations of the DNA and histones. Indeed, these modifications crucially regulate the accessibility of specific regulatory DNA elements for transcription machinery. Recent years have witnessed the emergence of growing evidence supporting an integral role for epigenetic mechanisms in neuronal gene expression, yet involvement of these mechanisms in brain ischemic preconditioning and neuroprotection is mostly unknown.

I.B. EPIGENETICS

Epigenetics has historically meant “on top of, or in addition to, genetics”. Epigenetics was initially considered to have a role mainly in development and cell differentiation, thus allowing cells with identical genomes to acquire distinct phenotypes based on genetic programming. Today, epigenetics is accepted as the study of modifications that result in heritable changes in gene expression independent of alterations in DNA sequence. These major epigenetic modifications include DNA methylation, histone modifications and, more recently, RNA interference, i.e. through non-coding microRNA (miRNA) (Konsoula and Barile, 2012).

I.B.1. Nucleosome

The nucleosome core particle is the fundamental unit of chromatin structure in all eukaryotes. It is an octamer, containing two copies of each of the four histone proteins, H2A, H2B, H3, H4, and around which 146 bp of DNA is wrapped in 1¾ superhelical turns (Luger et al., 1997). All core histone proteins have a similar structure with an N-terminal domain, a globular domain and a C-terminal domain. It has been known for many years that the histone N-terminal tails are exposed on the surface of the nucleosome and that selected amino acid residues could be subjected to a variety of enzyme-catalyzed, post-translational modifications. These include acetylation of lysines, phosphorylation of serines, and methylation of lysines and arginines, and addition of small peptide ubiquitin. The current epigenetics defines the nucleosome and its modified tail domains, not solely as a structural packer of DNA, but a carrier of epigenetic information that determines both how genes are expressed and how their expression patterns are maintained from one cell generation to the next.

I.B.2. Epigenetic Modifications

Covalent histone modifications, DNA methylation and RNA-associated post-transcriptional gene silencing have been so far the most investigated modifications that carry epigenetic information. The histone tail modifications are likely to act in concert with the rather more widely known mediator of chromatin structure and gene expression, namely methylation of cytosine residues in CpG dimers through the action of DNA methyltransferases. Long-term silencing, as found in imprinted genes or the female inactive X chromosome, is generally associated with relatively high levels of CpG methylation. Current knowledge on the exact mechanism(s) by which CpG methylation leads to gene silencing is still fragmentary, yet there is evidence that histone modifications are also involved. Notably, experiments in the filamentous fungus Neurospora crassa have shown that DNA methylation is dependent upon methylation of histone H3 lysine 9 (Tamaru and Selker, 2001). Recent studies have shed light on the complexity of epigenetic information that could possibly be carried in the histone tails. Currently, there are 50 different acetylated isoforms of the four core histones (H2B, H3, and H4 have 16 each and H2A has two). These isoforms can be modified further by methylation of selected lysines and arginines (H3 and H4) and phosphorylation of serine (H3, H4, H2B). Moreover, methylation can be attachment of one, two, or three methyl groups, and there are other modifications, such as ubiquitination and ADP-ribosylation (Hansen et al., 1998). The total number of possible histone isoforms, carrying different combinations of tail modifications can reach a total sum of many thousands. This vast information carrying potential of histone tail modifications have given rise to the concept of a histone code (Strahl and Allis, 2000) or an epigenetic code (Turner, 2000), which is expected to be much more sophisticated than the genetic code carried in DNA sequence.

I.B.3. Acetylation and Deacetylation

Acetylation and deacetylation of histone proteins associated with chromatin plays a pivotal role in the epigenetic regulation of transcription and other functions in cells, including neurons (Mai et al., 2005; Abel and Zukin, 2008). Histone acetyltransferases (HATs) and histone deacetylases (HDACs) catalyze the acetylation and deacetylation, respectively, of histone proteins at Lys (K) residues. The interplay between HATs and HDACs alters the net balance of histone acetylation levels, thereby remodeling chromatin structure (**Fig. 3**). In general, an increase in protein acetylation at histone tails results in a more open and relaxed chromatin conformation, thus facilitating transcription factor interaction with specific gene promoters and activating gene expression. HDACs often function as a component of the transcriptional repressor complex to silence gene expression and induce chromatin compaction through histone protein deacetylation. Accordingly, HDAC inhibition shifts the balance towards enhanced histone acetylation, chromatin relaxation and gene expression. Imbalance between the activities of HATs and HDACs could lead to disease states. For example, mutation and loss of activity of the HAT, cyclic AMP response element binding protein (CREB)-binding protein (CBP), is causative for Rubinstein–Taybi syndrome, a developmental disorder characterized by mental retardation (Bartsch et al., 2005). In addition to histones, HATs and HDACs also use a number of non-histone proteins as their substrates, notably tubulin and transcription factors such as the tumor suppressor p53, Sp1, Smad7, CREB, the pleiotropic transcription factor NF-kB, and signal transducers and activators of transcription-1 (STAT-1) (Chuang et al., 2009a).

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

Histone acetyltransferases (HATs) are a group of enzymes which transfer the acetyl moiety from acetyl coenzyme A (acetyl Co A) onto one or more lysine residues contained within the N-terminal tails of histone proteins. Sequence analysis of HAT proteins reveal that they fall into distinct families that show high sequence similarity (Kuo and Allis, 1998). Each HAT family appears to

have a distinct substrate preference, and different families tend to appear in different functional contexts Gcn5\PCAF, MYST, TAFII250 and CREB-binding protein (CBP)\p300 are some of the most studied HAT families. Among those, CBP\p300 family, including CBP and its close homologue p300, is fundamentally important in various signal modulated transcriptional events (Eckner et al., 1994). The ability of CBP\p300 to enhance transcription is believed to be accomplished in two modes. First, by acting as a bridging factor thus recruiting the RNA polymerase II holoenzyme via interaction with general transcription factors (Manteuffel-Cymborowska, 1999) and second, by acetylation of histones via their HAT activity (Ogryzko et al., 1996). Nucleosomal histones H3 and H4 are the preferred substrates. Both CBP and p300 proteins were shown to interact with a diverse set of sequence-specific transcription factors such as E2F, p53, MyoD, c-Myb and HIV-tat (Snowden and Perkins, 1998). Homozygous knockout mice of either CBP or p300 display, among several other malformations, defects in neural tube closing and are embryonic lethal (Yao et al., 1998).

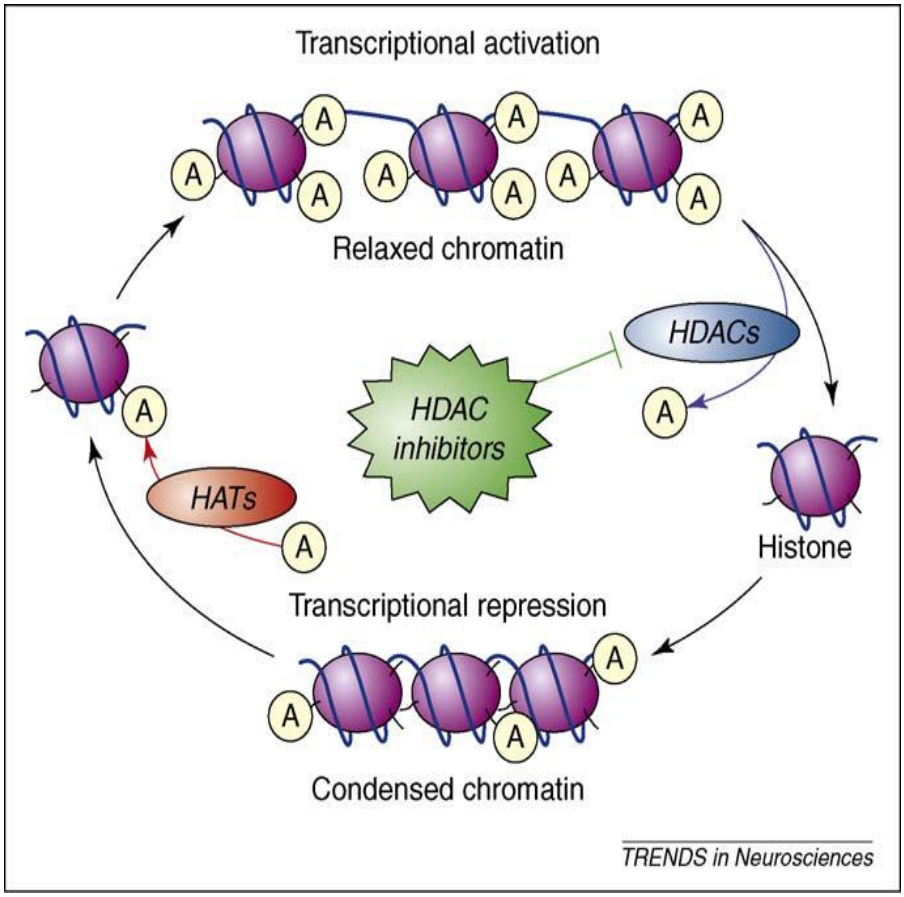

Figure 3. Effects of HDAC inhibitors on chromatin remodeling. Levels of histone acetylation at Lys residues on histone-tails are determined by interplays of acetylation and deacetylation catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. Inhibition of HDACs by HDAC inhibitors results in a net increase in histone acetylation levels and a more open, relaxed chromatin conformation that favors transcriptional activation. By contrast, chromatin with a compact conformation is transcriptionally inactive (Chuang et al. 2009).

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

The superfamily of HDACs consists of five main subtypes: classes I, IIa and IIb, IV, and the structurally distinct class III (Gregoretti et al., 2004; Butler and Bates, 2006). The name HDAC is a misnomer having a historic origin, as histones are not enzymatic substrates for some deacetylases in a given family, and are not exclusive substrates for other family members. Class I and class II HDACs include the Zn^{2+}-dependent deacetylases, which share significant structural homology, especially within the highly conserved catalytic domains (Thiagalingam et al., 2003).

Class I HDACs contain the ubiquitously expressed HDAC1, HDAC2 and HDAC3, and the muscle-specific HDAC8. HDAC1 and HDAC2 are predominantly localized in the nucleus, whereas HDAC3 shuttles between the nucleus and cytoplasm. All three of these deacetylases contain a nuclear localization signal within their protein sequences, and HDAC3 additionally has a nuclear export signal (Yang et al., 2002). The best-characterized enzymes HDAC1 and HDAC2 are components of three stable transcriptional complexes termed Sin3A, NuRD and CoREST. These complexes are recruited to gene promoters by DNA binding proteins, which suggests gene specific rather then global transcriptional regulation (Laherty et al., 1997; Wen et al., 2000).

Class IIa HDACs consist of four members — HDAC4, HDAC5, HDAC7 and HDAC9 — with distinct tissue specific patterns of expression, predominantly in muscle and heart (Majdzadeh et al., 2008). These proteins contain extended amino-terminal domains of ~600 aminoacids, which mediate interactions with HDAC3, myocyte enhancer factor 2 (MeF2), repressor complex NCoR2\SMRT and 14-3-3 proteins (Butler and Bates, 2006), followed by the Zn^{2+}-containing catalytic domain. Interestingly, some data indicate that HDAC4, HDAC5 and HDAC7 are unable to deacetylate histones themselves, but probably participate in gene-specific transcriptional regulation via an interaction with class I HDAC3 (Fischle et al., 2001). However, new results have demonstrated intrinsic deacetylase activity of HDAC4 and other class IIa deacetylases, and have showed that these enzymes are particularly active on class IIa-specific substrates *in vitro* (Jones et al., 2008). HDAC4, HDAC5, HDAC7 and MITR shuttle between the nucleus and cytoplasm, whereas full-length HDAC9 is localized in the nucleus (Bertos et al., 2001; Petrie et al., 2003). Retention in the cytoplasm is controlled by 14-3-3 anchor proteins, which bind exclusively to the phosphorylated from of HDACs and prevent entry into the nucleus, whereas nuclear re-entry occurs upon protein dephosphorylation (Zhang et al., 2001; Grozinger and Schreiber, 2000).

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 (Hubbert et al., 2002; Matsuyama et al., 2002). Its close structural homologue HDAC10 lacks the second functional catalytic domain (Tong et al., 2002). HDAC10 has been found in a complex with HDAC3, although the exact functions of this deacetylase are not known.

The class IV enzyme HDAC11 is structurally different from the class I and class II deacetylases. HDAC11 is predominantly localized in the nucleus. The function of this deacetylase is poorly understood, although region-specific and developmental expression patterns have been observed in the mouse brain, (Liu et al., 2008).

By contrast, the class III deacetylases, or sirtuins, are structurally and functionally different from other HDACs. Named after the Silent information regulator 2 (*Sir2*) gene — the first sirtuin identified in budding yeast — in humans, the class III HDACs include seven members. Sirtuins are markedly different in their absolute dependence on NAD+ to carry out catalytic reactions, which include both deacetylase and mono-ADP-transferase activities (Sauve et al., 2006). The predominant deacetylase activity has been shown for the class III HDACs SIRT1, SIRT2, SIRT3 and SIRT5 (Gan and Mucke, 2008). The deacetylation reaction mediated by sirtuins is coupled to the cleavage of NAD+, yielding nicotinamide and 2′-*O*-acetyl ADP-ribose, along with the deacetylated lysine residue within the protein substrate (Denu, 2005), (Denu J. M., 2005). Structurally, human SIRT1 is the closest analogue of yeast Sir2, which regulates cellular metabolism and ageing. SIRT2 has both nuclear and cytosolic localization, and interacts with numerous protein partners to execute multiple functions in cells. SIRT1 deacetylates a single lysine residue on several histones: K16 on histone 4 (H4), K14 on histone 3 (H3) and K26 on histone 1 (H1). Non-histone substrates of SIRT1 include the transcription factors p53, TAF_I68, p300 and peroxisome proliferator activated receptor-γ, coactivator 1α (PGC1α) (Michan and Sinclair, 2007). SIRT2 is a cytosolic protein that deacetylates α-tubulin and microtubules, a function that is redundant with that of HDAC6. Subsequently, it has been shown that SIRT2 also deacetylates K16 on H4 (Vaquero et al., 2006), and localizes to

neuronal nuclei. SIRT3, instead, is a mitochondrial protein. Acetyl-CoA synthetase (ACS2) has been identified as a SIRT3 substrate; however other data suggest a broad role for SIRT3 in regulating global mitochondrial lysine acetylation (Hallows et al., 2006; Schwer et al., 2006). Finally, SIRT6 has recently been shown to be involved in the regulation of telomomeric DNA during S phase where the putative enzymatic target is K9 on H3 (Kazantsev and Thompson, 2008).

I.B.6. HDACs Inhibitors

Intensive therapeutic development efforts have focused on targeting HDACs with small molecules. Initially, this interest was precipitated by the discovery of the anticancer potential of HDAC inhibitors. Subsequently, potential therapeutic applications were broadened to include other human illnesses, including CNS diseases, based initially on promising *in vivo* applications to polyglutamine-repeat diseases. Although cancer remains a primary target for HDAC-based therapy, significant efforts have been made to develop compounds for the treatment of brain disorders (Kazantsev and Thompson, 2008).

A variety of non-selective and selective HDAC inhibitors have been developed, both synthetically derived and from natural sources. Among the relatively non-selective HDAC inhibitors, trichostatin A (TSA) and suberoylanilide hydroxamic acid (vorinostat, also known as SAHA) inhibit most zinc-dependent HDACs and are permeable to the blood–brain barrier (BBB). The hydroxamate moiety of these compounds appears 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. However, butyrate does not appear to inhibit HDAC6 because the acetylation levels of α-tubulin, a substrate of HDAC6 (Hubbert et al., 2002), are unaffected by sodium butyrate treatment. Valproic acid, a fatty acid derivative with mood-stabilizing and anticonvulsant properties, is another HDAC inhibitor that binds to the active site of the enzymes; valproic acid inhibits class I and class IIa HDACs, but not class IIb (Gurvich et al., 2004). Butyrates and valproic acid are also known to readily cross the BBB (Butler

and Bates, 2006). Advances have been made to design more selective HDAC inhibitors. MS-275, a synthetic benzamide derivative, 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, can be administered orally, 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 HDACs (Khan et al., 2008). Romidepsin (FK-228), another cyclic tetrapeptide, also potently inhibits HDAC1 and 2 (Itoh et al., 2008). Tubacin is a catalytic domain-targeting small molecule inhibitor showing high selectivity for HDAC6 and for the deacetylation of α-tubulin, a microtubule component (Haggarty et al., 2003). Suramin, a symmetric polyanionic naphthylurea, and its structural analogs, inhibit human NAD+-dependent class III SIRT1 and SIRT2 activity (Trapp et al., 2007). Nicotinamide, also known as niacinamide, is a precursor of NAD+ and a competitive class III HDAC inhibitor that can be given orally (Green et al., 2008). **Table 1** lists the isoforms of these four classes of HDACs and their sensitivities to the key HDAC inhibitors discussed above (Chuang et al., 2009b).

Despite the substantial progress made in the study of HDAC inhibitors, very little has so far been done regarding pharmacological manipulation of HAT activity. Amongst the very few HAT inhibitors, garcinol and curcumin were shown to penetrate cellular membranes and inhibit acetylation of histones *in vivo* (Balasubramanyam et al., 2003).

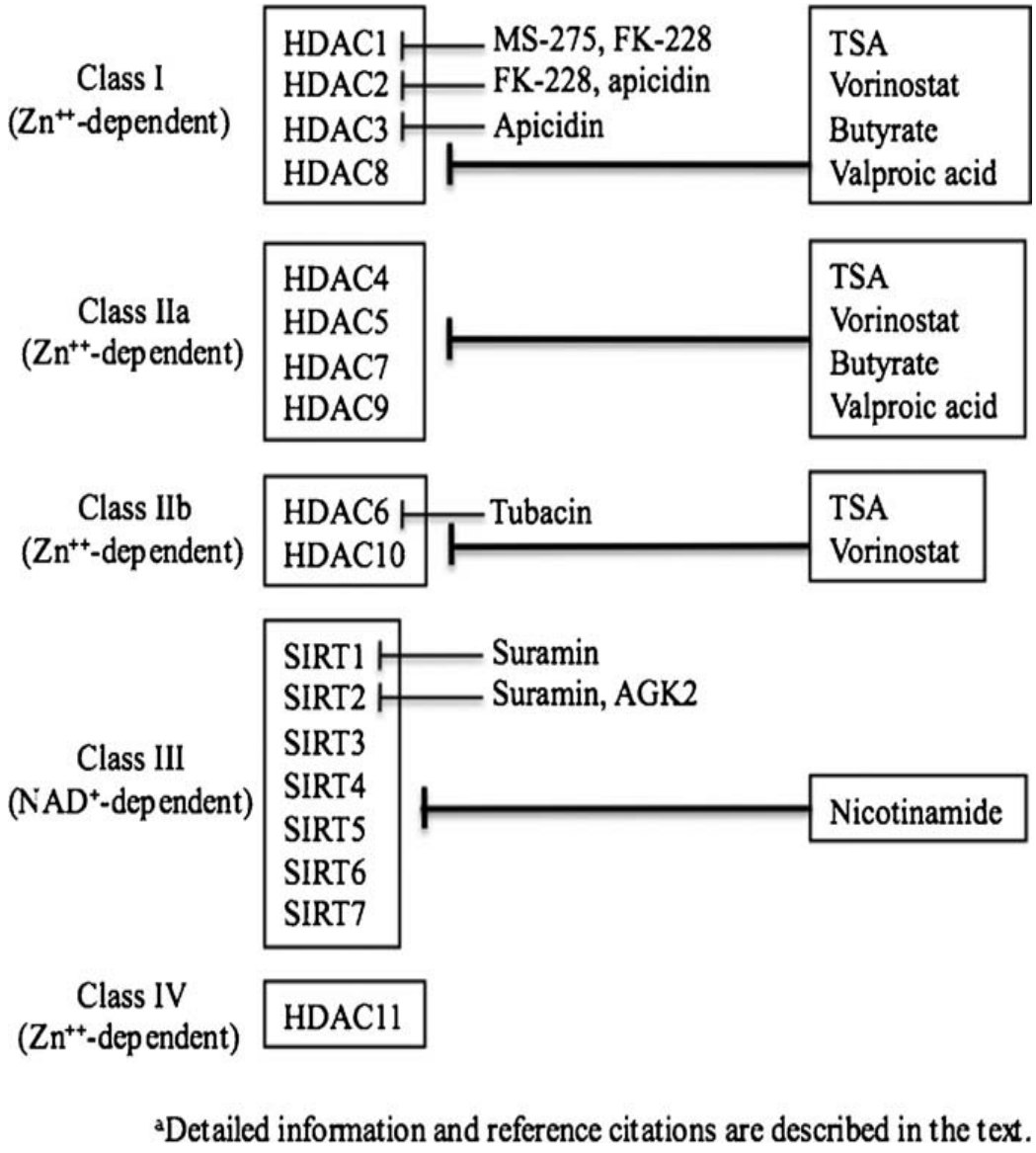

Table 1. HDAC isoforms and isoform-specific and nonspecific HDAC inhibitors (Chuang et al. 2009)

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

HDAC inhibition has neuroprotective effects in both *in vivo* and *in vitro* models of brain disorders. One pioneering study noted that levels of the HATs CBP\p300 and histone protein acetylation were decreased during apoptosis induced by potassium deprivation of cultured primary cerebellar granule cells, and during signal activation of β-amyloid precursor protein (APP) in cultured primary cerebral cortical neurons from rodents (Rouaux et al., 2003). 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 acetylation and activation of the DNA binding activity of Sp1 (Ryu et al., 2003c). 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 (Jeong et al., 2003). HDAC inhibitor-induced neurotoxicity could be partly due to 'derepression' of genes involved in apoptosis including Bim and B-myb (Biswas et al., 2005). 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 $p21^{waf1\backslash cip}$ (Langley et al., 2008). 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, 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 (Kontopoulos et al., 2006; Leng and Chuang, 2006; Monti et al., 2007). 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 (Feng et al., 2008). Taken together, the evidences 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 (Yasuda et al., 2009). 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 (Chen et al., 2006; Wu et al., 2008; Lin et al., 2011). 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 (Cao et al., 2013). 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 are also targets of HDAC inhibition and neuroprotection (Chuang et al., 2009b).

I.B.8. HDAC Inhibition in Animal Models of Neurodegenerative Disorders

I.B.8.1. Huntington's Disease (HD)

HD is an inherited, autosomal-dominant fatal neurodegenerative disease characterized anatomically by a predominant loss of striatal medium-sized spiny neurons and cortical neurons, and clinically by hyperkinetic involuntary movement, cognitive impairment and memory loss, as well as by psychosis and emotional deterioration. The genetic mutation responsible for HD is an expansion of a CAG trinucleotide repeat encoding polyglutamine (polyQ) in the first exon of the huntingtin (HTT) gene. Transcriptional dysregulation plays a central role in the pathogenesis\pathophysiology of HD (Sugars and

Rubinsztein, 2003; Hodges et al., 2006). For example, HTT with an expanded polyQ repeat has been shown to interact with and impair neuroprotective transcription factors, such as Sp1 and its co-activator TAFII130 (Hahnen et al., 2008). Importantly, a very recent report by Jeong and colleagues showed that HTT was acetylated at K444 by the HAT, CBP (Jeong et al., 2009). Enhanced K444 acetylation facilitated the trafficking of mutant HTT into autophagosomes for degradation and reduced the neurotoxicity of mutant HTT both in primary neuronal cultures and in a *C. elegans* HD model. These findings identify acetylation of HTT as a new mechanism for clearing accumulated HTT protein and suggest that increased HTT acetylation is a potential target for HDAC inhibition to elicit neuroprotective effects in HD. Using R6\2 mice, a known animal model of HD, Ferrante and colleagues reported that sodium butyrate treatment decreased the neurodegenerative phenotype and improved survival (Ferrante et al., 2003). Moreover, it has been suggested that the pathophysiology of HD is coupled intimately to deficiency of both BDNF and HSP70 in affected brain regions (Zuccato et al., 2001). Because the expression of both BDNF and HSP70 is regulated by class I and II HDAC inhibitors, it is conceivable that restoring BDNF and HSP70 to their normal levels contributes to the beneficial effects of HDAC inhibition in various HD models. In this context, it is notable that vorinostat and TSA increase vesicular transport of BDNF by inhibiting HDAC6, thereby increasing tubulin acetylation and compensating for the transport deficit in HD (Dompierre et al., 2007). However, the role of HDAC6 in HD pathology is clearly complex – for example, HDAC6-dependent retrograde transport on microtubules is crucial for the autophagic degradation of aggregated HTT (Iwata, A., 2005) and HDAC6 is therefore likely to be neuroprotective in this role. Further, expression of HDAC6 rescues polyQ-induced neurodegeneration associated with dysfunction of the ubiquitin–proteasome system in a fly model of spino-bulbar muscular atrophy (Pandey et al., 2007). The dual roles of HDAC6 in neurodegeneration and neuroprotection complicate the application of this subtype-specific inhibition in treating polyQ-induced neurodegenerative diseases.

I.B.8.2. Amyotrophic Lateral Sclerosis (ALS)

ALS is an adult-onset neurodegenerative disease characterized by progressive loss of motor neurons in the brain, brain stem, and spinal cord; these lead to generalized weakness, muscle atrophy, paralysis, and eventual mortality within five years of disease onset. Most ALS cases occur sporadically, with only about 10% of patients being categorized as having a familial form. Of these, approximately 20% are attributed to gain-of-function mutations in the gene encoding Cu\Zn superoxide dismutase1 (SOD1), a key antioxidant enzyme. Mice expressing mutant Cu\Zn SOD1 exhibit ALS-like phenotypes, including the formation of intracellular aggregates of SOD1 in the brain and spinal cord, behavioral abnormalities and premature death. Because transcriptional dysregulation may play a role in the pathophysiology of ALS, the effects of HDAC inhibitors have been examined in transgenic ALS mouse models. Using SOD1\G93A transgenic mice, Ryu and colleagues injected 4-phenylbutyrate starting before or shortly after symptom onset; this resulted in extended survival and improved pathological phenotypes (Ryu et al., 2005). Valproic acid treatment of SOD1 mutant mice had variable effects on disease symptom onset, duration, and survival. For instance, pre- or post-symptomatic valproic acid treatment in drinking water increased lifespan, but pre-symptomatic treatment had no effect on the onset of motor symptoms in G93A mice (Sugai et al., 2004). More recently, Feng et al. reported that valproic acid treatment of G93A mice had small, but significant, beneficial effects on motor dysfunction onset, motor deficits and survival time. In the latest study, combined treatment with valproic acid and lithium produced greater and more consistent benefits than valproic acid alone in delaying the onset of disease symptoms, prolonging lifespan, and decreasing neurological deficits (Feng et al., 2008). These and other studies highlight the potential effectiveness of combination treatments for ALS patients.

I.B.8.3. Spinal Muscular Atrophy (SMA)

SMA is an autosomal-recessive inherited motor neuron disease caused by degeneration of a-motor neurons in the anterior horn of the spinal cord and is characterized by weakness and atrophy of voluntary muscles. The genetic

basis of SMA, a leading hereditary cause of infant mortality, is homozygous deletion of the SMN1 gene on chromosome 5q13, that encodes the full-length survival motor neuron protein. A related gene located within the same chromosomal locus, SMN2, is ubiquitously expressed, but encodes an unstable SMN protein lacking C-terminal residues. Although SMA patients lacking SMN1 carry at least one copy of SMN2, the amount of functional SMN protein produced by SMN2 is insufficient to combat progressive motor neuron degeneration. Disease severity appears to be inversely correlated with SMN2 gene copy number and SMN expression, suggesting that SMN2 is a potential therapeutic target. A number of HDAC inhibitors increase SMN2 mRNA and protein levels *in vitro*, including sodium butyrate (Chang et al., 2001), 4-phenylbutyrate (Andreassi et al., 2004), valproic acid (Sumner et al., 2003), M344, vorinostat (Hahnen et al., 2006), TSA and romidepsin (FK-228) (Avila et al., 2007). Interestingly, the SMN2 promoter associates with HDAC1 and HDAC2, but not with HDAC3–5 (Kernochan et al., 2005), indicating that different HDAC isoforms play selective roles in regulating SMN gene expression. Chang and colleagues provided the first evidence that administering sodium butyrate to a transgenic mouse model of SMA (SMN1-\-SMN2), increased SMN protein expression, combated clinical symptoms, and increased their lifespan (Chang et al., 2001). Using a similar mouse model, Tsai and colleagues showed that oral administration of valproic acid in drinking water improved motor function, promoted neuromuscular junction formation and also suppressed spinal motor neuronal degeneration and muscular atrophy. The beneficial effects of valproic acid were associated with elevated levels of SMN mRNA and protein in spinal tissues, as well as increased levels of the anti-apoptotic proteins Bcl-2 and Bcl-XL (Tsai et al., 2008). 4-phenylbutyrate and valproic acid treatment increased SMN mRNA and protein in leukocytes of SMA patients and improved patients'muscle power (Brahe et al., 2005; Brichta et al., 2006). It should be noted that the beneficial effects of valproic acid and butyrate in both preclinical and clinical studies of SMA are limited. Future SMA studies using other HDAC inhibitors, notably class III inhibitors, seem warranted. Combinatorial treatment with an

HDAC inhibitor in conjunction with another drug exhibiting different actions would also be a rational approach.

I.B.8.4. Parkinson's Disease (PD)

PD is a prevalent neurodegenerative disease characterized by a relatively selective loss of dopaminergic neurons, predominantly in the substantia nigra. Most cases of PD occur sporadically. An established model of PD involves treatment of mice with the classical dopaminergic toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Pioneering work by Beal and colleagues (Gardian et al., 2004), showed in this model that administration of phenylbutyrate significantly attenuated both dopamine depletion and loss of substantia nigra neurons expressing the dopamine biosynthetic enzyme tyrosine hydroxylase. Using a related dopaminergic toxin, 1-methyl-4-phenylpyridinium (MPP+), in an *in vitro* model of midbrain neuron–glia cocultures, Hong and colleagues found that MPP+-induced death of dopaminergic neurons could be prevented by treatment with valproic acid, sodium butyrate, or TSA, as demonstrated by marked increases in dopamine uptake and the number of neurons staining positive for tyrosine hydroxylase (Chen et al., 2006; Wu et al., 2008). Mutations of a number of targets, including the presynaptic protein α-synuclein, have been linked to familial forms of PD. 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. By contrast, cytoplasmic accumulation of α-synuclein is neuroprotective both in cultured cells and in a transgenic Drosophila model of PD (Kontopoulos et al., 2006). More recently, Outeiro and colleagues reported that AGK2, a specific inhibitor of SIRT2, increased α-tubulin acetylation with the formation of large α-synuclein inclusions, and was able to rescue dopaminergic neurons both *in vitro* and in Drosophila PD model (Outeiro et al., 2007). Together, these findings suggest that both zinc-dependent HDACs, as well as SIRT2, are potential targets for therapeutic intervention in PD.

I.B.8.5. Alzheimer's Disease (AD)

AD is the fourth most common cause of death in the USA and affects ~50% of the population over 85 years of age in industrialized countries. Clinically, the disease is characterized by progressive memory loss and personality changes, ultimately leading to dementia. Its neuropathological hallmarks include accumulation of extracellular β-amyloid (Aβ), an aberrant processing product of normal APP, and intracellular neurofibrillary tangles resulting from hyperphosphorylation of Tau protein. A follow-up study by Guan et al. convincingly demonstrated that mice overexpressing HDAC2, but not HDAC1, exhibit decreased dendritic spine density, synaptic number and synaptic plasticity, and impaired memory formation (Guan et al., 2009). Conversely, *Hdac2* knockout mice show memory improvement. Further, HDAC2 regulates synaptic formation and plasticity in the mouse hippocampus and binds to promoters of a spectrum of genes involved in neuronal activity, synaptic formation and plasticity. The memory impairment in mice overexpressing HDAC2 was ameliorated by treatment with the HDAC inhibitor vorinostat. These findings underscore the involvement of chromatin modification by HDAC2 in regulating synaptic plasticity and memory formation. In the Tg2576 mouse model of AD, daily injections of 4-phenylbutyrate reversed spatial memory deficits by normalizing Tau hyperphosphorylation in the hippocampus, but without affecting Aβ levels (Barrett and Wood, 2008). In the APP23 transgenic mouse model of AD, daily injections with a relatively low dose of valproic acid (30 mg\kg, i.p.) robustly reduced Aβ plaque number and improved memory deficits when administered early (starting at seven months) (Qing et al., 2008). In another study, nicotinamide, a class III HDACs inhibitor, induced a chronic but low-level, increase in endogenous p25, which surprisingly was linked to improved learning and memory. Although nicotinamide could exhibit its beneficial effects through both sirtuin-dependent and -independent mechanisms, these results suggest that class III HDACs are involved in the pathology of AD, and that oral nicotinamide potentially might prove useful as a treatment for this disease.

I.B.8.6. Stroke

Stroke, an acute neurological\neurodegenerative disease, is the third leading cause of death in the USA. Most stroke cases are caused by cerebral ischemia. In a middle cerebral artery occlusion (MCAO) stroke model, reduced bulk histone acetylation was found at Lys residues in the ischemic brain of rats or mice; these changes were restored by treatment with HDAC inhibitors, with a concomitant decrease in infarct volume (Faraco et al., 2006). In a rat MCAO model, Chuang and colleagues showed that post-insult treatment with valproic acid, sodium butyrate or TSA also improved behaviors (Ren et al., 2004; Kim et al., 2007b). Recently, neuroprotection was reproduced, by Lanzillotta et al., in mice subjected to MCAO and treated with the new class I HDAC inhibitor, MS-275 (20 μg\kg and 200 μg\kg) or resveratrol (6800 μg\kg), individually. Notably, the administration of lowest doses of MS-275 (2 μg\kg) and resveratrol (68 μg\kg) synergistically reduced infarct volume and neurological deficits. Importantly, the treatment was effective even when administered 7 h after the stroke onset (Lanzillotta et al., 2012). Moreover, in the study of Cao et collegues, they evaluated the neuroprotective potential of the class I HDAC inhibitor, MS-275, on behavioral performance and histological outcome of acute neuronal degeneration (Cao et al., 2013). In addition, administration of 4-phenylbutyrate in mice subjected to hypoxia–ischemia protected against endoplasmic reticulum (ER) stress (Kim et al., 2007b), evidenced by decreased eIF2a phosphorylation and expression of the eIF2a-regulated proapoptotic protein CHOP. It is increasingly recognized that neuroinflammation plays a causative role in neurodegeneration following ischemic injury. Kim et al. demonstrated that post-insult treatment with valproic acid or sodium butyrate suppressed permanent MCAO-induced activation of microglia and monocytes\macrophages and proinflammatory iNOS and COX-2 overexpression (Kim et al., 2007a). Treatment with HDAC inhibitors also markedly inhibited ischemia-induced p53 overexpression and superinduced heat shock protein 70 (HSP70) in the ischemic brain. It is likely that superinduction of endogenous HSP70 by HDAC inhibition contributes to these anti-inflammatory effects. In support, one recent study noted that

HSP70 overexpression inactivated NF-kB by stabilizing a complex of HSP70–IkBa–NF-kB in a mouse MCAO model (Zheng et al., 2008). The expression of cytoskeletal proteins has also been implicated in neuroprotection by HDAC inhibition under ischemic conditions. For instance, HDAC inhibition upregulated gelsolin, a protein involved in actin filament organization, and by this route contributed to neuroprotection from ischemic brain injury (Yildirim et al., 2008; Zheng et al., 2008). In addition, valproic acid was neuroprotective in an intracerebral hemorrhagic model of stroke by HDAC inhibition and transcriptional activation, and displayed anti-inflammatory actions by down-regulating proinflammatory factors including Fas-L, IL-6, and MMP-9 (Sinn et al., 2007). The identity of the HDAC isoform(s) involved in HDAC inhibitor-mediated neuroprotection remains unclear. However, from the related cardiac field, it is noteworthy that knockdown of HDAC4 reduced infarct size following myocardial ischemia-induced reperfusion injury (Chuang et al., 2009b).

I.C. SODIUM CALCIUM EXCHANGER

I.C.1. State of Art of $Na^+\backslash Ca^{2+}$ Exchanger

The $Na^+\backslash Ca^{2+}$ exchanger (NCX) consists of 9 transmembrane segments that can mediate Ca^{2+} and Na^+ fluxes across the plasma membrane (Blaustein & Lederer, 1999) (**Fig. 4**). NCX was discovered and characterized in the late 1960s, when Baker et al. (Baker et al., 1969), Reuter and Seitz (Reuter and Seitz, 1968), and Martin and De Luca (Martin and DeLuca, 1969) realized the presence of countertransport mechanism that exchanged Na^+ and Ca^{2+} ions across the plasma membrane of different excitable and non excitable cells. However, the most crucial advancement in NCX research was made in 1988 (Philipson et al., 1988) and 1990 (Nicoll et al., 1990) when Philipson and his colleagues purified and cloned the first isoform of this antiporter: NCX1. Remarkably, few years later, the same group cloned NCX2 (Li et al., 1994) and NCX3 (Nicoll et al., 1996), two isoforms selectively expressed in the brain and in the skeletal muscle.

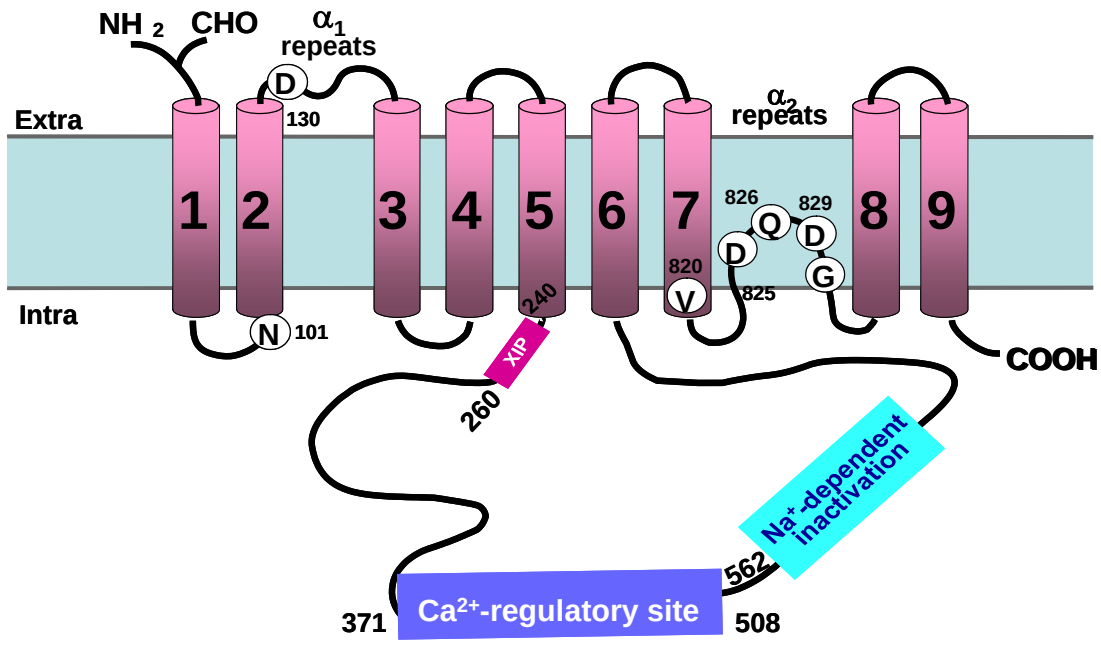

Figure 4. **Molecular topology of NCX** (Annunziato et al., 2004).

The regulation of intracellular concentration of Ca^{2+} and Na^{+} ions in excitable cells is a relevant physiological phenomenon that maintains cellular homeostasis. The $Na^{+}\backslash Ca^{2+}$ exchanger, in parallel with selective ion channels and ATP-dependent pumps, maintains the physiological cytosolic concentrations of these ions (Blaustein and Lederer, 1999). Depending on the intracellular concentrations of Ca^{2+} $[Ca^{2+}]_i$, and Na^{+}, $[Na^{+}]_i$, NCX can operate either in the *forward mode*, coupling the uphill extrusion of Ca^{2+} to the influx of Na^{+} ions, or in the *reverse mode*, mediating the extrusion of Na^{+} and the influx of the Ca^{2+} ions (Blaustein and Lederer, 1999; Philipson and Nicoll, 2000). The stechiometry of NCX is generally accepted to be three Na^{+} ions\one Ca^{2+} ion; however, at a later time, in addition to the major 3:1 transport mode, it has been demonstrate that ion flux ratio can vary from 1:1 to a maximum of 4:1, depending on $[Na^{+}]_i$ and $[Ca^{2+}]_i$ (Fujioka et al., 2000; Kang and Hilgemann, 2004).

I.C.2. Molecular Biology of NCX and its Distribution

The $Na^{+}\backslash Ca^{2+}$ exchanger belongs to the superfamily of membrane proteins comprising the following members:

1. the NCX family, which exchanges three Na^{+} ions for one Ca^{2+} ion or four Na^{+} ions for one Ca^{2+} ion depending on $[Na^{+}]_i$ and $[Ca^{2+}]_i$ (Fujioka et al., 2000; Kang and Hilgemann, 2004)
2. the $Na^{+}\backslash Ca^{2+}$ exchanger K^{+}-dependent family, which exchanges four Na^{+} ions for one Ca^{2+} plus one K^{+} ion (Lytton et al., 2002)
3. the bacterial family which probably promotes $Ca^{2+}\backslash H^{+}$ exchange (Cunningham and Fink, 1996)
4. the nonbacterial $Ca^{2+}\backslash H^{+}$ exchange family, which is also the Ca^{2+} exchanger of yeast vacuoles (Pozos et al., 1996)
5. the $Mg^{2+}\backslash H^{+}$ exchanger, an electrogenic exchanger of protons with Mg^{2+} and Zn^{2+} ions (Shaul et al., 1999)

These membrane proteins are all peculiarly characterized by the presence of α-repeats, the regions involved in ion translocation. About the NCX family, three dominant genes coding for the three different NCX1, NCX2 and NCX3 proteins have been identified in mammals. These three genes appear to be

dispersed, since NCX1, NCX2 and NCX3 have been mapped in mouse chromosomes 17, 7, and 12, respectively (Nicoll et al., 1996). At the post-transcriptional level, at least 17 NCX1 and 4 NCX3 proteins are generated through an alternative splicing of the primary nuclear transcripts (Quednau et al., 1997). These variants arise from a region of the large intracellular f loop, are encoded by six small exons defined A to F, and are used in different combinations in a tissue-specific manner (Lee et al., 1994). To maintain an open reading frame, all splice variants must include either exon A or B, which are mutually exclusive. Excitable tissues, such as those of the brain and heart, are usually characterized by the presence of exon A, whereas kidney, stomach, and skeletal muscle tissues comprise NCX with exon B (Quednau et al., 1997).

NCX1 is composed of 938 aminoacids, in the canine heart, having a theoretical molecular mass of 120 KDa and containing nine transmembrane segments (TMS). NCX1 amino-terminus is located in the extracellular space, whereas the carboxyl terminus is located intracellularly (**Fig. 4**). The nine transmembrane segments can be divided into an N-terminal hydrophobic domain, composed of the first five TMS (1-5), and into a C-terminal hydrophobic domain, composed of the last four TMS (6-9). These two hydrophobic domains are important for the binding and the transport of ions. The first (1-5) TMS are separated from the last four (6-9) TMS through a large hydrophilic intracellular loop of 550 amino acids, named the f loop (Nicoll et al., 1999). Although the f loop is not implicated in Na^+ and Ca^{2+} translocation, it is responsible for the regulation of NCX activity elicited by several cytoplasmic messengers and transductional mechanisms, such as Ca^{2+} and Na^+ ions, NO, phosphatidylinositol 4,5 bisphosphate (PIP2), protein kinase C (PKC), protein kinase A (PKA), and ATP. In the center of the f loop, a region of approximately 130 amino acids in length has been reported to exert a Ca^{2+} regulatory function. This region is characterized by two Ca^{2+} binding domain (CBD1 and CBD2) that undergo conformational changes upon binding of Ca^{2+} and look very similar in the Ca^{2+} bound form, whereas in the absence of Ca^{2+}, the domains show dramatic structural differences. CBD1 binds Ca^{2+} with K_d values of 120 and 240 nM, whereas the respective

values of CBD2 are 820 nM and 8,6 µM (**Fig. 5**) (Hilge et al., 2006). At the N-terminal end of the f loop near the membrane lipid interface, an autoinhibitory domain, rich in both basic and hydrophobic residues, named exchange inhibitory peptide (XIP) (Matsuoka et al., 1997), has been identified. The f loops also characterized by alternative splicing sites named β1-repeat and β2-repeat. These β-repeats are characterized by similar regions comprising 60 to 70 amino acids for which no functional role has been proposed (Hilgemann, 1990).

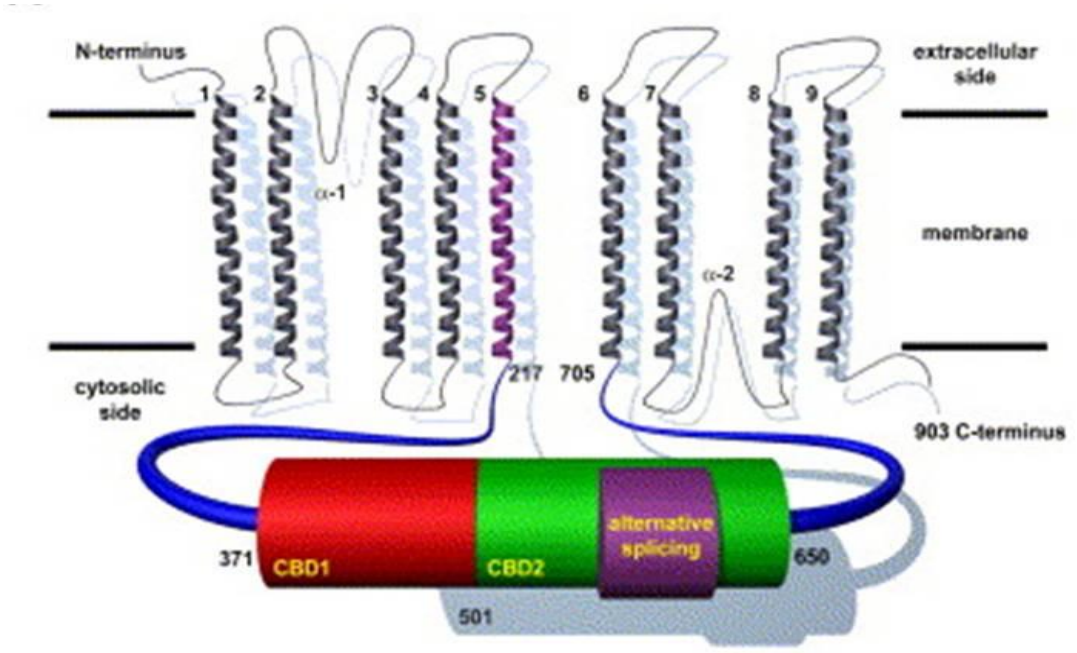

Figure 5.Ca^{2+} binding in CBD1 and CBD2 domains. (Hilge et al., 2006)

The NCX protein aminoacid sequence found between TMS2 and TMS3 is called α-1 repeat, whereas the one found between TMS7 and TMS8 is named α-2 repeat. Both these regions are located on the opposite site of the membrane and include two segments composed of 12 and 9 highly conserved residues separated by a non conserved segment of 18 to 20 amino acids (Nicoll et al., 2002). Since the putative α-helices of the α-repeats are amphipathic, the hydrophilic faces of these helices may form a portion of the ion translocation pathway (Nicoll et al., 1996).

Interestingly, NCX2 and NCX3 have been found only in the brain and in the skeletal muscle. These two gene products consist of 921 and 927 amino acids and are characterized by molecular masses of 102 and 105 KDa, respectively. In addition, NCX2 displays a 65% sequence identity with NCX1, whereas NCX3 possesses a 73% sequence identity with NCX1 and 75% sequence identity with NCX2 (Nicoll et al., 1996). All three NCX gene products share the same membrane topology.

In the central nervous system (CNS), the $Na^+\backslash Ca^{2+}$ exchanger plays a fundamental role in controlling the changes in the intracellular concentrations of Na^+ and Ca^{2+} ions. These cations are known to regulate neurotransmitter release, cell migration and differentiation, gene expression, and neurodegenerative processes.

I.C.3. Regulation of $Na^+\backslash Ca^{2+}$ Exchanger Activity

Several factors are involved in the regulation of $Na^+\backslash Ca^{2+}$ exchanger activity: the intracellular pH; metabolic related compounds, ATP, PA, PIP2, PKA, and PKC; redox agents, hydroxyl radicals, H_2O_2, dithiothreitol (DTT), O_2^- ,Fe^{3+}, Fe^{2+}, Cu^{2+}, OH, glutathione reduced (GSH), and glutathione oxidized (GSSG); and the gaseous mediator, NO. The site level at which $[Ca^{2+}]_i$ regulates NCX activity is different from the one required for Ca^{2+} transport (Levitsky et al., 1994). The removal of intracellular Ca^{2+} ions completely blocks NCX activity (Philipson and Nicoll, 2000). The location of such regulatory site has been identified in the 134-amino acid region, situated in the center of the f loop. In addition to intracellular Ca^{2+} regulatory site, an increase in $[Na^+]_i$ can also regulate the $Na^+\backslash Ca^{2+}$ exchanger. In particular, when intracellular Na^+ increases, it binds to the transport site of the exchanger molecule, and after an initial fast outward the $Na^+\backslash Ca^{2+}$ current, an inactivation process occurs (Hilgemann et al., 1992). This inactivation process, very similar to the phenomenon occurring in voltage-dependent ionic channels, is named Na^+-dependent inactivation. The region of the intracellular f loop, in which this regulatory site is located, has been identified in a 20-aminoacid portion of the N-terminal part of the loop named XIP (Matsuoka et al., 1997). Regarding on the mechanism by which XIP inhibits

NCX activity, it has been proposed that when the XIP-binding site is ligand-occupied, a conformational change is induced in C-terminal portion of the f loop, thus resulting in the inhibition of the ion transport (Li et al., 1991). XIP is provided with relevant pharmacological implications. In fact, those exogenous peptides, having the same amino acid sequence as XIP, act as potent inhibitors of NCX activity (Annunziato et al., 2004; Pignataro et al., 2004). Interestingly, Ca^{2+} ions, at low micromolar concentrations, binding its regulatory site, decrease the extent of this Na^{+}-dependent inactivation. In fact, mutations in the Ca^{2+} regulatory binding site alter the activation and inactivation kinetics of exchange currents by modulating Na^{+}-dependent inactivation (Matsuoka et al., 1995).

H^{+} strongly inhibits NCX activity under steady-state conditions (Doering and Lederer, 1993). The action exerted by H^{+} ions is pathophysiologically relevant with regards to brain and heart ischemia. In fact, when intracellular H^{+} and Na^{+} ion homeostasis is deregulated, the anoxic conditions resulting in these cells may selectively interfere with the activity of the different NCX gene products.

ATP may increase the activity of the exchanger in a number of ways. Firstly, ATP directly participates in the NCX molecule phosphorylation process by PKA and PKC (Caroni and Carafoli, 1983). Secondly, it increases PIP2 production. This mechanism of activation is related to the relevant PIP2 influence on Na^{+}-dependent inactivation of NCX. In fact, PIP2 directly interacts with the XIP region of the exchanger, thus eliminating its inactivation and stimulating NCX function (Hilgemann and Ball, 1996). Finally, by activating G-protein-coupled receptors, via endogenous and exogenous ligands, ATP can stimulate NCX activity through the pathway involving PKC or PKA activation (DiPolo and Beaugé, 1998). The mechanism underlying the phosphorylating effect on the exchanger seems to be related to an increase in its affinity for both internal Ca^{2+} and external Na^{+} and to a decrease in its inhibition by internal Na^{+}. In addition, ATP cellular depletion inhibits NCX1 and NCX2, but does not affect NCX3 activity (Secondo et al., 2007a).

Moreover, phosphoarginine (PA) can stimulate NCX activity. In particular, PA present in millimolar concentrations in the cytosol, activates $Na^{+}\backslash Ca^{2+}$

exchanger function in the forward mode of operation by intracellular Mg^{2+}- and Ca^{2+}-dependent way (Di Polo et al., 2004).

Several groups of investigators also have found that NCX is sensitive to different combinations of redox agents (Amoroso et al., 2000; Santacruz-Toloza et al., 2000). In particular, the stimulation of the exchanger activity requires the combination of a reducing agent with an oxidizing agent. The effects of both agents are mediated by metal ions. The antiporter's sensitivity to changes in the redox status can assume particular relevance during oxidative stress. In fact, in this condition, the modulation of reactive oxygen species (ROS) could affect the transport of Na^+ and Ca^{2+} ions through the plasma membrane (Santacruz-Toloza et al., 2000).

The ubiquitous gaseous mediator NO seems to be involved in the modulation of NCX activity. In fact, there are several evidences that NO, released by NO donors, is able to stimulate NCX in the *reverse mode* of operation in neuronal preparations and astrocytes. By contrast, in C6 glioma cells, the stimulatory action on NCX *reverse mode* of operation, elicited by the sodium nitroprusside (SNP), is not elicited by NO release but by the presence of iron in SNP molecule (Amoroso et al., 2000). In addition, the relationship between the constitutive form of nitric oxide synthase (NOS) and NCX has recently been demonstrated. Indeed, heat stress by inducing NOS phosphorylation causes NOS complexation with NCX, thus decreasing its activity (Kiang et al., 2003).

I.C.4. Pharmacological Modulation of Na^+\Ca^{2+} Exchanger Activity: Inhibitors and Activators

A great deal of interest has been devoted to the pharmacological modulation of NCX. The reasons for this enormous interest lay in the hope of finding clinically effective drugs for those pathophysiological conditions in which a stimulation or an inhibition of the NCX might have achieved beneficial effects. A lot of drugs that could specifically modulate NCX activity, have been developed.

I.C.4.1. Inhibitors

1. A great interest has been oriented to the endogenous exchanger inhibitor peptides. Since it was demonstrated that a 20-amino acid sequence of the intracellular f loop of the exchanger molecule, XIP, played an autoinhibitory function through an Na^+-dependent inactivating mechanism and that the synthetic peptides with the same amino acid sequence could exert an inhibitory action on NCX function (Li et al., 1991; DiPolo and Beaugè, 1994), a great effort has been made to synthesize and to characterize the molecular pharmacology of different XIP analogs. XIP, an anphipathic molecule, potently inhibits Na^+\Ca^{2+} exchanger activity in both modes of operation. Since XIP hardly penetrates the cell membrane because of its prevalent hydrophilia, a XIP bearing a molecule of glucose attached to the Tyr-6 residue has recently been synthesized (**Fig. 6**). Interestingly, this Tyr-6-glycosilated form of XIP, intracerebroventricularly infused in male rats bearing permanent middle cerebral artery occlusion (pMCAO), caused a dramatic increase in infarct volume (Pignataro et al., 2004). These results suggest that NCX plays a pivotal role in the mechanisms that lead to neuronal death under ischemic conditions. Therefore, a pharmacological modulation of its activity may represent one of the possible therapeutic strategies for stroke treatment.

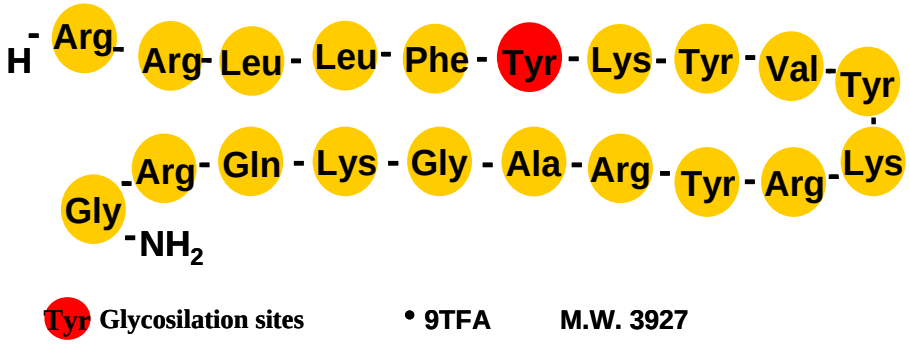

Figure 6. Structure of the 6-tyrosine glycosilated form of the exchange inhibitor peptide XIP (Annunziato et al., 2004).

2. As inhibitors of NCX activity, amiloride and its derivates have been used. In attempt to evaluate NCX activity, amiloride has been used as a probe to block NCX function (Sharikabad et al., 1997). However, two major drawbacks have limited its use. Firstly, micromolar concentrations are required for its NCX inhibitory activity; secondly, it lacks specifity, for it can also inhibit both the epithelial Na^+ channel at micromolar concentrations and the $Na^+\backslash H^+$ exchanger in the millimolar range. More recently, to overcome these hindrances, two classes of amiloride analogs have been developed. The compounds of first class have inhibitory effect on the epithelial Na^+ channel and on the plasma membrane NCX, even though that display great affectiveness in inhibiting the $Na^+\backslash H^+$ exchange in the 1 to 10 µM range (Amoroso et al., 1990; Taglialatela et al., 1990). The compounds of second class, having no inhibitory effect on the $Na^+\backslash H^+$ exchanger, bear substituents on the terminal guanidine nitrogen atom and behave as specific inhibitors of the epithelial Na^+ channels and NCX. Among these compounds, dimethyl-benzamylamiloride (DMB) has been shown to be a selective inhibitor of the $Na^+\backslash Ca^{2+}$ exchanger in exitable cells, such as neurons, in which the kidney epithelial Na^+ channels are not expressed (Taglialatela et al., 1990). In contrast, [N-(4-chlorobenzyl)]2,4-dimethylbenzamyl (CB-DMB) appears to be the most specific inhibitor of NCX activity, for it has no inhibitory properties against the $Na^+\backslash H^+$antiporter and the epithelial Na^+ channels (Sharikabad et al., 1997). The amiloride derivatives are able to reversibly inhibit NCX activity either when the antiporter operates in the forward (Taglialatela et al., 1990) or in the reverse mode of operation (Amoroso et al., 1997). It was hypothesized that these derivatives act as Na^+ analogs, interacting at an Na^+-binding site on the carrier, presumably, the region to which the third Na^+ binds, and reversibly tie up the transporter in an inactive complex (Kaczorowski et al., 1985). Because of their pharmacological properties, these amiloride derivative compounds have been shown to interfere with the

release of neurotransmitters from the brain, under both physiological and pathophysiological conditions.

3. Bepridil is a diarylaminopropylamine derivate having multiple inhibitory effects on ionic currents, including the L-type (Yatani et al., 1986) and T-type Ca^{2+} currents (Cohen et al., 1992), the delayed-rectifier K^+ current, the transient outward current (Berger et al., 1989), as well as the K^+ current, activated by intracellular Na^+ (Mori et al., 1998). In addition to these pharmacological properties, bepridil can also block NCX activity (Watanabe and Kimura, 2001). This inhibitory action is dependent on the mode of operation of the antiporter. In fact, NCX operating in the forward manner is more strongly inhibited than in the reverse mode of action. The site of action of bepridil may be located on the cytoplasmic side of the exchanger, since the intracellular treatment with trypsin attenuates its inhibitory action (Watanabe and Kimura, 2001). In *in vivo* models of ischemia, bepridil, by blocking NCX, enhances glial and neuronal injury elicited by pMCAO (Pignataro et al., 2004).
4. KB-R7943, an isothiourea derivative, was identified by Shigekawa's group (Iwamoto and Shigekawa, 1998), because they screened a compound library for the inhibition of Na^+-dependent Ca^{2+} uptake. A particular feature of this compound is that it inhibits the antiporter with a different potency, depending on NCX mode of operation. In fact, when NCX operates in the forward mode, the IC_{50} value needed for NCX inhibition is much lower than the IC_{50} value needed for the inhibition of reverse mode of operation (Iwamoto et al., 1996). KB-R7943 interacts with the exchanger molecule extracellularly at the α-2-repeat, between the TMS7 and TMS8 of the exchanger (Iwamoto et al., 2001; Shigekawa et al., 2002). According to peculiar pharmacological properties, KB-R7943 has aroused a great deal of interest among investigators working on NCX activity. It has been demonstrated that KB-R7943, in rats bearing pMCAO, reduces infarct volume (Papa et al., 2003). This neuroprotective action of this compound does not seem the result of NCX blockade, since other

antiporter inhibitors, such as GLU-XIP, CB-DMB, and bepridil, do indeed aggravate brain injury (Tortiglione et al., 2002; Annunziato et al., 2004; Pignataro et al., 2004; Sirabella et al., 2009). To explain the neuroprotective effect of KB-R7943 in pMCAO, it should be noted that this drug is also able to produce a remarkable and long-lasting hypothermic effect (Pignataro et al., 2004), which itself exerts a relevant neuroprotective action in cerebral ischemia (Yanamoto et al., 2001). In addition, recent reports have shown that KB-R7943, besides its peculiar NCX blocking properties, also exerts an inhibitory effect on several other ionic transport mechanisms, such as L-type VGCC. Moreover, this compound inhibits receptor-operated ion channels, such as NMDA (Matsuda et al., 2001) whose blockade may also lead to neuroprotective actions (Paule et al., 2003).

5. In 2001, the newly synthesized ethoxyaniline derivative SEA0400, was reported as being the most potent NCX inhibitor available at the time (Matsuda et al., 2001). More recent studies, however, demonstrated that it was also able to interfere with Ca^{2+} movement across the cell membrane (Reuter et al., 2002). This compound, similarly to KB-R7943, inhibits the antiporter's Na^{+} efflux-Ca^{2+} influx mode of operation. However, at variance with the isothiourea derivative, it predominantly blocks NCX1, it has lower affinity for NCX2, and it has no effect on NCX3 (Iwamoto et al., 2004b).

I.C.4.2. Activators

The availability of pharmacological agents capable of stimulating the activity of NCX, either in the reverse or in the forward mode of operation, may represent a useful strategy to adopt in some pathophysiological conditions, such as cardiac or brain ischemia. The pharmacological stimulation of the antiporter could, in fact, contribute to the re-establishment of intracellular Na^{+} and Ca^{2+} ion homeostasis.

1. Li^{+}, a monovalent cation, is able to stimulate the Na^{+}_{i}-dependent Ca^{2+} uptake of all three NCX gene products with low affinity. Its exent of stimulation is, however, somewhat smaller in NCX1 than in NCX2 and

NCX3 (Iwamoto and Shigekawa, 1998). Some groups of investigators have provided evidence that a long-term Li^+ exposure robustly protects cultured cerebellar, cortical, and hippocampal rat neurons against glutamate-induced excitotoxicity (Nonaka et al., 1998), suppresses the protein and mRNA expression of the two pro-apoptotic factors p53 and BAX, and increases the antiapoptotic mediator Bcl-2 (Chen and Chuang, 1999). However, the possible interplay between the activation of NCX and Li^+ neurobenefical effect still remains to be demonstrated.

2. As previously mentioned, changes in the redox state are capable of stimulating the NCX activity. It was proposed that these agents could activate the exchange activity by promoting thiol-disulfide interchange in the protein carrier (Reeves et al., 1986). More recently, the cysteine residues involved in this disulfide bond have been identified. However, the analysis of mutated exchangers has indicated that cysteines are not responsible for the stimulation of the exchanger activity induced by a mixture of redox agents (Fe-DTT). Therefore, it has been suggested that the stimulation of NCX by Fe-DTT is mainly due to the removal of the Na^+-dependent inactivation process (Santacruz-Toloza et al., 2000).

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. In this regard, evidence that the stimulation of NCX activity by oxidant agent Fe^{3+} may exert a neuroprotective effect both in *in vitro* a and in *in vivo* models of hypoxia and ischemia has been provided. It has been demonstrated that SNP, by stimulating NCX activity through its $K_3Fe(CN)_6$ portion-containing iron, is able to significantly reduce cellular injury elicited by chemical hypoxia (Amoroso et al., 2000). That Fe^{3+} should be responsible for this SNP neuroprotective effect is demonstrated by the fact that deferoxamine, an iron ion chelator, reverses the neuroprotection. This neuroprotective effect of iron has recently been confirmed in rats bearing pMCAO. In fact, $FeCl_3$,

intracerebroventricularly perfused after ischemia induction, has been shown to reduce the extension of brain infarct volume (Pignataro et al., 2004). Collectively, these results suggest that a stimulation of NCX activity during stroke might help neurons and glial cells to survive.

I.C.5. $Na^+\backslash Ca^{2+}$ Exchanger Intervention in Physiological Conditions

The $Na^+\backslash Ca^{2+}$ exchanger protein may play a relevant function in different neurophysiological conditions. In neuron, the level of NCX expression is particularly high in those sites where a large movement of Ca^{2+} ions occurs, as it happens at level of synapses (Canitano et al., 2002). In particular, during an action potential or after glutamate-activated channel activity, there is a large increase in cytosolic free Ca^{2+} concentration $[Ca^{2+}]_{cyt}$. Ca^{2+} is removed from the contractile apparatus and sequestrated in the intracellular store by the sarcoplasmic reticulum (SR) Ca^{2+} (SERCA) pump and\or extrusion to the extracellular fluid (ECF). Ca^{2+} is extruded by the plasma membrane Ca^{2+} ATPase and by NCX. The $Na^+\backslash Ca^{2+}$ exchanger becomes the dominant Ca^{2+} extrusion mechanism when $[Ca^{2+}]_i$ is higher than 500nM, as it happens when a train of action potentials reaches the nerve terminal. It has been calculated that for these $[Ca^{2+}]_i$ values (500nM), more than 60% of Ca^{2+} extrusion is mediated by $Na^+\backslash Ca^{2+}$ exchanger families. In addition, because the three NCX isoforms are differentially expressed in several CNS regions, it seems that each isoform may play a different functional role in distinct regions of the brain (Papa et al., 2003). Essentially, the primary function of NCX is to extrude Ca^{2+} from the cell, in the forward mode, using $[Na^+]$ gradient. However, under some physiologic conditions, such as the occurrence of an action potential or of spontaneous $[Ca^{2+}]_i$ oscillations, NCX could revert its mode of operation, thus participating in endoplasmic reticulum (ER)-Ca^{2+} refilling. In fact, it has been demonstrated that spontaneous $[Ca^{2+}]_i$ oscillations induced by inositol 1,4,5-triphosphate receptor stimulation might lead to the activation of non-selective cation channels, which causes Na^+ influx into the junctional cytosol microdomains. This influx facilitates the entrance of Ca^{2+} through NCX working in the reverse mode (Fameli et al., 2007).

I.C.6. Pathophysiological Implications of the $Na^+\backslash Ca^{2+}$ Exchanger in CNS Functions

The dysregulation of $[Ca^{2+}]_i$ and $[Na^+]_i$ homeostasis is involved in neuronal and glial injury, occurring in *in vitro* and *in vivo* models of hypoxia-anoxia and in several neurodegenerative diseases. In fact, during anoxic conditions, due to the compromission of the two plasmamembrane ATP-dependent pumps – $Na^+\backslash K^+$ ATPase and Ca^{2+} ATPase – NCX assumes a relevant role in controlling the intracellular homeostasis of these two cations. The role played by NCX in neurons and glial cells involved in cerebral ischemia should be differentiated according to the anatomical regions involved in the ischemic pathological process. In particular, it is conceivable that, since in the *penumbral region* ATPase activity is still preserved, NCX may likely work in a *forward mode*; as a result, by extruding Ca^{2+} ions, the exchanger favors the entry of Na^+ ions. Therefore, the inhibition of NCX in this area reduces the extrusion of Ca^{2+} ions, thus enhancing Ca^{2+}-mediated cell injury. In contrast, in the *ischemic core region*, in which ATP levels are remarkably low and $Na^+\backslash K^+$ ATPase activity is reduced, intracellular Na^+ ions massively accumulate because of $Na^+\backslash K^+$ ATPase failure. Hence, the intracellular Na^+ loading promotes NCX to operate in the *reverse mode* as an Na^+ efflux-Ca^{2+} influx pathway. Consistently, biochemical studies have clearly demonstrated that whereas NCX1 and NCX2 activity is strictly dependent on ATP levels, which are lowered during the development of brain ischemia, NCX3 is the only NCX gene product that is independent of ATP (Secondo et al., 2007b). In conclusion, the NCX pharmacological inhibition in this *core region*, further worsens the necrotic lesion of the surviving glial and neuronal cells as the loading of the intracellular Na^+ increases (Pignataro et al., 2004). More specifically, according to the time step of the ischemic pathological process, in the early phase of neuronal anoxic insult, the $Na^+\backslash K^+$-ATPase blockade increases $[Na^+]_i$, which in turn induces NCX to reverse its mode of operation. Although during this phase NCX causes an increase in $[Ca^{2+}]_i$, its effect on neurons appears beneficial for two reasons. First, by promoting Ca^{2+} influx, NCX favors Ca^{2+} refilling into the ER, which is depleted by anoxia followed by reoxygenation, thus allowing neurons to delay ER stress (Sirabella et al.,

2009). Second, by eliciting the decrease in $[Na^+]_i$ overload, NCX prevents cell swelling and death (Annunziato et al., 2007). Conversely, in the later phase of neuronal anoxia, when $[Ca^{2+}]_i$ overload takes place, NCX *forward mode* of operation contributes to the lowering of $[Ca^{2+}]_i$, thus protecting neurons from $[Ca^{2+}]_i$-induced neurotoxicity (Annunziato et al., 2004). Furthermore, NCX is involved in some serious diseases characterized by a loss of ion homeostasis control, including Alzheimer's disease, multiple sclerosis, and epilepsy (Craner et al., 2004; Ketelaars et al., 2004; Pannaccione et al., 2012). Recently, experiments in ischemic rats treated with inhibitors of NCX activity or synthesis, together with experiments in knockout mice for the *ncx1*, *ncx2* or *ncx3* gene, have demonstrated that the blockade of NCX protein synthesis or activity worsens ischemic brain damage by dysregulating Na^+ and Ca^{2+} homeostasis (Pignataro et al., 2004). Therefore, in recent years there has been great interest in the identification of new compounds capable of increasing NCX activity to limit the extension of ischemic brain damage.

I.C.7. Neurounina-1: a Novel Compound That Increases Na^+\Ca^{2+} Exchanger Activity

Until a few years ago, only non-selective NCX activators have been reported to stimulate NCX activity, including lithium (Iwamoto and Shigekawa, 1998), redox agents (Reeves et al., 1986; Secondo et al., 2011), agonists of G-protein-coupled receptors (Eriksson et al., 2001; Woo and Morad, 2001; Annunziato et al., 2004), diethylpyrocarbonate (Ottolia et al., 2001), concanavalin A, nerve growth factor and insulin (Makino et al., 1988; Formisano et al., 2008). Molinaro et al. obtained an activator of the NCX isoforms, modifying the structure of one of the most potent NCX inhibitors, SM-15811, thereby synthesizing a new compound, 7-nitro-5-phenyl-1-(pyrrolidin-1-ylmethyl)-1H-benzo[e][1,4]diazepin-2(3H)-one, that they named neurounina-1. In their study, it was demonstrated that neurounina-1 is the first molecule that enhances NCX1 and NCX2 activity and also exerts a remarkable neuroprotective effect in stroke. It is noteworthy that neurounina-1 displayed a potent and reversible stimulatory effect on NCX1 and NCX2 in both *forward* and *reverse modes* of operation, with an estimated EC50 in the

low nanomolar range (1- 0.1 nM). By contrast, this compound did not affect the activity of the NCX3 isoform in the range of 0.001 to 10 mM. It is noteworthy that the results obtained *in vivo* showed that the intraperitoneal administration of neurounina-1 in single doses ranging from 0.003 to 30 mg\kg significantly reduced the infarct volume in a mouse model of tMCAO. Remarkably, neurounina-1 was effective even when administered up to 5 hours after ischemia induction, a therapeutic window that, along with its viable route of administration, may have interesting clinical perspectives. Collectively, these results showed that neurounina-1 is provided with high potency for NCX1 and NCX2, a high lipophilicity index, low toxicity, and a remarkable neuroprotective effect in experimental model of cerebral ischemia, with a wide therapeutic window and easy route of administration (Molinaro et al., 2013).

I.C.8. Transcriptional Regulation of *ncx1* Gene in the Brain

Although much research has focused on the mechanisms of regulation of *ncx1* at the post-translational level, few studies have directly addressed the regulation of *ncx1* expression at the transcriptional level. Specifically, little is known on the mechanisms responsible for the changes in *ncx1* expression caused by a variety of signals such as glucocorticoids, growth factors, depolarization, Ca^{2+} influx, and adrenergic stimulation (Golden et al., 2000; Li et al., 2000; Smith and Smith, 1994) or occurring under physiological or pathophysiological conditions such as post-natal development (Gibney et al., 2002) and cerebral ischemia (Boscia et al., 2006; Li et al., 2006; Lu et al. 2002; Pignataro et al., 2004). *ncx1* gene expression appears to be directed by three alternative regulatory regions, named heart (Ht), kidney (Kd), and brain (Br) promoters (Lee et al., 1994). These promoters include different binding sequences for several transcriptional factors that control the tissue-specific expression of *ncx1* in the heart, kidney and brain (Barnes et al., 1997; Lee et al., 1994; Nicholas et al., 1998). Specifically, in cardiomyocytes, the expression of *ncx1* is controlled by the Ht promoter that includes serum response factor (SRF), GATA-4, activator protein 1 (AP-1), and Nkx2.5 binding sites (Nicholas and Philipson, 1999). In particular, the cardiac-

specific homeodomain transcription factor Nkx2.5 induces the expression of *ncx1* and enhances SRF-induced *ncx1* gene transcription in neonatal cardiomyocytes (Muller et al. 2002). In kidney, the *ncx1* expression is controlled by the Kd promoter region that includes a typical TATA box preceded by two tandem GATA elements, NF-Y binding site, and two CAAT\enhancer binding protein-like (C\EBP-like) sites. Furthermore, Kd promoter has two putative binding elements for CREB, one for the activating transcription factor (ATF) and one for AP-1. Although each of these transcriptional binding sites is not specific to the kidney, their unique combinations may lead to kidney-specific transcription regulation (Nicholas et al., 1998).

Br *ncx1* promoter is an ubiquitous GC-rich TATA-less promoter, giving rise to the majority of *ncx1* transcripts in the brain. This region contains several putative binding sites for transcriptional factors including Specificity protein 1 (Sp1), AP-1, AP-2, and nuclear factor kB (NF-kB). It has been hypothesized that Sp1 and NF-kB binding sites could be responsible for the ubiquitous transcription of *ncx1* in all tissues, whereas the high level of expression of NCX1 in the brain could be dependent on AP-2 (Nicholas et al., 1998). Consistent with the hypothesis that the constitutive expression depends on Sp1 and NF-kB, the region spanning from 312 bp upstream the transcription start site (TSS) to 151 bp downstream the TSS of Br promoter, which contains the binding sites for these transcriptional factors, is sufficient to induce the expression outside the brain, in rat vascular smooth muscle cells (VSMC) (Scheller et al. 1998). To date, the promoter of *ncx2* gene has not yet been identified, and moreover, no extensive data are available for its transcriptional regulation. On the other hand, it has been shown that *ncx2* expression is rapidly downregulated after membrane depolarization in a calcineurin-dependent manner, suggesting the possible involvement of the transcriptional factor CREB (Li et al. 2000). The promoter of *ncx3* is located immediately upstream of first exon (Gabellini et al., 2003) and includes binding sites for AP-1, AP-2, CREB, downstream regulatory element antagonist modulator (DREAM), Egr-1, KROX24, MyoD, GATA2\3, and Sp1. Two of these transcription factors, CREB and DREAM, seem to be the most

important regulators of *ncx3* gene transcription in response to changes in the intracellular concentrations of Ca^{2+} and cAMP (Gabellini et al. 2003;Gomez-Villafuertes et al. 2005).

During the last 10 years, many evidences demonstrated that NCX1 exerts a neuroprotective activity in experimental models of stroke, and suggested that ischemic neurons could be rescued from cell death by enhancing the expression or the activity of NCX1 isoform with neurotrophic factors or neuroprotective drugs (Annunziato et al., 2004; Pignataro et al., 2004; Boscia et al., 2006; Secondo et al., 2007a; Sirabella et al., 2009). Such a therapeutic approach appears rational also considering that *ncx1* gene expression was found to be downregulated during stroke by unknown mechanisms (Boscia et al., 2006). Recently there is a growing interest in unraveling the transcriptional regulation of *ncx1* during brain ischemia and to identify possible tools to increase its transcription.

I.C.8.1. ncx1 Is Transcriptionally Upregulated by NF-kB Under Anoxic Conditions

NF-*k*B is a family of transcription factors that exists as either a hetero- or homodimer of several subunits called RelA (p65), RelB, cRel, p50 and its precursor p105 (NF-*k*B1), and p52 and its precursor p100 (NF-*k*B2). Some dimers are more prevalent than others, and they shuttle between the cytoplasm and nucleus, but are predominantly sequestered in the inactive state in the cytoplasm by members of the inhibitor of NF-*k*B (I*k*B) (Hayden and Ghosh, 2008). Upon stimulation by tumor necrosis factor-α (TNF-α), oncogenes, or UV light, I*k*B is firstly phosphorylated by a kinase cascade and then degraded by a ubiquitination-mediated proteasomal pathway. As a result, NF-*k*B is released and translocates into the nucleus where it binds target DNA sequences and regulates the transcription of several genes (Perkins and Gilmore, 2006). Interestingly, a putative NF-*k*B binding site was identified on Br *ncx1* promoter at -306\-297 bp from the TSS (Nicholas et al., 1998). Recently, we demonstrated that under hypoxic conditions, NF-*k*B increases the protein expression of NCX1 in cortical neurons. Moreover, the pharmacological blocker SN-50 or the silencing of the NF-*k*B subunit p65

prevents hypoxia-induced NCX1 upregulation (Sirabella et al., 2009). This suggests an involvement of the transcriptional factor NF-*k*B in NCX1 upregulation in the ischemic brain.

I.C.8.2. *ncx1 is a Novel Target Gene for HIF-1*

HIF-1 is a nuclear factor required for transcriptional activation in respons to hypoxia. It consists of two subunits: an oxygen-sensitive HIF-1α and a constitutively expressed HIF-1β subunit (Wang and Semenza, 1993; Bruick, 2003). Both contain two motifs named Per-ARNT-Sim (PAS) and basic helix-loop-helix (bHLH). Whereas both motifs are required for heterodimer formation between HIF-1α and HIF-1β subunits, only the bHLH region affords specific binding to a region of HIF-1 target genes called hypoxia-responsive element (HRE) (Crews, 1998). Under normoxic conditions, HIF-1α protein is rapidly hydroxylated by a proline-hydroxylase, and this, in turn, allows the recognition by Von Hippel–Lindau tumor suppressor protein that targets HIF-1α for ubiquitination and proteasomal degradation (Tanimoto et al., 2000) (**Fig. 7**). By contrast, during hypoxia or in the presence of iron chelators and divalent cations, such as cobalt, HIF-1α protein scapes proteosomal degradation, dimerizes with HIF-1β, generates HIF-1, and translocates into the nucleus (Kallio et al., 1997) (**Fig. 7**). A large variety of genes involved in erythropoiesis, iron metabolism (Semenza et al., 1991), angiogenesis (Levy et al., 1995), and glucose metabolism (Semenza et al., 1996) are regulated by HIF-1. In addition, HIF-1 is upregulated in rat brain during cerebral ischemia (Chavez and LaManna, 2002; Matrone et al., 2004), in repetitive episodes of hypoxia–reoxygenation (Semenza, 2009), or in ischemic preconditioning, a condition in which a period of sublethal ischemia is able to protect the brain from a subsequent lethal insult (Pignataro et al., 2009; Taie et al., 2009). Thus, in an attempt to unravel the mechanisms involved in the regulation of *ncx1* expression at the transcriptional level, our collegues investigated whether *ncx1* gene might be a target of the transcription factor HIF-1 in the brain. They reported that: (1) in neuronal cells, NCX1 increased expression after oxygen and glucose deprivation or cobalt-induced HIF-1 activation was prevented by silencing HIF-1; (2) the brain NCX1 promoter

contained 2 regions of HIF-1 target genes called hypoxia-responsive elements that are sensitive to oxygen and glucose deprivation or cobalt chloride; (3) HIF-1 specifically bound hypoxia-responsive elements on brain NCX1; (4) HIF-1 silencing prevented NCX1 upregulation and neuroprotection induced by ischemic preconditioning; and (5) NCX1 silencing partially reverted the preconditioning-induced neuroprotection in rats. NCX1 gene is a novel HIF-1 target, and HIF-1 exerts its pro-survival role through NCX1 upregulation during brain preconditioning (Valsecchi et al., 2011).

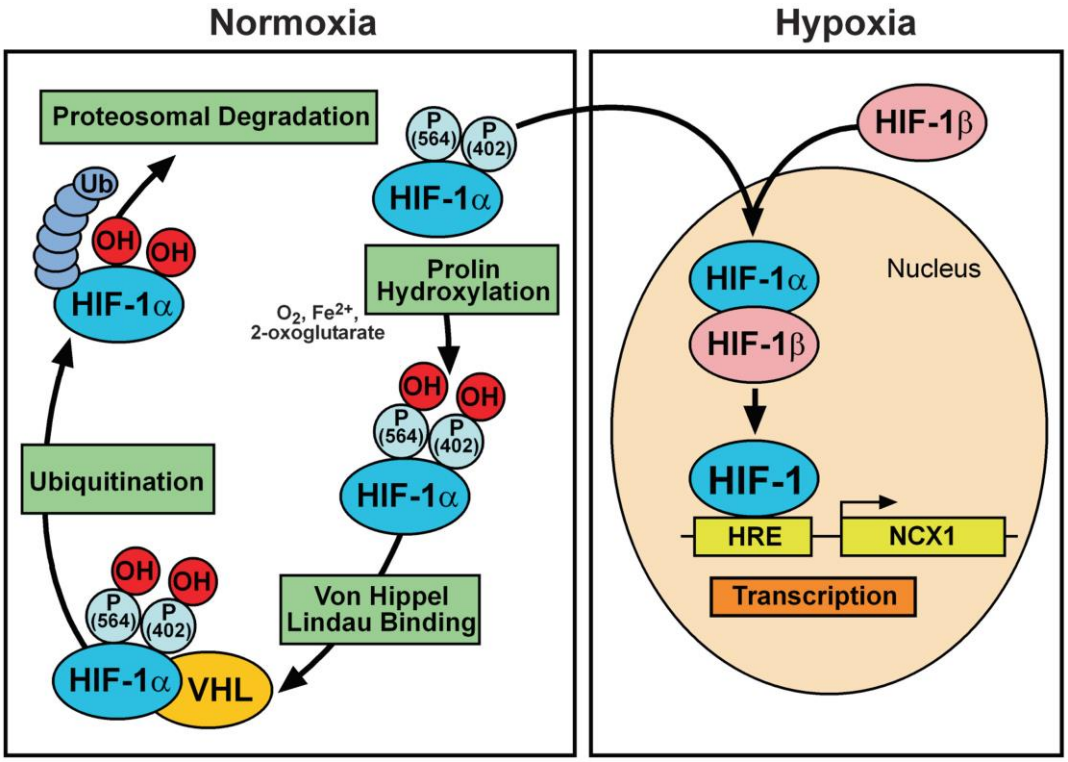

Figure 7. Regulation and activation pathways of HIF1α (Valsecchi et al., 2013).

I.C.8.3. ncx1 Is Transcriptionally Activated by Akt1 via CREB

CREB is a member of a large multigene family encompassing at least ten different structurally related cAMP-responsive transcription factors (the ATF\CREB family). All these bZip class transcription factors form homo- or hetero-dimers that could bind, through their leucine zipper domain, to CRE elements present on the gene promoters. In particular, the transcriptional factor CREB is activated by the phosphorylation of a serine residue,

stimulated by a cyclic AMP dependent kinase, Ca^{2+}, growth factors, and stress signals (De Cesare et al., 1999). More important, CREB has a distinct pro-survival activity and mediates the neuroprotective effect of NGF. Indeed, this neurotrophin, upon binding to its trkA receptors, causes the activation of the Ser\Thr kinase Akt which ultimately phosphorylates CREB and causes its translocation in the nucleus (Autio et al., 2011). Interestingly, Nicholas et al. in 1998 reported the presence of a putative CRE binding site in Kd *ncx1* promoter, whereas its existence in the Br *ncx1* promoter remained unaddressed (Nicholas et al., 1998). Recently, Formisano et al. found another CREB binding site on the Br *ncx1* promoter, that is responsible for the increase in *ncx1* expression and activity upon the activation of the neuroprotective kinase Akt1. In particular, the activation of the Akt1 by the nerve growth factor (NGF) leads to the phosphorylation of CREB and causes its translocation into the nucleus that, in turn, binds the CRE sequence at the level of the Br *ncx1* promoter increasing the transcription of this gene. Furthermore, the silencing of CREB prevents the Akt1-induced increase in *ncx1* protein expression (Formisano et al., 2008). These data demonstrated that CREB controls the gene expression of *ncx1* and that *ncx1* could participate to the multiple pro-survival actions of this transcriptional factor.

I.C.8.4. ncx1 is a Novel Target Gene for REST

The transcriptional repressor REST is a zinc finger protein that binds to a conserved 21-bp motif known as RE1 (repressor element 1, also called NRSE). Of these base pairs, only 14 are relatively important for its function (core REST) (Li et al., 2008). REST perturbation has been described in several neurological conditions and in the mechanism of neuropathic pain (Ooi and Wood, 2007; Mucha et al., 2010). In brain ischemia, REST is a master transcriptional regulator and is associated with selective and delayed cell death of hippocampal CA1 neurons. Its main repressive action occur through the repression of its two target genes, μ-opioid receptor 1 (Opmr1) (Formisano et al., 2007) and AMPA receptor (AMPAR) subunit GluR2 (Calderone et al., 2003). Recently, it has been evaluated the association of *ncx1*-RE1 sequence with REST and its role in the modulation of NCX1

promoter activity by REST. It has been found that: (1) REST binds in a sequence specific manner and represses through H4 deacetylation *ncx1* gene in neuronal cells by recruting CoREST; (2) in neurons and in SH-SY5Y cells REST silencing by siRNA and site-direct mutagenesis of REST consensus sequence on NCX1 brain promoter determined an increase in NCX1 promoter activity; (3) by contrast, REST overexpression caused a reduction in NCX1 protein expression and activity; (4) interestingly, in rats subjected to transient middle cerebral artery occlusion (tMCAO) and in organotypic hippocampal slices or SH-SY5Y cells exposed to oxygen and glucose deprivation (OGD) plus reoxygenation (RX), the increase in REST was associated with a decrease in NCX1; however, this reduction was reverted by REST silencing; (5) REST knocking down, along with the deriving NCX1 overexpression in the deep V and VIb cortical layers, caused a marked reduction in infarct volume after tMCAO. Notably, double silencing of REST and NCX1 completely abolished neuroprotection induced by siREST administration. Collectively, these results demonstrate that REST, by regulating NCX1 expression, may represent a potential druggable target for the treatment of brain ischemia (Formisano et al., 2012)

I.D. SP TRANSCRIPTION FACTORS

I.D.1. The Sp\XKLF Transcription Factor Family

The Sp\XKLF (Specificity protein\Kruppel-like factor) family of transcription factors is united by a particular combination of three conserved Cys2His2zinc fingers that form the DNA-binding domain of these factors (Philipsen and Suske, 1999). Kruppel-like factors have been named after the Drosophila segmentation gene Kruppel that shows a similar arrangement of zinc fingers. In human, the Kruppel-like three zinc finger motif was first found in Sp1 (Kadonaga et al., 1987). Family founder Sp1 is thought to contact DNA with the amino acids KHA in the first, RER in the second and RHK in the third zinc finger (**Fig. 8**). As a consequence of the conserved DNA binding motif, Sp\XKLF members recognize the same GC-(GGGGCGGGG) and GT-(GGTGTGGGG) boxes albeit with different affinities due to the substitutions of aminoacids in the zinc fingers. GC and GT boxes are important for the expression of many different ubiquitous as well as tissue-specific cellular and viral genes (Philipsen and Suske, 1999). In addition, these motifs are involved in the maintenance of the methylation-free status of the CpG islands, as has been shown for the APRT gene.

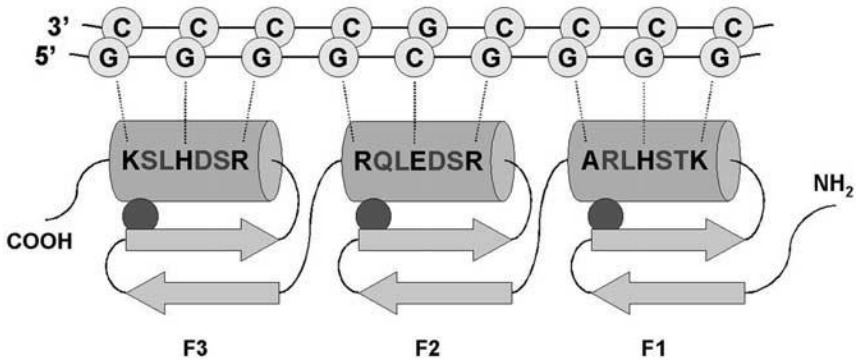

Figure 8. The zinc fingers of Sp\XKLF transcription factors. The individual fingers (F1, F2, and F3) are depicted as a β-sheet (arrows) and a α-helix (cylinder) held together by a zinc ion (dark grey sphere). DNA containing a classical GC box (5'-GGGGCGGGG-3') is shown as a double array of beads on strings with the nucleotide pairs partially overlapping. The aminoacids in the α-helices are indicated, and interactions between critical residues (black) in each finger and a specific triplet of nucleotides of the GC box are shown with dotted lines (Bouwman et al. 2002).

The name of the family indicates that it is subdivided into two major subgroups. First, the Sp proteins named after transcription factor Sp1, that are not only virtually identical in their zinc finger region but also have similar N-terminal motifs (**Fig. 9**), and second, a more heterogeneous group known as KLFs (Philipsen and Suske, 1999). The Sp\XKLF family comprises a large number of homologous transcription factors. At present, about 23 members of the Sp\XKLF family have been identify: 8 Sp factors and 15 KLF factors. Sp1-8 are allocated adjacent to a HOX gene cluster; Sp1 and Sp7 (Osterix) are on 12q13.13 (HOX C); 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). Obviously, the regulation of transcription via GC and GT boxes by these proteins is a complex process that needs to be tightly controlled. Factors like EKLF (Erythroid Kruppel-like Factor), Sp4 and Sp7 show tissue-restricted expression patterns and their *in vivo* requirement in those tissues became apparent after gene targeting experiments in mice (Göllner et al., 2001b; Nakashima et al., 2002). Others like Sp1 and Sp3 are ubiquitously expressed but also fulfil distinct functions, as has been indicated by gene ablation studies (Göllner et al., 2001a).

I.D.2. Structural Characteristics of the Sp Factors

Sp1, the first cloned member of the Sp factors, was identified as a transactivator of the SV40 (Simian Virus 40) early promoter (Dynan and Tjian, 1983). It was long thought that Sp1 would be essential for the transcriptional control of all of the genes that are regulated via GC or GT boxes. However, that view changed dramatically when closely related transcription factors, now called Sp2, 3 and 4, were cloned. This was followed by the identification of Sp5, Sp6 or KLF14, Sp7 or Osterix and Sp8 (Bouwman et al. 2002).

Sp5-8 have several small domains in common with Sp1-4 (**Fig. 9**). Within the Sp factors, Sp1, Sp2, Sp3 and Sp4 form a subgroup based on their similar modular structure (**Fig. 9**). Sp1, Sp3 and Sp4 contain two major glutamine-rich transactivation domains A and B (**Fig. 9**) that are essential for transcriptional activation. Next to these A and B domains, serine\threonine-

rich sequences are located, that may be a target for post-translational modification. While Sp2 has only one glutamine-rich domain, it does share a highly charged domain C and a serine\threonine-rich region with the other factors. Sp2 has a different consensus-binding site due to the substitution of a critical histidine residue by a leucine residue in zinc finger 1 (Kingsley and Winoto, 1992). The Sps all contain a so-called Buttonhead box immediately N-terminal to the zinc finger domain (Harrison et al., 2000) (**Fig. 10A**). This conserved stretch of 11 amino acid residues may contribute to the transactivation potential of the factors, since a deletion of an overlapping region results in reduced activity of Sp1 *in vitro* (Courey and Tjian, 1988). Furthermore, domain C, and more specifically the Btd element within domain C (Athanikar et al., 1997), is involved in synergistic activation by Sp1 or Sp3 with sterol-regulatory element-binding proteins (SREBP). Harrison et al. (2000) identified another stretch of conserved amino acids consisting of the sequence SPLALLAATCSR\KI (Sp box), that is located at the N-terminus of the proteins (**Fig. 10B**). This element contains an endoproteolytic cleavage site and is situated close to a region at the N-terminus of Sp1 that targets proteasome-dependent degradation *in vitro* (Su et al., 1999).

Although not required to direct cleavage, the fact that the Sp box is highly conserved indicates that it may have a function in regulation of proteolysis of Sp factors. Another possible role for the Sp box may lie in the control of transactivation potential via interaction with a putative repressor (Murata et al., 1994). Although the functions of the Btd and Sp boxes are not clear at the moment, their absence in the XKLF subgroup confirms the relationship between the Sp transcription factors. With the exception of the Btd and the Sp boxes, the N-terminal regions of Sp5-8 are completely different from those of Sp1-4 and more closely related to each other.

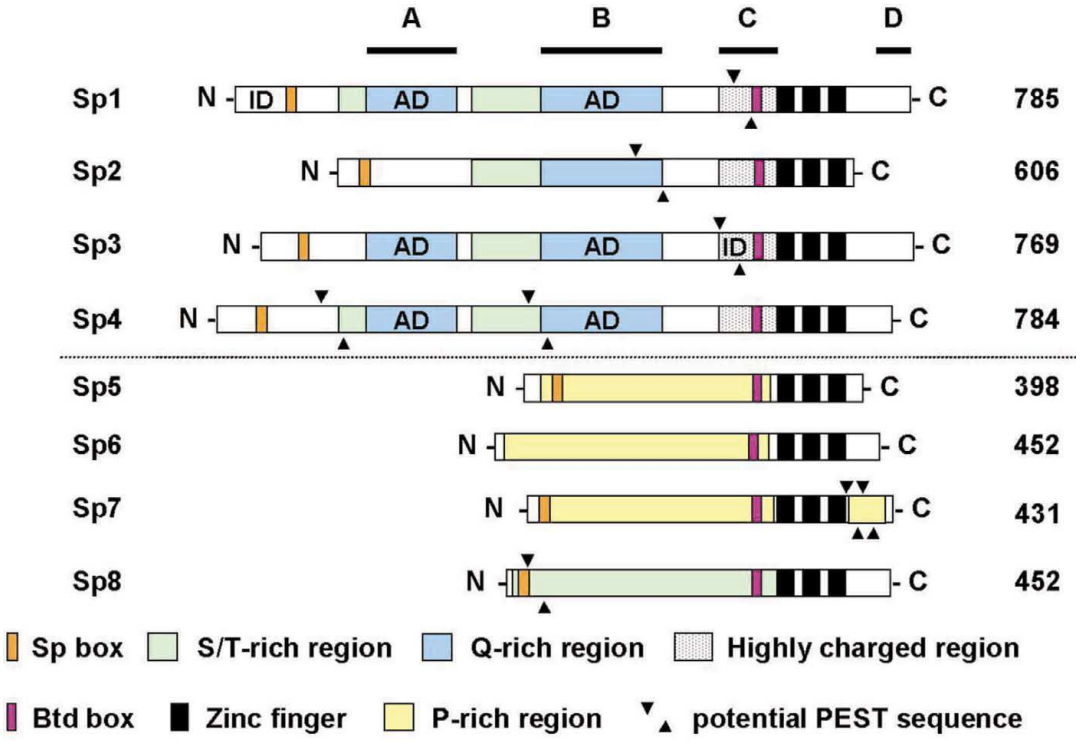

Figure 9. **Structural motifs in Sp factors.** Sp and Btd boxes, serine\threonine-rich, glutamine rich and highly charged regions, and zinc fingers are indicated, as well as activation (AD) and inhibitory (ID) domains. A, B, C, and D modules of Sp1 (Courey and Tjian, 1988) are marked with black bars. Each pair of arrow heads points at a PEST domain with a significant PEST-find score (>5.0) (Bouwman et al. 2002).

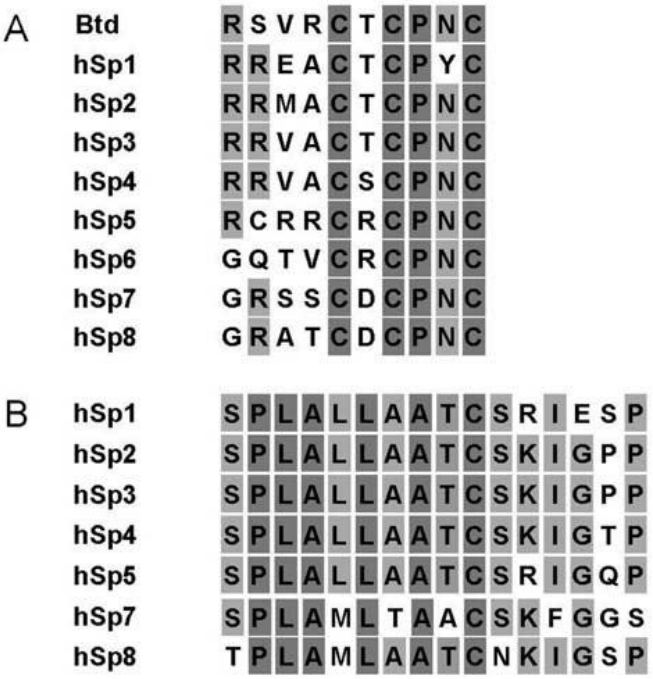

Figure 10. The Btd and Sp boxes are conserved in Sp1-8. (A) Alignment of Btd boxes (Wimmer et al., 1993) from Drosophila Btd and human Sp factors. Strictly and highly (>75%) conserved amino acids are indicated with a dark grey and a light grey background, respectively. (B) Alignment of Sp box-containing sequences from human Sp factors. Strictly and highly (>75%) conserved amino acids are indicated with a dark grey and a light grey background, respectively (Bouwman et al. 2002).

I.D.3. Functional Analysis of Sp factors: Transactivation Properties

Traditionally, studies on the transactivation potential of Sp factors have been performed in Drosophila SL2 cells, since Sp-like proteins were thought not to be present in Drosophila. Although this has been challenged by the cloning of the Drosophila Sp1 homologues Buttonhead and D-Sp1, SL2 cell extracts are reportedly devoid of Sp-like activities (Courey and Tjian, 1988; Santoro et al., 1988).

I.D.3.1. Sp1

Sp1 can stimulate the transcription from proximal promoters but also from distal enhancers (Courey et al., 1989). *In vitro* experiments suggest that Sp1 tetramers are involved in the synergistic activation via distant sites. For the multimerization, activation domain B appeared to be of critical importance (Pascal and Tjian, 1991). Together with domain A, domain B also mediates superactivation of Sp1-dependent transcription which can be achieved by non-DNA-binding mutants in case of multiple binding sites (Courey et al., 1989; Hagen et al., 1995). For synergistic activation via binding to multiple sites, domain D is required in addition to both transactivation domains (Pascal and Tjian, 1991)

I.D.3.2. Sp2

Since the binding site specificity of Sp2 differs from that of the other Sp proteins (Kingsley and Winoto, 1992), the inability of Sp2 to activate promoters containing GC boxes can be readily explained. Data from the only report of a promoter that is affected by co-transfected Sp2 indicate that this transcription factor may function in a cell-type-dependent manner. Sp2 represses Sp1- and Sp3-driven activation of a construct containing the murine CTP: phosphocholine cytidylyl-transferase *γ* promoterin Drosophila cells, but activates the same construct in C3H10T1\2 mammalian cells (Bakovic et al., 2000). It is likely that Sp2 has different characteristics than Sp1, 3 and 4 since it has only one glutamine rich transactivation domain, whereas two domains are required for superactivation and synergistic activation by Sp1 (Pascal and Tjian, 1991).

I.D.3.3. Sp3

Although Sp3 was found to be highly homologous to Sp1 with similar affinities for GC and GT boxes, it soon became clear that there are some striking functional differences. Sp3 can activate transcription from different promoters in Drosophila SL2 cells and also in certain mammalian cell lines (Galvagni et al., 2001). Upon co-transfection with Sp1, additive and synergistic effects were noticed. However, under other circumstances Sp3 is at best weakly active, and in the case of promoters containing multiple adjacent binding sites Sp3 can repress transcription driven by Sp1 or other transcription factors (Birnbaum et al., 1995; Dennig et al., 1996; Majello et al., 1997). Intriguingly, it should be noted that some promoters could be activated by Sp3 in Drosophila cells but not in certain mammalian cells (Hansen et al., 1999) and viceversa (Sjottem et al.,1996), which further demonstrates the complex nature of this transcription factor.

I.D.3.4. Sp4

As was shown for Sp3, the functional properties of Sp4 turned out to be different from those of Sp1, despite obvious structural similarities. Compared with Sp1, Sp4 shows similar transactivation potential through its glutamine-rich activation domains. In addition, Sp4 can be superactivated by fingerless Sp1 and repressed by Sp3. However, whereas Sp1 can synergistically activate promoters containing multiple binding sites, transactivation by Sp4 only occurs in an additive manner (Hagen et al., 1995). The transactivation potential of Sp4 with respect to different promoters and cell types has not been studied as intensively as in the case of Sp1 or Sp3. Several promoters could be activated by Sp4 in mammalian cell lines as well as in Drosophila cells, but others only appeared to respond to different family members (Bouwman et al. 2002).

I.D.4. Specificity in the Regulation of Transcription by Sp Factors

Sp factors have different functional properties and fulfil specific roles in the regulation of biological processes. Unique functions *in vivo* are demonstrated by the clear and different phenotypes of Sp1, Sp3, Sp4 and Sp7 knockout mice (Bouwman et al., 2000). On the other hand, under certain circumstances apparently depending on cellular conditions and promoter context, Sp factors are at least partly redundant. However, various control mechanisms could determine the combined effect of these highly homologous proteins on transcription regulation.

I.D.4.1. Expression Levels

The presence of Sp1, Sp3, and perhaps also Sp2, in many if not all different cell types, does not imply that the levels of these proteins are not subject to specific regulation. Whereas there are no expression data available for Sp2, there are several reports on the control of Sp1 and Sp3 expression. Expression of Sp1 differs between the cell types during development, and is down-regulated in many fully differentiated cells (Saffer et al., 1991). Murine Sp1 mRNA can undergo alternative splicing, which during spermatogenesis leads to an N-terminally truncated protein that lacks the first glutamine-rich transactivation domain and both serine\threonine-rich domains (Persengiev et al., 1995). Although the available amount of Sp1 protein is regulated to a large extent at the mRNA level, there are also other control mechanisms. Sp1 undergoes proteasome-dependent degradation under conditions of nutrient starvation and adenylate cyclase stimulation. Initiation of this process is thought to be determined by a low glycosylation state of Sp1 and consists of an endoproteolytic cleavage triggered by an N-terminal region of Sp1 (Saffer et al., 1999). The cleavage site is situated in the Sp box that is conserved in Sp1-8, which may indicate that proteolysis of other Sp proteins is regulated in a similar fashion. Treatment of full-length Sp1 with the serine protease myeloblastin, that is downregulated during differentiation, yields a fast migrating binding activity similar to that in undifferentiated cells. Therefore, myeloblastin or a related protease might provide a switch that regulates Sp1-dependent transcription through limited proteolysis. In such a

scenario, the small C-terminal peptide could act as a transcription inhibitor, since it lacks the transactivation domains but does contain the DNA-binding zinc finger region. Apoptosis also coincides with Sp1 proteolysis and the action of a caspase-3-like protease in B cells can produce a similarly truncated DNA-binding Sp1 isoform (Rickers et al., 1999). The caspase-3 cleavage site is contained in a region of domain C (**Fig. 9**) that has earlier been identified as a PEST sequence, a putative target motif for inducible proteolysis (Mortensen et al., 1997). Although this specific sequence is not conserved in the other Sp proteins, they all contain PEST motifs at different positions (**Fig. 9**). Sp1 is also degraded by a cathepsin-like protease termed SPase that is expressed in the green monkey kidney cell line CV-1. SPase displays some specificity in its action, since it targets Sp1 and the phosphorylated retinoblastoma-susceptibility gene product (Rb) but not other nuclear factors such as c-Jun or c-Fos. Although not assessed directly, it is likely that SPase also degrades Sp3 -and perhaps other Sp\XKLF factors- given the complete depletion of different GC box-binding proteins from extracts treated with this protease (Nishinaka et al., 1997). Often, for several cell types and promoters, Sp1 and Sp3 have been identified as the major GC\GT box binding activities. Variation in especially the expression of Sp1 and Sp3 may have important consequences for transcription activation, given the dual nature of Sp3 that can function as an activator as well as an inhibitor. There are a number of reports that show variations in the ratio Sp1\Sp3 under different cellular conditions. One report suggests that in human umbilical vein endothelial cells, hypoxia enhances the amount of Sp1 protein while Sp3 levels remain unaltered (Xu et al., 2000). Hypoxia induces a similar change in the Sp1\Sp3 ratio in myoblasts, albeit in these cells this is achieved via the post-transcriptional down-regulation of the Sp3 protein level (Discher et al., 1998). In addition, a relatively high Sp1\Sp3 ratio is seen in epithelial cells compared to fibroblasts (Apt et al., 1996) and in endothelial cells compared to non-endothelial cells (Hata et al., 1998). In most cases, the increase of the Sp1\Sp3 ratio has been correlated with the increased expression of response genes. In these cellular contexts, those genes might be activated by Sp1 and repressed by Sp3, suggesting that transcription is

regulated via the co-operative action of both transcription factors. Transactivation by Sp3 may also be dependent upon the regulation of alternative translation initiation. The nucleotides surrounding the first two internal AUG codons allow reasonably efficient translation initiation, giving rise to N-terminal truncated Sp3 isoforms (Kennett et al., 1997). Since these truncated Sp3 molecules lack part of the transactivation domains, it is likely that they are less potent activators, and repress transcription by competing for binding sites. It was then suggested that the contradictory results obtained with Sp3 in transactivation assays are a consequence of differences in Sp3 expression constructs, leading to different ratios of full-length versus truncated isoforms (Kennett et al., 1997). This may indeed explain some results, but not all, since full-length Sp3 can also repress Sp1-mediated transcription (Fandos et al., 1999).

I.D.4.2. Tissue Specificity

Whereas Sp1, Sp3 and Sp6 are widely expressed, and Sp2 mRNA has been detected in various cell lines, Sp4 shows a more restricted expression pattern (Scohy et al., 2000). A high level of Sp4 mRNA is predominantly found in brain. The robust expression of Sp4 in the central nervous system may correlate with the impaired mounting behaviour of Sp4-\- mutant males (Nguyên-Trân et al., 2000). Another aspect of the Sp4 knockout phenotype is sudden cardiac arrest, and this could be linked to specific expression of Sp4 in the conductive system of the heart (Nguyên-Trân et al., 2000). Sp5 has a highly dynamic expression pattern during mouse embryogenesis in the developing brain, the spinal cord, the trigeminal ganglia, the somites and additional sites outside the nervous system (Treichel et al., 2001). Surprisingly, mice homozygous for a targeted mutation in Sp5 show no overt phenotype, but the enhancement of the phenotype of the Brachyury mutation (T allele) in compound mutant mice (Sp5 null, T\+) indicates a genetic interaction between Sp5 and Brachyury. Sp7 (Osterix) is specifically expressed in all developing bones. In Sp7 null mutants no bone formation occurs due to a defect in osteoblast differentiation (Nakashima et al., 2002). Finally, the expression pattern of Sp8 has not been studied yet.

I.D.4.3. Binding Site Specificity

The binding site specificity of Sp2 is different from all the other Sp\XKLF factors. *In vitro* translated Sp2 binds weakly to a GT box of the T cell antigen receptor variable gene segment Vα11.1 promoter, and not at all to an Sp1 consensus GC box (Kingsley and Winoto, 1992). Based on these data, it is likely that Sp2 is involved in regulation of a different set of genes than Sp1, Sp3 and Sp4, that all bind to GC and GT boxes with similar affinities (Hagen et al., 1992). There are several examples of promoter or enhancer sequences that are specifically bound by distinct Sp factors. For instance, Sp2 has been shown to bind to a GC box containing region that appears to be essential for the activity of the methionine adenosyl-transferase II (MATII) promoter in Jurkat cell extracts (Halim et al., 2001). Strikingly, whereas Sp3 and Sp4 were also part of the complexes bound to this region, Sp1 could not be detected. Another example is a GT motif in the neuronal nicotinic acetylcholine receptor β4 promoter that specifically interacts with Sp1 and Sp3 in extracts from a neuronal cell line and from rat brain (Bigger et al., 1997). Although Sp2 and Sp4 are present in these cells, there was no evidence that they could bind to this motif, while Sp4 antibodies did show binding of this factor to a consensus GC box. At present, there is no physical or biochemical explanation for this apparent binding site specificity.

I.D.4.4. Post-translational Modifications

Like many other transcription factors, Sp1 is subject to post-translational modifications which can influence its activity. The two major types of post-translational modifications that are thought to be involved in transcription regulation by Sp1 are glycosylation and phosphorylation.

I.D.4.4.1. Glycosylation

There are no published data that show glycosylation of Sp proteins other than Sp1, although they do contain putative glycosylation sites (Hagen et al., 1992). *O*-glycosylation has been related to the nuclear localization, the stability and\or the transactivation potential of Sp1. However, direct evidence for the effect of glycosylation on Sp1 function has not been obtained, and

with respect to transactivation potential, it has recently been shown that glycosylation can also have adverse effects (Yang et al., 2001). One of the suggested explanations for increased transactivation was that GlcNAc residues might act as ligands for recognition by components of the transcription machinery (Jackson and Tjian, 1988). In contrast, the opposite has been observed for the glycosylation of the carboxy-terminal part of Sp1 activation domain B. Interactions between this domain and dTAF(II)110 and full-length Sp1 are markedly decreased upon glycosylation, correlating with a decreased transactivation potential *in vitro* (Roos et al., 1997) and *in vivo* (Yang et al., 2001). Glucose deprivation in combination with adenylate cyclase stimulation results in reduced glycosylation of Sp1, associated with an increased susceptibility to proteasome-dependent degradation. The process is blocked in cells treated with glucosamine, a metabolic derivative of glucose that is used primarily asa substrate for protein glycosylation (Han and Kudlow, 1997). It has therefore been suggested that glycosylation blocks protein interactions and prevents Sp1 from entering into protein complexes that are readily degraded by proteasomes (Roos et al.,1997).

I.D.4.4.2. Phosphorylation

O-glycosylated proteins are also phospho-proteins. Sp1 becomes phosphorylated at its N-terminus by DNA-dependent protein kinase upon binding to DNA (Gottlieb and Jackson, 1993). The C-terminus of Sp1 can also be phosphorylated; this has been linked to cell cycle progression from G0 to G1. *In vitro* data suggest that the unknown kinase that mediates this phosphorylation specifically targets serine residues in the most N-terminal zinc fingers 1 and 2 (Black et al., 1999). Several additional reports link Sp1 phosphorylation with decreased binding activity (Zhu and Liao, 2000), but phosphorylation can also result in increased binding (Haidweger et al., 2001). In some cases, increased binding through phosphorylation has been correlated with enhanced transactivation. For instance, phosphorylation of Sp1 by protein kinase C-γ (PKC-γ) in smooth muscle cells stimulates platelet-derived growth factor β-chain (PDGF-β) expression. Interestingly, PKC-γ, that can directly interact with the Sp1 zinc finger region, has no apparent

effect on Sp3 (Rafty and Khachigian, 2001). Apparently, certain kinases can specifically regulate the transcriptional activity of distinct Sp proteins. Nevertheless, it is likely that there are also forms of phosphorylation that have a similar effect on different Sp factors. Sp1 interacts with cyclin A and can be phosphorylated via a cyclin A-associated kinase. As a result, DNA binding is increased, concomitantly with transactivation mediated via Sp1-binding sites. Since Sp3 DNA binding is similarly affected, and Sp3 as well as Sp4 have been reported to interact with cyclin A, this suggests a common regulatory pathway (Haidweger et al., 2001).

I.D.4.4.3. Acetylation of the Inhibitory Domain of Sp3

Transactivation assays with mutant proteins revealed that the transactivation potential of Sp3 is influenced by an inhibitory domain (Dennig et al., 1996). This domain resides in a highly charged stretch of amino acids that is not present in the comparable region of Sp1 (domain C; **Fig.9**). The presence of a repressive module explains the earlier inactivity of the N-terminal region of Sp3, despite the presence of glutamine-rich domains that resemble the Sp1 transactivation domains A and B (Hagen et al., 1994). Mutation of a critical KEE amino acid triplet results in relief of repression and potentates Sp3 transactivation, especially of promoters containing multiple binding sites (Dennig et al., 1996). Recent data show that the lysine residue in this KEE triplet is highly acetylated *in vivo*. A mutant of Sp3 lacking this lysine revealed that it is responsible for the low transcriptional activity of Sp3 *in vivo*. Since this Sp3 mutant is no longer highly acetylated *in vivo*, these results indicate that the transcriptional activity of Sp3 is regulated by acetylation (Braun et al., 2001).

I.D.5. Physiological Function of Sp-Proteins

In the past, a large variety of biological functions have been assigned to Sp1-binding sites and to Sp1. However, the identification of the three paralogous proteins Sp2, Sp3 and Sp4 raises the question as to which tasks are performed by which protein. This question is particularly interesting for Sp1 and Sp3 because both proteins are present in the same cell and are

indistinguishable in their DNA-binding specificity. Gene disruption in mice is a powerful tool for obtaining information on specific functions of individual Sp-proteins. Given that Sp1 is implicated in the activation of a very large number of genes, such as housekeeping, tissue-specific and cell cycle-regulated genes, and is required to prevent methylation of CpG islands (Macleod et al., 1994; Brandeis et al., 1994), one would expect that cells lacking Sp1 would not survive. Surprisingly, this is not the case. S. Philipsen and his coworkers found that Sp1-deficient embryonic stem cells (ES cells) are viable, have normal growth characteristics and can be induced to differentiate and form embryoid bodies as efficiently as wild type ES cells (Marin et al., 1997). Nevertheless, Sp1 is essential for normal mouse embryogenesis. The *Sp1*-knockout embryos are severely retarded in development and they all died around day 11 of gestation. They displayed a marked heterogeneity in phenotype indicating that Sp1 has indeed a general function in many cell types. Interestingly, the defects in *Sp1*-\-mice are caused by a cell autonomous mechanism. *Sp1*-\- ES cells injected into blastocysts contributed efficiently to chimaeric embryos at early stages but after day E 11 they rapidly declined with no contribution to newborn mice. Thus, Sp1 appears to be a transcription factor whose function is essential for differentiated cells after day 10 of development. Independently of the severe developmental defects of the Sp1 null mice, the embryos express many putative Sp1 target genes at normal levels, including housekeeping and cell-cycle regulated genes. In addition, CpG-islands remained methylation free. So far, the only genes which were found to be expressed at a lower level in *Sp1*-\-mice are the thymidine kinase and the methyl-CpG binding protein 2 (*MeCP2*) genes (Marin et al., 1997). The *Sp1*-knockout demonstrated that Sp1 is not essential for the expression of many genes previously shown be activated in cell culture transfection experiments. One could speculate that other Sp-family members compensate at early embryonic stages, at least in part for the loss of Sp1 activity. Sp3 would be a good candidate because it is also ubiquitously expressed, has the potential to activate transcription and its DNA-binding activity is indistinguishable from Sp1. In that regard, the phenotype of *Sp3* knockout mice will be of great interest. Sp4, the tissue

restricted member of the Sp-family, is predominantly expressed in the brain but also detectable in epithelial tissues, testis and developing teeth (Hagen et al., 1992). Disruption of the mouse *Sp4* gene revealed that it is important for early post-natal survival (Supp et al., 1996). Approximately two thirds of the *Sp4*-\- mice die within a few days of birth. The cause of the early death remains unknown. Those mice which survive are significantly smaller than their wild type littermates. The reduced body weight appears to result from an unknown, but growth hormone independent, mechanism (Supp et al., 1996). Interestingly, surviving mice exhibit a striking sex-specific abnormality. While fertility of the female mutants appears normal, males do not breed although their reproductive organs are fully developed and apparently normal. It appears that male *Sp4*-\- mice are unable to copulate. The most likely cause of this abnormal behavior is a neurological defect. The hypothalamus and the vomeronasal organ are known to play important roles in reproductive physiology and behavior. However, both structures are histologically normal in *Sp4*-\- mice. Thus, we await further investigation to understand the role of Sp4 and to identify its target genes. In that context, it should be noted that for both the *Sp1* and the *Sp4* knockouts, the zinc finger regions have been chosen to inactivate the genes. In both cases the N-terminal part encoding the transactivation domains are still expressed (Marin et al., 1997; Supp et al., 1996). One could speculate that the activation domains on their own might act as a gain-of-function or might interfere with other Sp-family members. In the case of the *Sp4* knockout such a scenario does not seem to be the case. Knockout *Sp4*-\- mice which do not express the N-terminal part of the protein manifest the same phenotype (Suske et al. 1999).

I.D.6. Sp1 and Sp3 Are Oxidative Stress-Inducible, Antideath Transcription Factors in Brain

Sp1 activities have been shown to change in response to apoptosis inducing stimuli. Indeed, polyglutamine expansions in the huntingtin protein can induce neuronal toxicity, in part, by sequestering Sp1 and one of its coactivators, TATA binding protein-associated factor (TAF)II130, suggesting a role for Sp1 in neuronal survival (Dunah et al., 2002). Notably, Sp1 has

been shown to regulate prosurvival proteins [e.g., the inhibitor of apoptosis (IAP) protein, survivin, and manganese superoxide dismutase], as well as prodeath proteins [e.g., Fas ligand and 12-lipoxygenase (Ryu et al., 2003a). Like other transcription factors, the role of Sp1 in regulating cell death may depend on a number of factors, such as the cell type and the death stimulus (Lin et al., 1998). Ryu et colleagues (2003) demonstrated that oxidative stress significantly induces Sp1 and Sp3 protein levels and DNA binding in neurons *in vitro* and *in vivo*. Moreover, they found that the enforced expression of Sp1 or Sp3 is neuroprotective. These findings established Sp1 and Sp3 as redox-regulated transcriptional activators that enhance survival in cortical neurons. Sp1 and Sp3 are therefore sufficient components of the protective, homeostatic response to oxidative stress and one potential consequence of oxidative stress, DNA damage, in neurons (Ryu et al., 2003b).

Indeed, recent studies suggest that Sp1 motifs are responsible for the regulation of the IAP protein survivin (Li and Altieri, 1999). Members of the IAP protein family have been shown to suppress apoptosis induced by oxidative stress by directly suppressing the activity of terminal caspase-3 and caspase-7 (Tamm et al., 1998; Suzuki et al., 2000). Interestingly, survivin is expressed not only in common human cancers, but also in some types of embryonic neurons (Adida et al., 1998), suggesting that this protein is poised to act as an inhibitor of apoptosis in the cortical neurons. Bcl-2 and Bcl-xL, two other general inhibitors of apoptosis, also have essential Sp1 sites in their promoters (Grillot et al., 1997; Dong et al., 1999), and p53 has been shown to repress the expression of the antiapoptotic factor telomerase by binding to cognate Sp1 motifs in the telomerase promoter (Kanaya et al., 2000). Taken together, these observations support an antiapoptotic role for Sp1.

The data are consistent with a model in which Sp1 is a part of a compensatory genetic program to oxidative stress in neurons. A prediction of this model is that neurons with acquired or inherited defects in Sp1 signaling will be more vulnerable to oxidants. Indeed, autopsy tissue from HD patients shows prominent defects in Sp1 DNA binding despite increased Sp1

levelsand increased markers of oxidative damage (Browne et al., 1999). Furthermore, toxicity of cultured neurons induced by the forced expression of mutant huntingtin with pathological numbers of polyglutamine repeats can be abrogated by the coexpression of Sp1 and its coactivator TAFII130 (Dunah et al., 2002; Li et al., 2002) or by the exogenous addition of small-molecule antioxidants (Wyttenbach et al., 2002).

Recently it has been found that enhanced neuronal protection by histone deacetylase inhibitors in response to oxidative stress is mediated by Sp1 acetylation in primary neurons and in an animal model of Huntington's disease. The fact that acute oxidative stress induces Sp1 acetylation represents the protective role of Sp1 as the frustrated attempt of neurons to protect themselves from oxidative stress-induced cell death. These studies strongly imply that Sp1 promotes neuronal survival in response to oxidative stress (Lee et al., 2006).

Nevertheless, these findings suggest that small-molecule activators of Sp1-dependent gene expression may be propitious therapeutic targets for a host of neurodegenerative conditions, including HD, Parkinson's disease, amyotrophic lateral sclerosis, and stroke, which in some cases have been associated with expanded polyglutamine repeats and in all cases have been associated with oxidative stress. (Ryu et al., 2003a).

Aim of the study

II. AIM OF THE STUDY

Changes in Na^{+}-Ca^{2+} exchanger 1 (*ncx1*) gene expression, an ubiquitous plasma membrane protein regulating cellular calcium and sodium homeostasis in the brain is important to the progression of cerebral ischemia (Annunziato et al., 2004). Indeed, studies have shown that *ncx1* knocking down, caused a marked increase in infarct volume after brain ischemia (Pignataro et al., 2004; Boscia et al., 2006), and partially reverted the preconditioning-induced neuroprotection (Pignataro et al., 2011a). It is known that NCX1 reduction in brain ischemia is due to the transcriptional repressor REST (Formisano et al., 2012), that regulates global gene expression after stroke (Noh et al., 2012; Schweizer et al., 2013), while NCX1 increase in ischemic preconditioning (PC), is determined by the transcriptional activator HIF-1 (Valsecchi et al., 2011). Interestingly, *ncx1* brain promoter (ncx1-Br) sequence contains several consensus binding sites for Specificity protein 1 (Sp1). Sp transcription factors is a family comprising of four isoforms Sp1, 2, 3 and 4 (Suske, 1999), that bind to the same Sp1 recognition sequence with similar affinities (Hwang et al., 2001), except Sp2 that does not bind to the classical Sp1 sequence, but to a GT-rich element (Philipsen and Suske, 1999). Interestingly, Sp1 and Sp3 can both act as activators (Ammanamanchi et al., 2003; Ravache et al., 2010) or repressors (Ammanamanchi and Brattain, 2001; Law et al., 2011) of gene expression, Sp4 generally acts as transcription activator (Ishimaru et al., 2007). It has been recently reported that Sp1 is involved in NGF-induced regulation of *ncx1* (Sirabella et al., 2012). On the basis of these results, a first aim of this study was to evaluate the association of Sp1 sequences with Sp transcription factor family and its role in the modulation of NCX1 expression during brain ischemia and ischemic preconditioning. We found in cortical neurons that Sp1 acts as activator while Sp3 acts as repressor of *ncx1* expression. Furthermore, considering that REST reduces NCX1 expression during cerebral ischemia (Formisano et al., 2012) and that HIF-1 increases NCX1 expression during ischemic preconditioning plus ischemia, in the present study we investigated the possible interaction of REST with Sp3 and HIF-1 with Sp1 in brain ischemia and ischemic preconditioning plus ischemia,

respectively. In addition, since it has been reported that both REST and Sp3 can modulate their target genes via the histone deacetylases family (HDAC) (Won et al., 2002; Rodenas-Ruano et al., 2012) and that histone acetyl transferase (HAT) is involved in HIF-1 and Sp1 induced gene expression (Billon et al., 1999; Ke and Costa, 2006), we investigated: (1) the interaction between REST and Sp3 to form a protein complex which in turn recruit HDAC1 and HDAC2 repressing *ncx1*expression in an *in vivo* model of brain ischemia, and (2) the interaction between HIF-1 and Sp1 to form a protein complex which recruit the HAT p300 to activate *ncx1* expression in an *in vivo* model of brain ischemic preconditioning. Finally, we assessed the effect of the HDAC class I inhibitor MS-275 (Lanzillotta et al., 2012) and histone acetyltransferase (HAT) p300 inhibitor C646 (Min et al., 2010) on cell survival and its correlation with NCX1 expression in cortical neurons subjected to OGD plus Reoxygenation (OGD\Rx), and to IPC plus OGD\Rx.

Materials and Methods

III. MATERIALS AND METHODS

III.1. Materials

All restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs (Ipswich, MA) or Promega (Milan, IT). Luciferase reporter kits and luciferase vectors were from Promega (Milan, IT). Synthetic oligonucleotides were from Primm (Milan, IT). siRNAs against rat REST and HIF-1 were already published (Valsecchi et al., 2011; Formisano et al., 2012), whereas siRNAs for Sp1 (siSp1) (SI02039044), Sp3 (siSp3) (SI05434387), Sp4 (siSp4) (SI02039114), p300 (sip300) (SI02989693) and negative control siCONTROL (siCTL) (1027280) were from Qiagen (Milan, IT). To knock-down rat HDAC1 and HDAC2 MISSION siRNAs from Sigma (Milan, IT) were used. The sequences of siRNAs used are as follows: for HDAC1 Forward, 5′-CUUUGAAUACUUUGGACCA(dT) (dT)-3′ and Reverse, 5′-UGGUCCAAAGUAUUCAAAG(dT)(dT)-3′; for HDAC2 Forward, 5′-GAUAUCGGGGAAUUAUUAUU(dT) (dT)-3′ and Reverse, 5′-AAUAAUAAUUCCCGAUAUC(dT) (dT)-3′. Two sets of siRNAs were tested for Sp1, Sp3, Sp4, p300, HDAC1 and HDAC2 and the set that was more effective was chosen for the experimentation. The construct to silence NCX1 (siNCX1) and mismatch sequence cloned in the same vector (MS siNCX1) were used as previously described (Formisano et al., 2008; Sirabella et al., 2009). The constructs pN3 and pN3-Sp1,3,4, carrying Sp1,3,4 cDNAs, were kindly provided by Prof. G. Suske (Marburg, Germany). The constructs pKCRH-NCX1 overexpressing NCX1 (NCX1) and the empty vector (pKCRH) were kindly provided by Prof. Iwamoto (Fukuoka, Japan) (Iwamoto et al., 2004a). The HDAC inhibitor MS-275 (EPS002) and HAT-inhibitor C646 (SML002) were obtained from Sigma (Milan, IT). Both 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 $1x10^6$ cells\well for 24-well plate for LDH assay, $2x10^6$ 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, siSp1, siSp3 or siSp4. To overexpress pN3, Sp1, Sp3, Sp4, pKCRH, pKCRH-NCX1 and to silence NCX1 with siNCX1 and MS siNCX1, neurons were transfected with above mentioned constructs in the following amounts: 0.5 µg for 24-well plates, 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 2 µg of total DNA vectors; the reporters (560 ng each) were

the following: (1) the pGL3 construct, (2) the pGL3-ncx1 (*short ncx1 promoter*), (3) the pGL3-ncx1\Sp1Bmut (GGCGGCGGGC), (4) the pGL3-ncx1\Sp1Cmut (CGGGCGGGG), (5) the pGL3-ncx1\Sp1Dmut (GGGAGGGG), (6) the pGL3-ncx1\Sp1Emut (GGCCCCGGC), (7) the pGL3-ncx1\Sp1CDEmut (that contain Sp1 sites C,D and E simultaneously mutated), in which underlined bases represent mutated sequences in pGL3. Mutagenesis of the Sp1 sites in the promoter was performed using the QuickChange site-directed mutagenesis kit from Stratagene. To overexpress Sp1, Sp3 and Sp4 we used the expression vectors and the empty vector pN3 (1.3 µg each). For RNA interference, 50nM of specific siCTL, siSp1, siSp3 or siSp4 were used. 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 (Formisano et al., 2012).

III.4. Combined Oxygen and Glucose Deprivation (OGD) and Reoxygenation (Rx)

OGD in neurons was performed in a medium previously saturated with 95% N_2 and 5% CO_2 for 20 min and containing: NaCl 116 mmol\L, KCl 5.4 mmol\L, $MgSO_4$ 0.8 mmol\L, $NaHCO_3$ 26.2 mmol\L, NaH_2PO_4 1 mmol\L, $CaCl_2$ 1.8 mmol\L, glycine 0.01 mmol\L, and 0.001 w\v phenol red, and then placed in a hypoxia chamber for 3 hours (Billups Rothemberg Inc. Del Mar., CA, USA) (temperature 37°C, atmosphere 5% CO_2 and 95% N_2). To terminate OGD, cells were removed from the hypoxic chamber, and then changed with normal medium for 24 hours of Reoxygenation (Rx). For preconditioning (PC), the cortical neurons were exposed to 30 min of OGD, as described above, and then placed in normal medium. 24 hours after the PC stimulus, the cultures were again subjected to OGD for 3 hours followed by 24 hours of Rx. Transfections with pKCRH-NCX1, pKCRH, siNCX1 and MS siNCX1 were performed at 7 DIV; after 24 hours cells were subjected to OGD\Rx or IPC+OGD\Rx. MS-275 1 µM was added at the end of OGD

period for 2 hours whereas C646 20 μM was added to the medium 30 min before PC, and throughout the IPC phase.

III.5. Quantitative Real-Time PCR (qRT-PCR) Analysis

The first-strand cDNA and quantitative real-time PCR was carried out as previously described (Formisano et al., 2007; Formisano et al., 2012). 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 ©) (Formisano et al., 2007). The oligonucleotide sequences for NCX1, NCX3 and Hypoxanthine phosphoribosyltransferase (HPRT) were already published (Pignataro et al., 2011b; Formisano et al., 2012).

III.6. Western Blotting

For Western blot analysis, cells (or tissues) were collected in ice-cold lysis buffer (Formisano et al., 2012) containing anti-protease cocktail (P8340 Sigma, Milan, IT). For HDAC1, HDAC2 and Acetyl-histone H3 expression, proteins (50 μg) were separated on 12% SDS polyacrylamide gels, whereas for NCX1, Sp1, Sp3, Sp4, REST, HIF-1 and p300 expression, proteins (100 μg) were separated on 8% 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 HDAC1 (5356) and HDAC2 (5113) (Cell-Signaling, EuroClone, Milan, IT), p300 (sc-48343) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), HIF-1(MAB5382) (Millipore, Milan, IT) and 1:2000 β-actin (A 4700) (Sigma, Milan, IT), either with the 1:1000 polyclonal antibodies against REST (07-579) and Acetyl-histone H3 (06-866) (Millipore, Milan, IT), NCX1 (p 11-13) (Swant, Bellinzona, Switzerland), Sp1 (sc-14027), Sp3 (sc-644) and Sp4 (sc-645) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). 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.7. Chromatin Immunoprecipitation (ChIP) and re-ChiP Assay

Brain tissue and cortical neurons were processed into chromatin by use of published protocols (Formisano et al., 2007; Formisano et al., 2012) 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 Sp1 (sc-14027), Sp3 (sc-644), Sp4 (sc-645), p300 (sc-48343) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), REST (07-579), acetyl-Histone H4 (06-866), Hypoxia Inducible Factor 1 α (MAB5382) (Millipore, Milan, IT), HDAC1 (5356), HDAC2 (5113)

(Cell-Signaling, EuroClone, Milan, IT), RNA POL II (R1530) (Sigma, 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 (Renthal et al., 2007). The following oligonucleotides were used for the amplification of immunoprecipitated DNA: ncx1-Br (A) Forward 5'-CCGCTGGGGAAACCCCTGCC-3' and Reverse 5'-GCGCTGCAACTTTTCTTTTGAACG-3', ncx1-Br (B) Forward 5'-GGGTGCAGAAGAGAGCGCTGGC-3' and Reverse 5'-GCACAAAGCGCGGCGGCCCG-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. Re-ChIP experiments were employed to detect the simultaneous presence of Sp3 and Sp1 with REST or HIF-1, respectively. Beads from the first ChIP with anti-Sp1 and anti-Sp3 were incubated with an equal volume of 10 nM dithiothreitol at 37 °C for 30 min, centrifuged at 12000 rpm for 1 min to elute DNA-bound proteins. The elution was repeated twice. The final elute was diluted 1:10 in lysis buffer containing a protease inhibitor cocktail and re-immunoprecipitated with the anti-REST, the anti-HIF-1 antibodies, or IgG.

ChIP and input DNA were analyzed by PCR. Denaturation 10 min @ 95ºC, 35 cycles of 30 sec @ 95ºC; 1 min @ 60ºC; 1 min @ 72ºC. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. For ChIP and Re-ChIP experiments we used ncx1-Br (B) primers, since the amplified sequence of ncx1-Br contains the binding sites for REST (-18\+1) (Formisano et al., 2012), HIF-1 (-331\-327 and -164\-160) (Valsecchi et al., 2011), Sp1 and Sp3.

III.8. Quantitation of neuronal injury

Neuronal injury was assessed by measurement of LDH efflux into the medium after OGD\Rx or IPC+OGD\Rx. In neuronal cultures, LDH activity in the medium is correlated with the number of damaged cells (Koh and Choi, 1987). Cytosolic levels of LDH in the extracellular medium were measured by using an LDH Cytotoxicity Kit (1000882 Cayman, DBA, Milan, IT). Briefly, after induction of OGD\RX or PC+OGD\Rx, the medium was removed and sampled for LDH content by measuring the absorbance at 490 nm using a spectrophotometer BioPhotometer (Eppendorf, Hamburg, Germany). The results were expressed as a percentage of LDH release versus a control obtained in untreated sister cultures.

III.9. In vivo studies

III.9.1. 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.9.2. Transient Focal Ischemia and Ischemic Preconditioning

Transient focal ischemia was induced as previously described (Valsecchi et al., 2011), 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 4 experimental groups: (1) sham-operated (CTL); (2) preconditioned rats (PC); (3) ischemic, subjected to transient MCA occlusion (tMCAO); and (4) preconditioned ischemic rats (PC+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; in the preconditioned ischemic group, rats were subjected to 30 minutes of tMCAO 72 hours before 100 minutes of tMCAO. The siRNAs, used in ischemic and preconditioned ischemic rats were intracerebroventricularly (icv) administered (1 µl) as previously reported at the concentrations of 20 µM for REST (Formisano et al., 2012), 3 µM for HIF-1 (Valsecchi et al., 2011) and 10 µM for Sp1, Sp3, HDAC1, HDAC2 and p300. For tMCAO and PC+tMCAO experiments, icv injections were performed three times, i.e., 18 h and 6 h before and 24 h after ischemia induction and 24, 18, and 6 hours before ischemic preconditioning induction. 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.10. 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. Sp1 and Sp3 are bound *in vivo* to a specific region of ncx1-Br promoter sequence

Since Sp1 and Sp3 both recognize GC-rich sequences known as GC boxes (Isomura et al., 2005), we searched for Sp1 sequences at the level of ncx1-Br sequence (GenBank accession no. U95138), and subjected ncx1-Br sequence to computational prediction of transcriptional factors using the database TF SEARCH version 1.3 (Cassimere et al., 2009). As shown in **Fig 11a**, five binding sites named Sp1 A-E located at -439/-431, -179/-170, -129/-92, -111/-104 and -67/-58, from the transcriptional start site (TSS) on the ncx1-Br sequence have been identified. All these sequences have the TF search threshold score above 85.0. Experiments of ChIP assay were performed on extracts from cortical neurons that basally express Sp1, Sp3 (Ryu et al., 2003a) and Sp4 (Ishimaru et al., 2007) to prove a direct association of Sp transcription factors with the ncx1-Br in the nuclear environment. To this aim ncx1-Br was divided in two different fragments named ncx1-Br (A) (-702/-387) ncx1-Br(B) (-366/+58), and experiments were performed using specific primers recognizing these two parts of ncx1-Br. As shown in **Fig 11b**, when chromatin was precipitated using antibodies for Sp1, Sp3 and Sp4, we were able to amplify only ncx1-Br (B), but not ncx1-Br (A) with Sp1 and Sp3 antibodies, compared to the IgG. Importantly, no signal was detected with the Sp4 antibody in the ncx1-Br (A) and (B) fragments (**Fig 11b**). This result suggests that Sp4 does not bind ncx1-Br and that ncx1-Br (B), but not ncx1-Br (A) sequence is important for Sp1 and Sp3 binding in cortical neurons.

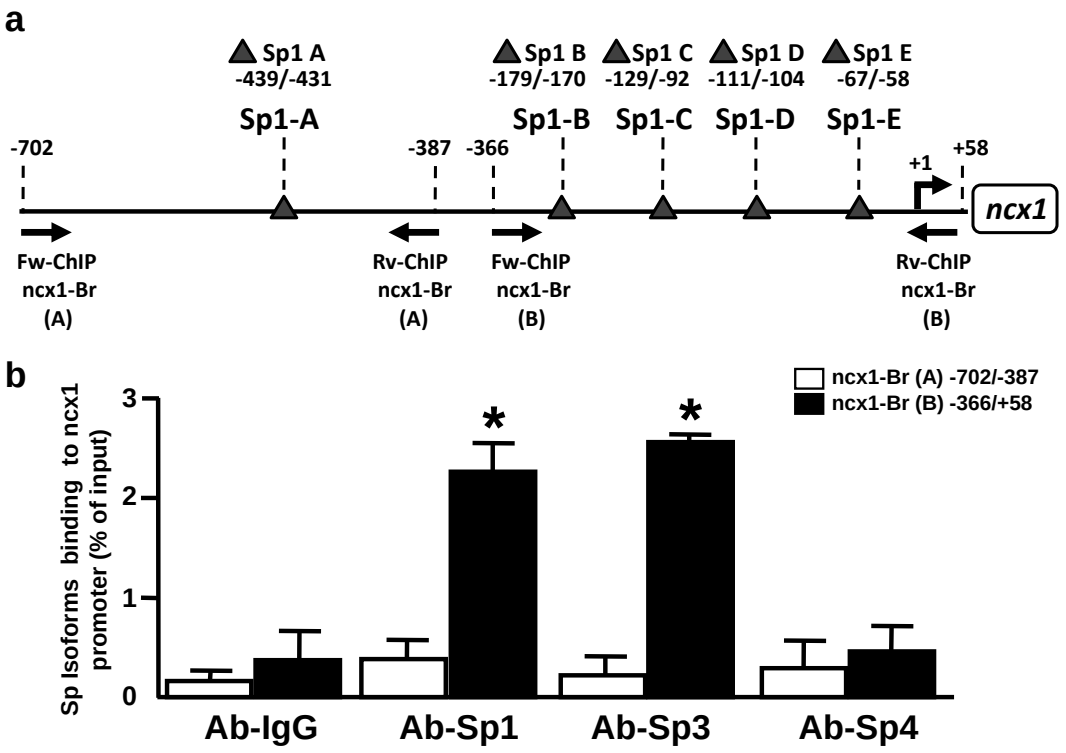

Figure 11. Transcription factors Sp1 and Sp3, but not Sp4, bind *ncx1* brain promoter sequence from -380 to +58. (a) Map of the rat *ncx1* gene indicating location of Sp1 sequences contained in distal (ncx1-Br A) and proximal (ncx1-Br B) regions of *ncx1* brain promoter and of PCR primers used to detect the presence of specific DNA sequences in ChIP complexes. Triangles represent 5 Sp1 identified motives in ncx1-Br, named Sp1 A-E. **(b)** ChIP analysis of ncx1-Br A and ncx1-Br B regions carried out with anti-Sp1, anti-Sp3 and anti-Sp4. Anti-IgG was used as negative control. The binding activity of Sp1, Sp3 and Sp4 is graphically represented as the percentage of total input of chromatin DNA. *P<0.05 vs IgG. Each column represents the mean ± s.e.m (n=3).

IV.2. Sp1 and Sp3 have an opposite effect on *ncx1* luciferase activity, mRNA and protein expression

To evaluate the effect of Sp transcription factors on *ncx1* luciferase activity, mRNA and protein expression, neurons were silenced by specific siRNAs named siSp1, siSp3 and siSp4, that were able to decrease Sp1, Sp3 and Sp4 protein expression by 68%, 63% and 70%, respectively. In particular, for luciferase experiments we used a pGL3 construct contained the 5'-flanking region upstream of brain *ncx1* transcriptional start site (+1), named *short ncx1 promoter* (pGL3-ncx1), already used in a previously paper (Valsecchi et al., 2011), including the native Sp1 consensus sequences from B to E, present in the ncx1-Br (B) fragment. As shown in **Fig 12 a-c**, at 24 hours luciferase assay, Real Time PCR (qRT–PCR) and at 48 hours western blot analysis revealed that in neurons *ncx1* luciferase activity, transcript and protein decreased of 45%, 48% and 37% respectively with siSp1 and increased by 68%, 65% and 53% respectively with siSp3, whereas they did not modify by transfection of siSp4 or by co-transfection of double silencing for Sp1\Sp3, when compared to cells transfected with siCTL. To further confirm the role of Sp transcription factors in modulating NCX1 expression, neurons were transfected with constructs overexpressing Sp1, Sp3 and Sp4. that were able to increase Sp1, Sp3 and Sp4 protein expression by 69%, 74% and 64%, respectively. As shown in **Fig 12 d-f**, in neurons overexpressing Sp1, luciferase activity and mRNA levels at 24 hours and protein levels at 48 hours of *ncx1* were increased by 64%, 77% and 61% respectively, whereas cells overexpressing Sp3, luciferase activity and mRNA (24 hours) and protein levels (48 hours) were decreased by 76%, 58% and 42%, respectively. As expected Sp4 overexpression and co-transfection of Sp1 and Sp3 constructs did not cause any change of NCX1 expression. To identify the critical Sp1 sequences within *ncx1* promoter, the Sp1 binding sites B-E were mutated in the pGL3-ncx1 construct by site-direct mutagenesis in order to generate four different luciferase reporter constructs named Sp1 B mut, Sp1 C mut, Sp1 D mut and Sp1 E mut (**Fig.13 a**) and co-transfected with an expression plasmid of Sp1 or of Sp3 in cortical neurons (**Fig.13 b,c**). When Sp1 was overexpressed, the luciferase activity of Sp1 C

mut, Sp1 D mut, Sp1 E mut was significantly inhibited by almost 30% (**Fig.13 b**), whereas it was significantly increased by almost 35% when Sp3 was overexpressed (**Fig.13 c**), as compared with activity of pGL3-ncx1 in neurons overexpressing Sp1 or Sp3, respectively. For both the experimental conditions, mutation of Sp1 sequence B has no effect on *ncx1* transcriptional activity (**Fig.13 b,c**). Interestingly, in neurons overexpressing Sp1 or Sp3, mutation of all three Sp1 sites C, D, E was able to further reduce by 65% or increase by 85% *ncx1* promoter luciferase activity, compared to pGL3-ncx1 alone, respectively (**Fig.13 b,c**). Altogether, these results demonstrate that Sp1 and Sp3 by three specific sequences interact with ncx1-Br promoter, determining an increase or a reduction of its expression, respectively.

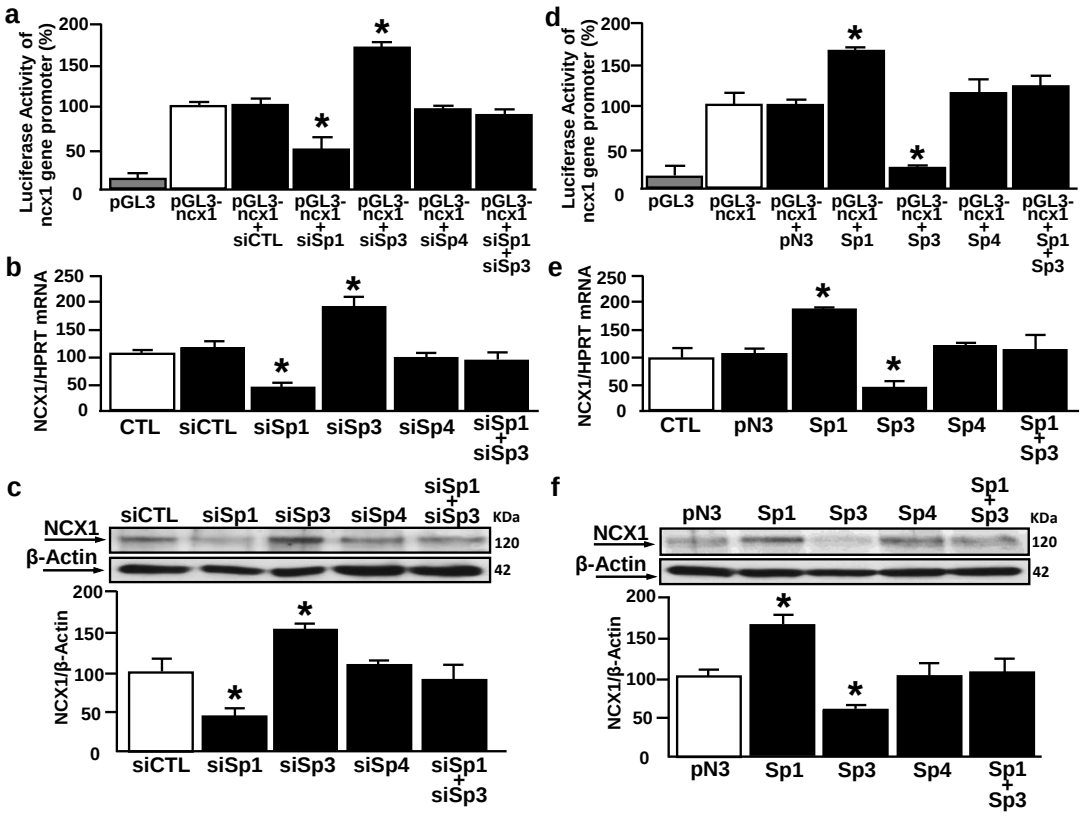

Figure 12. Sp1 is a transcriptional activator whereas Sp3 is a transcriptional repressor of *ncx1* in cortical neurons. (a-c) luciferase assay of ncx1-Br, qRT–PCR and representative WB with quantification of NCX1 in control conditions (CTL) and after treatment with siCTL, siSp1, siSp3, siSp4, and in combination of siSp1 and siSp3 (n=4). Each column represents the mean ± s.e.m. (*P<0.05 vs pGL3 ncx1, siCTL). **(d, f)** luciferase assay of ncx1-Br, qRT–PCR and representative WB with quantification of NCX1 in control conditions (CTL) and after after transfection with pN3 or overexpression of Sp1, Sp3, Sp4, and after Sp1 and Sp3 together (n=3). Each column represents the mean ± s.e.m. (*P<0.05 vs pGL3 ncx1, pN3).

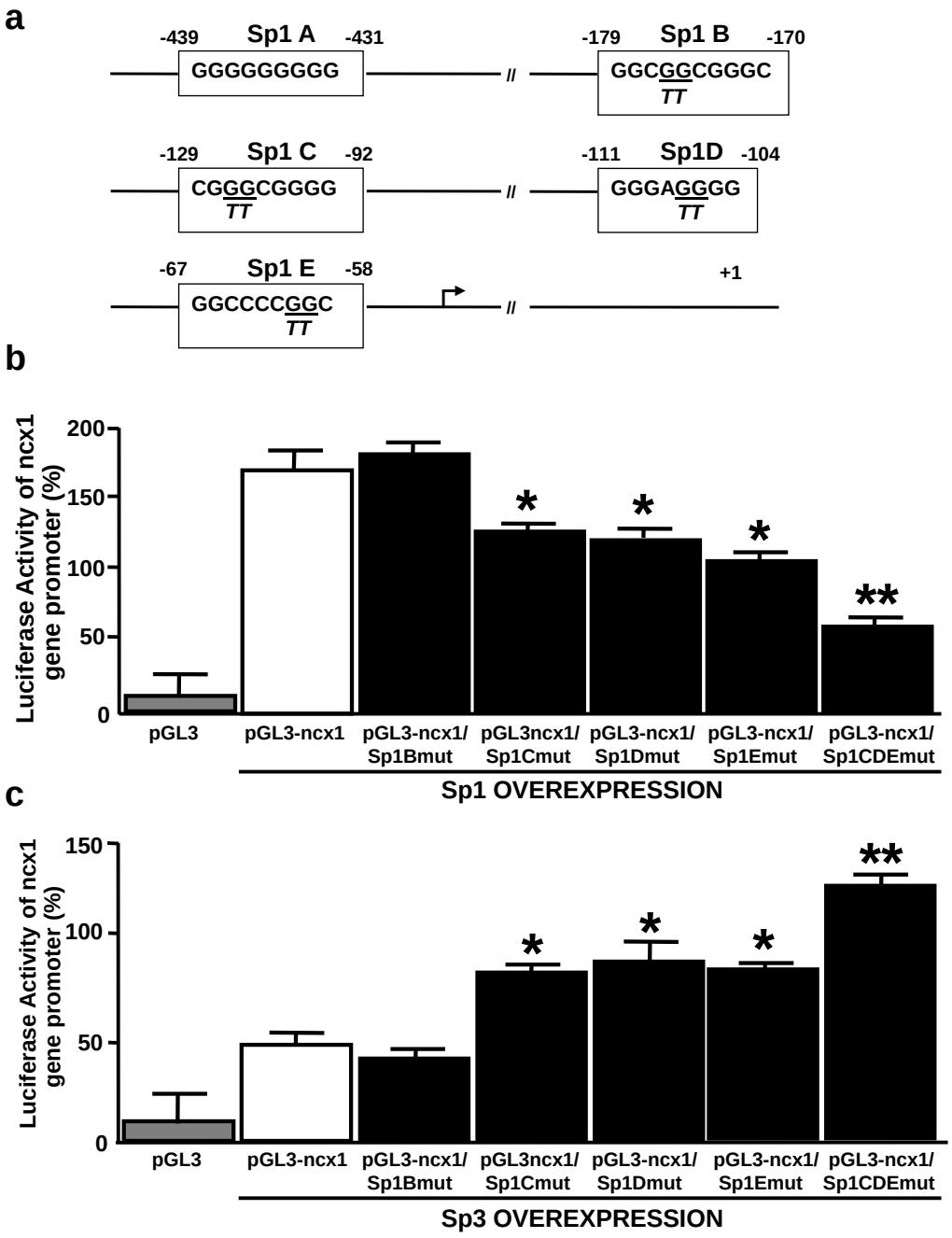

Figure 13. Effect of Sp1 and Sp3 on the transcriptional activity of the ncx1-Br after site-directed mutagenesis of the putative Sp1-binding sites. (a) The core consensus sequences of five putative Sp1 A–E sites are boxed and the locations relative to the reported transcription start site are separately numbered. Mutated nucleotides of the particular sequences are underlined and replaced nucleotides shown in italic below the wild-type sequences. The bent arrows indicate the reported transcription start site. **(b, c)** Cortical neurons transiently overexpressing Sp1 or Sp3 were co-transfected with pGL3-ncx1 or pGL3-ncx1/Sp1Bmut, pGL3-ncx1/Sp1Cmut, pGL3-ncx1/Sp1Dmut, pGL3-ncx1/Sp1Emut and pGL3-ncx1/Sp1CDEmut constructs. 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=4 independent experiments (*P<0.05 vs pGL3-ncx1, **P<0.05 vs all).

IV.3. REST\Sp3 and HIF-1\Sp1 complexes co-localize on *ncx1* brain promoter after tMCAO and PC+tMCAO, respectively

The identified Sp1 sequences on the ncx1 Br are near the binding sites of previously characterized *ncx1* transcriptional repressor REST, responsible for NCX1 decrease in cerebral ischemia (tMCAO) (Formisano et al., 2011) and of *ncx1* transcriptional activator HIF-1, responsible of NCX1 increase after ischemic preconditioning followed by tMCAO (PC+tMCAO) (Valsecchi et al., 2011). WB experiments were performed in the ipsilesional temporoparietal cortex of rats at 24 hrs after preconditioning (PC) alone, after tMCAO or PC+tMCAO, since REST, HIF-1 and NCX1 have already been investigated at this time point (Pignataro et al., 2011a; Valsecchi et al., 2011; Formisano et al., 2012). Interestingly, tMCAO induced a 44% reduction of NCX1, in parallel with an increase of HIF-1 (75%), REST (180%), and Sp3 (100%), but not of Sp1, when compared to the CTL (**Fig.14 a-c, e**). In addition, PC+tMCAO determined a remarkable increase of NCX1 by 140%, HIF-1 by 450% and Sp1 by 180%, compared to the CTL, whereas REST and Sp3 were reduced by 150% and by 154% respectively, compared to the cerebral ischemia values (**Fig.14 a-e**). Furthermore, PC+tMCAO-induced Sp3 decrease was also significantly reduced compared to the CTL (**Fig.14 e**). PC alone did not significantly modify the expression of REST, HIF-1, Sp1, Sp3 and NCX1, compared to the CTL (**Fig.14 a-e**). In all the experimental conditions Sp4 protein expression resulted not modified (**Fig.14 f**). Therefore, we evaluated the possible interaction of REST with Sp3 and of HIF-1 with Sp1, after tMCAO and PC+tMCAO, respectively. Interestingly, in rats subjected to cerebral ischemia, the binding to the ncx1-Br sequence was increased for RESTand Sp3 but not for HIF-1 and Sp1, compared to the CTL (**Fig.15 a-d**). On contrary, PC+tMCAO induced the binding of HIF-1 and Sp1 on ncx1-Br sequence, but not of REST and Sp3, compared to the to the cerebral ischemia alone (**Fig.15 a-d**). We further conducted re-ChIP assay to test whether the transcriptional repressors REST and Sp3 or the transcriptional activators HIF-1 and Sp1 co-localized on the ncx1 Br sequence during tMCAO and PC+tMCAO respectively. Re-ChIP assay showed that Sp3 with REST (**Fig.15e, lane 4**) and HIF-1 with Sp1 (**Fig.15f,**

lane 4) were bound together on the *ncx1* promoter after tMCAO, and after PC+tMCAO, respectively. These results suggest that Sp3 and REST form a functional complex on ncx1-Br sequence during tMCAO, whereas Sp1 and HIF-1 form another functional complex during PC+tMCAO.

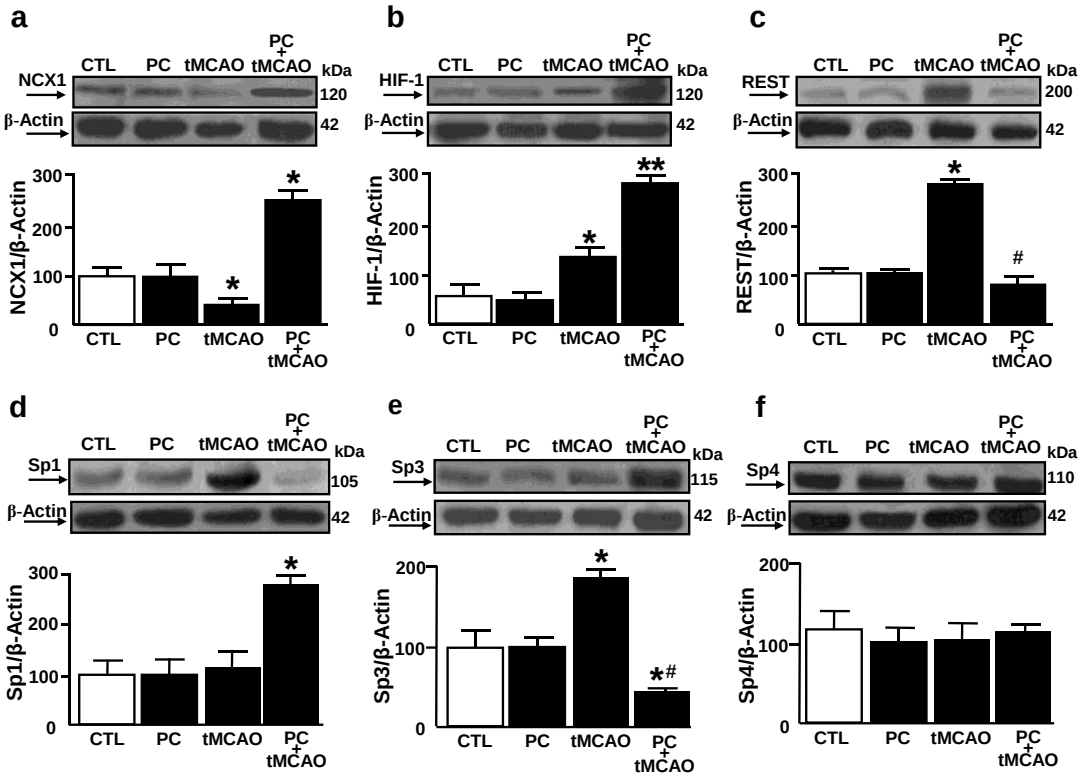

Figure 14. Effect of tMCAO and PC+tMCAO rat models on NCX1, HIF-1, REST, Sp1, Sp3 and Sp4 in the ipsilateral temporoparietal cortex. (a-f) Representative WB with quantification of NCX1, HIF-1, REST, Sp1, Sp3 and Sp4 protein expression in: (1) control group (CTL), (2) PC, (3) tMCAO and (4) PC+tMCAO.Each column represents the mean ± s.e.m. (n= 5 animals for each column) (*P<0.05 vs CTL, **P<0.05 vs all, $^{\#}$P<0.05 vs tMCAO).

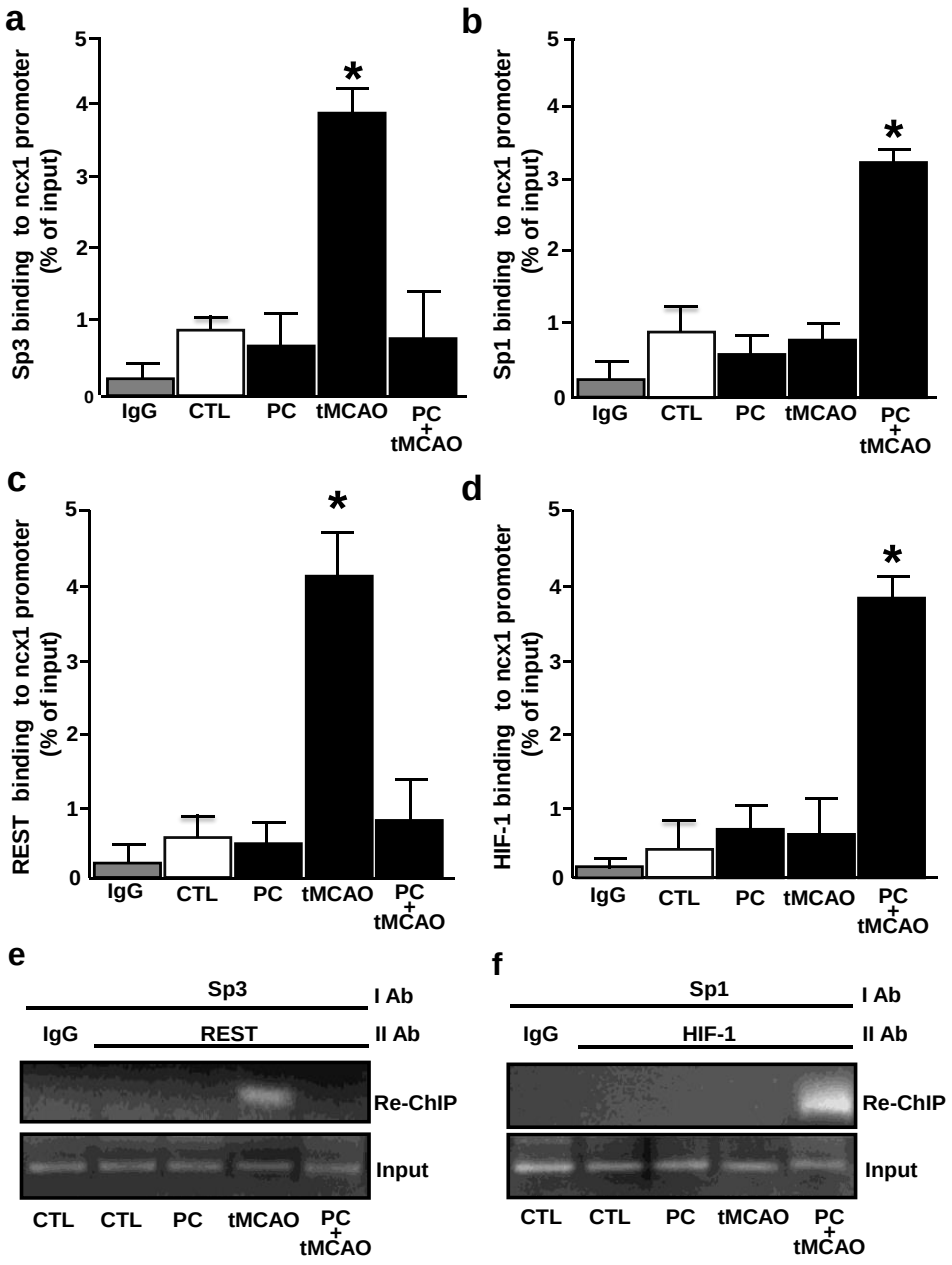

Figure 15. tMCAO and PC+tMCAO rat models promote REST/Sp3 and HIF-1/Sp1 colocalization on ncx1-Br in the ipsilateral temporoparietal cortex, respectively. (a-d) ChIP analysis of ncx1-Br in the ipsilateral temporoparietal cortex in: (1) CTL, (2) PC, (3) tMCAO and (4) PC+tMCAO. The binding activity of HIF-1, REST, Sp1 and Sp3 are graphically represented as the percentage of total input of chromatin DNA. Anti-IgG was used as negative control. Each column represents the mean ± s.e.m. (n= 6 animals for each column) (*P<0.05 vs CTL,). **(e-f)**Re-ChIP analysis of ncx1-Br in the ipsilateral temporoparietal cortex in: (1) CTL, (2) PC, (3) tMCAO and (4) PC+tMCAO. Primary ChIP products for anti-Sp3 and anti-Sp1 (I Ab) were subjected to re-ChIP with anti-REST, anti-HIF-1, respectively, or with

anti-IgG (II Ab). The input DNA lane represents 5% of the precleared chromatin used in each ChIP reaction. The figure is representative of two independent experiments.

IV.4. Epigenetic remodeling of ncx1-Br promoter by HDAC1 and HDAC2 after tMCAO and by p300 after PC+tMCAO, respectively

Since the transcription factors REST, HIF-1, Sp1, and Sp3 regulate their target genes by epigenetic modifications, such as acetylation (Won et al., 2002; Formisano et al., 2007; Azahri et al., 2012; Noh et al., 2012; Pawlus and Hu, 2013). We evaluated by ChIP assay the modification of the acetylated histone protein H3 and the binding of HDAC1, HDAC2, histone acetyltransferase p300 and RNA polymerase II (RNA-Pol II) on ncx1-Br promoter sequence in the ipsilesional temporoparietal cortex of the following groups: (1) control group (CTL), (2) PC, (3) tMCAO, (4) PC+tMCAO.As shown in **Fig.16a,b**, tMCAO reduced the abundance of *ncx1* promoter associated with acetylated H3 and RNA-Pol II in parallel with an increase of the binding of HDAC1 and HDAC2 (**Fig.16 c,d**), that are recruited by REST to promote gene silencing, compared to the CTL. During tMCAO, p300 binding on *ncx1* promoter was absent, as shown in **Fig. 16 e**. Interestingly, there was no HDAC1\2 binding on ncx1-Br when the animals were exposed to PC+tMCAO (**Fig.16 c,d**). In contrast, PC+tMCAO increased the abundance of ncx1-Br associated with acetylated H3, in parallel with an increase of RNA-Pol II and p300 binding, that is known to mediate the activation of HIF-1 target genes, compared to the CTL (**Fig.16 a, b, e**). To better understand the role of REST, Sp3 and HDAC1\2 on the tMCAO-induced NCX1 reduction, experiments of ChIP, qRT-PCR and WB were performed to evaluate the acetylation status of ncx1-Br, ncx1 gene and protein expression, by icv injection of siSp3, siREST, alone or in combination, and by double silencing for HDAC1\2. As shown in **Fig.17 a-c**, tMCAO-induced ncx1-Br deacetylation and ncx1 gene and protein decrease were reverted by the silencing of the all above mentioned proteins, compared to the CTL. Next, we studied the role of HIF-1, Sp1 and p300 in the PC+tMCAO-induced NCX1 increase by ChIP, qRT-PCR and WB analysis, after icv injection of siSp1, siHIF-1, alone or in combination, and by silencing for p300. As shown in **Fig.17 d-f**, PC+tMCAO-induced hyperacetylation of

histone H3 on ncx1-Br and *ncx1* gene and protein increase were reverted by knocking down with specific siRNA of Sp1, HIF-1, alone or in combination, and p300, compared to the CTL.

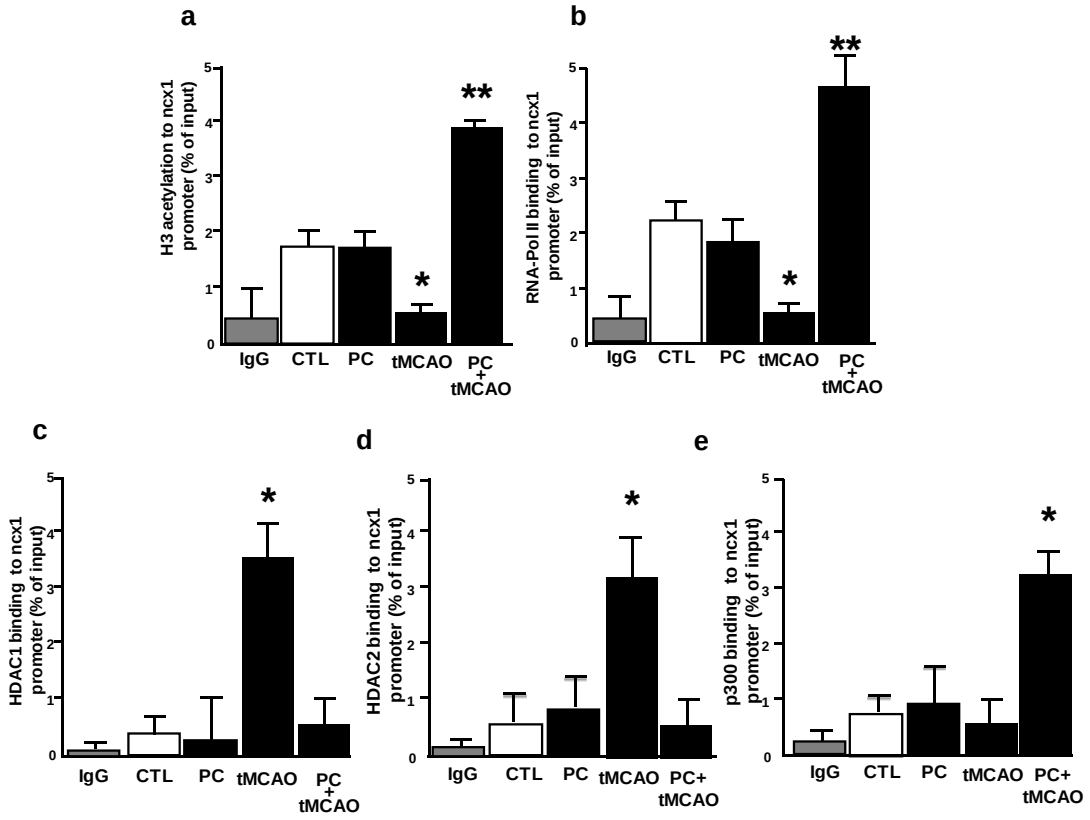

Figure 16. Effect of tMCAO and PC+tMCAO rat models on H3 acetylation, RNA-Pol II, HDAC1, HDAC2 and p300 binding to ncx1-Br. (a-e) ChIP analysis of ncx1-Br in the ipsilateral temporoparietal cortex in: (1) CTL, (2) PC, (3) tMCAO and (4) PC+tMCAO. The binding activity of H3 acetyl, RNA-Pol II, HDAC1, HDAC2 and p300 are graphically represented as the percentage of total input of chromatin DNA. IgG was used as negative control. Each column represents the mean ± s.e.m. (n= 6 animals for each column) (*P<0.05 vs CTL, **P<0.05 vs all).

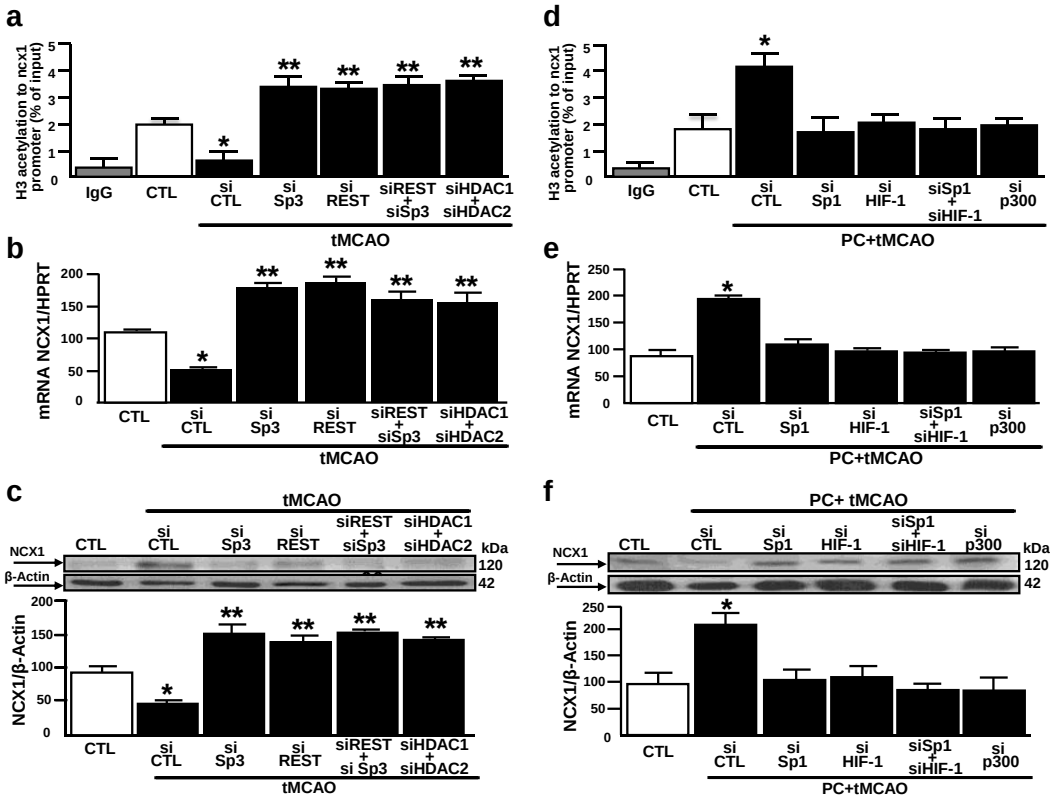

Figure 17. NCX1 is epigenetically modulated by Sp3/REST/HDAC1/2 complex in tMCAO and by Sp1/HIF-1/p300 in PC+tMCAO. (a) ChIP analysis with anti-H3 acetyl of ncx1-Br in CTL and in tMCAO after icv injection of siSp3, siREST, siREST+siSp3, siHDAC1+siHDAC2. IgG was used as negative control. Each column represents the mean ± s.e.m. (n= 5 animals for each column) (*P<0.05 vs CTL, **P<0.05 vs all). **(b, c)** qRT–PCR and representative WB with quantification of NCX1 in CTL and in tMCAO after icv injection of siCTL, siSp3, siREST, siREST+siSp3, siHDAC1+siHDAC2. Each column represents the mean ± s.e.m. of n=6 independent experiments (*P<0.05 vs CTL, **P<0.05 vs all). **(d)** ChIP analysis with anti-H3 acetyl of ncx1-Br in CTL and in PC+tMCAO after icv injection of siCTL, siSp1, siHIF-1, siSp1+siHIF-1 and sip300. IgG was used as negative control. Each column represents the mean ± s.e.m. (n= 6 animals for each column) (*P<0.05 vs CTL). **(e, f)** qRT–PCR and representative WB with quantification of NCX1 in CTL and PC+tMCAO after icv injection of siCTL, siSp1, siHIF-1, siSp1+siHIF-1 and sip300. Each column represents the mean ± s.e.m. (n= 6 animals for each column) (*P<0.05 vs CTL).

IV.5. A class I HDAC inhibitor MS-275, by increasing NCX1 expression during OGD/Rx, reduces neuronal cell death

Since HDAC1 and HDAC2 belong to class I histone deacetylases (Chuang et al., 2009a), we evaluated the effect of class I HDAC inhibitor MS-275, used at the concentration of 1μM in cortical neurons exposed to OGD/Rx (Lanzillotta et al., 2012). Firstly, the specificity of MS-275 to inhibit class I HDAC was validated by measuring the levels of acetylated forms of histone

H3 (indicating activity of class I) (Simonini et al., 2006; Galmozzi et al., 2013). Results showed that MS-275 increased the acetylation of histone H3 (**Fig.18 a**). As shown in **Fig.18 b**, MS-275 exerted a remarkable neuroprotective effect, by preventing cell death observed in OGD/Rx and siNCX1 (1µg) counteracted this neuroprotective effect. To identify the role of NCX1 in MS-275-induced neuroprotection, western blot analysis were performed for NCX1 in the same experimental conditions. Furthermore, exposure of cortical neurons to OGD/Rx+MS-275 induced an increase in NCX1 protein expression compared to the OGD/Rx+vehicle (**Fig.18 c**). As expected, MS-275-induced NCX1 increase was counteracted by siNCX1 (**Fig.18 c**).

IV.6. Hystone acetyl transferase p300 inhibitor C646 blocks PC-induced neuroprotection, by counteracting NCX1 increase

We investigated the hypothesis that PC exerts a neuroprotective effect in cortical neurons exposed to OGD/Rx via the increase of p300 and NCX1 expression. The specificity of C646 to inhibit Hystone acetyl transferase p300 was validated by measuring the levels of acetylated forms of histone H3 (Federman et al., 2013). Results showed that C646 decreased the acetylation of histone H3 (**Fig.18 d**). As shown in **Fig.18 e**, in neurons exposed to PC+OGD/Rx, the p300 inhibitor C646 at 20 µM (Min et al., 2010) was able to reduce cell survival compared to PC+OGD/Rx+vehicle. Interestingly, NCX1 overexpression was able to counteract C646-induced cell death, compared to PC+OGD/Rx+vector (**Fig. 18 e).** To confirm the role of NCX1 in PC-induced neuroprotection, experiments of WB were performed to analyze NCX1 expression. As shown in **Fig. 18 e**, C646 prevented the increase of NCX1, in cortical neurons exposed to PC+OGD/Rx and this effect was counteracted by NCX1 overexpression.

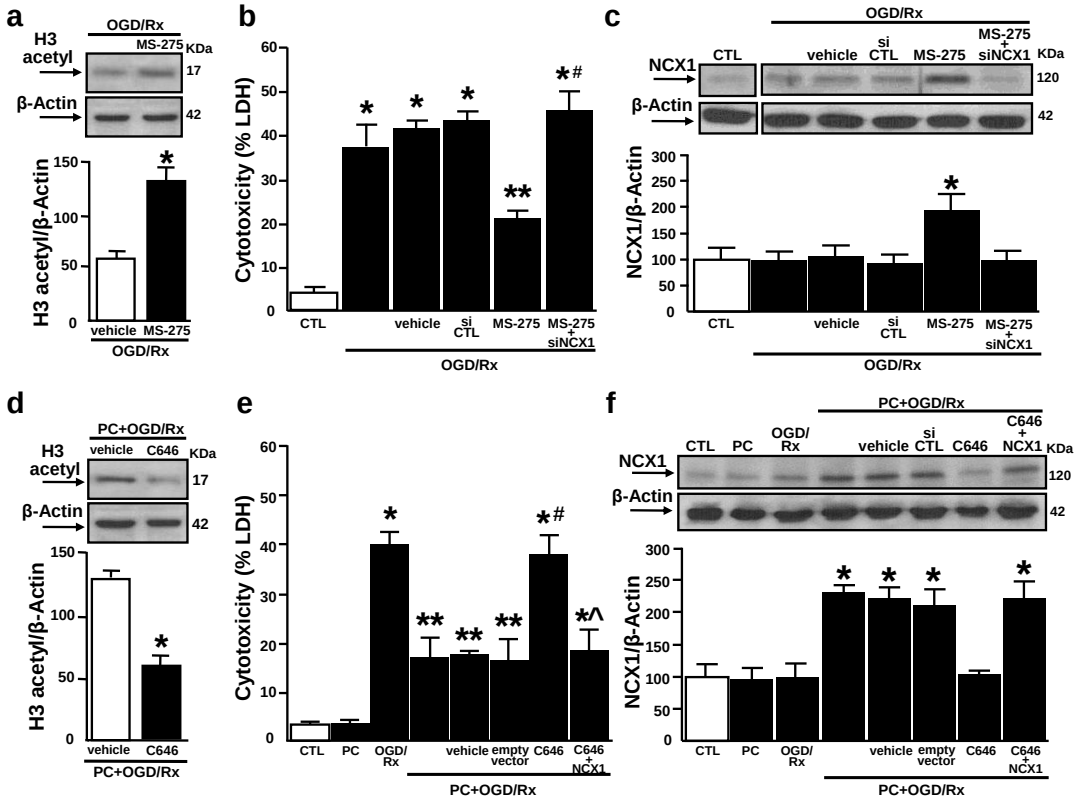

Figure 18. Effect of class I HDAC inhibitor MS-275 and of HAT p300 inhibitor C646 on cell survival and NCX1 protein expression in cortical neurons exposed to OGD/Rx or to PC+OGD/Rx, respectively. (a) representative WB with quantification of H3 acetyl in neurons subjected to OGD/Rx alone or with MS-275. Each column represents the mean ± s.e.m. of n=3 independent experiments (*P<0.05 vs OGD/Rx). **(b, c)** LDH assay and NCX1 protein expression with quantification in (1) control condition (CTL), (2) OGD/Rx (3) OGD/Rx+vehicle, (4) OGD/Rx+siCTL, (5) OGD/Rx+MS-275 (1μM), (6) OGD/Rx+MS-275+siNCX1. Each column represents the mean ± s.e.m. of n=4 independent experiments for LDH assay, and n=3 for WB. (*P<0.05 vs CTL, **P<0.05 vs all, $^{\#}$P<0.05 vs OGD/Rx+MS-275). **(d)** representative WB with quantification of H3 acetyl in neurons subjected to PC+OGD/Rx alone or with C646. Each column represents the mean ± s.e.m. of n=3 independent experiments (*P<0.05 vs OGD/Rx). **(e, f)** LDH assay and NCX1 protein expression with quantification in (1) CTL, (2) PC, (3) OGD/Rx, (4) PC+OGD/Rx, (5) PC+OGD/Rx+vehicle, (6) PC+OGD/Rx+Empty vector, (7) PC+OGD/Rx+C646, (8) PC+OGD/Rx+C646+NCX1. Each column represents the mean ± s.e.m. of n=3 independent experiments for LDH assay, and n=3 for WB. (*P<0.05 vs CTL, **P<0.05 vs all, $^{\#}$P<0.05 vs PC+OGD/Rx alone or with vehicle and empty vector, $^{\wedge}$P<0.05 vs PC+OGD/Rx+C646).

Discussion

V. DISCUSSION

This study has evidenced for the first time that NCX1 expression is epigenetically down-regulated in brain ischemia by Sp3\REST complex and up-regulated in brain ischemic preconditioning by Sp1\HIF-1 complex. Particularly, our results suggest that Sp3 with REST and Sp1 with HIF-1 form a functional complex on ncx1-Br sequence in brain ischemia and in brain ischemic preconditioning, respectively. In fact, we showed that REST\Sp3-induced NCX1 down-regulation after tMCAO and HIF-1\Sp1-induced NCX1 up-regulation after PC+tMCAO are associated to their expression, since icv injection of siREST and siSp3, alone or in combination, in brain ischemia and of siHIF-1 and siSp1, alone or in combination, in brain ischemic preconditioning completely reverts NCX1 mRNA and protein decrease or increase, respectively. On the other hand, a cross-talk between Sp1, Sp3 and REST to regulate their target genes has been already proposed. In particular, Sp1 acts as an activator for the expression of REST target genes (Plaisance et al., 2005), while Sp3 by binding GC box interacts with REST to repress the mu opioid receptor expression (Kim et al., 2006). Furthermore, hypoxic condition induced expression of Regulated in development and DNA damage response 1 (Redd1), that is mediated by coactivation of Sp1 and HIF-1 (Jin et al., 2007). Interestingly, in the present paper we have identified five Sp1 motifs in the rat ncx1-Br, located at -439, -179, -129, -111, -67 from the TSS, named Sp1 A-E, as the possible molecular determinants involved in Sp1 isoforms-induced *ncx1* regulation. Furthermore, we found that among the family of transcription factors Sp, only Sp1 and Sp3, but not Sp4, bind to ncx1-Br in a very selective manner, as revealed by ChIP analyses. Our results are in line with data showing that Sp1 and Sp3 regulate target genes transcription via binding to identical cognate DNA elements with similar affinities (Hagen et al., 1992). Indeed, transfection in cortical neurons of specific siRNAs for Sp1 and Sp3 produced, respectively, a decrease or an increase in the *ncx1* promoter activity, mRNA and protein expression and conversely, combined transfection of siRNAs of Sp1\Sp3 did not induce any change in *ncx1* promoter activity, mRNA and protein expression. These results demonstrate that Sp1 is an activator while Sp3 is a repressor of *ncx1*

transcription, a mechanism also reported for other Sp1 isoforms gene target (Stains et al., 2003). Notably, in the present paper we identified three Sp1 binding sites (C-E) on ncx1-Br that show the highest promoter activity and found that mutation analysis at the level of Sp1C-E sites in neurons overexpressing Sp1 and Sp3 significantly reduces or increases the activity of *ncx1* brain promoter, respectively, whereas Sp1-B mutation has no effect. Collectively, these results show that three of the five Sp1 elements present on ncx1-Br sequence modulate the promoter activity of the *ncx1* gene. It is known that global transcriptional repression upon ischemia is similarly reflected on the decrease of histones acetylation, and that HDAC inhibition reduces ischemia-induced alterations in HDAC expression, by determining neuroprotection (Abel and Zukin, 2008; Noh et al., 2012). Data emerging from our study seem to suggest that both REST during ischemia and HIF-1 during preconditioning interfere with NCX1 gene expression through the participation of HDAC1, HDAC2 and p300. In particular, in brain ischemia by ChIP analysis we found that REST and Sp3 together with the corepressors HDAC1 and HDAC2 are recruited to the ncx1-Br decreasing H3 acetylation and RNA-Pol II binding and determining a reduction of *ncx1* mRNA and protein expression. Regarding the relationship between HIF-1 and p300 during preconditioning, our results showed that PC+tMCAO increases the abundance of ncx1-Br associated with acetylated H3, in parallel with an increase of RNA-Pol II, HIF-1 and p300 binding, determining an increase of NCX1 mRNA and protein expression. All these results together suggest that the complex REST\Sp3\HDAC1,2 decreases NCX1 expression during brain ischemia, whereas the complex HIF-1\Sp1\p300 increases the expression of this gene during preconditioning. Interestingly, this regulation regards exclusively NCX1 isoform, but not NCX3 isoform, that is also involved in the mechanisms of brain ischemia and of ischemic brain preconditioning (Pignataro et al., 2011a). Indeed, changes in NCX3 mRNA and protein expression were not caused by REST\Sp3\HDAC1,2 complex in brain ischemia and by HIF-1\Sp1\p300 complex in ischemic brain preconditioning. Since it has been widely demonstrated by our group that NCX1 expression is involved in the pathophysiology of brain ischemia and in the protection

exerted by preconditioning, we investigated the role of HDAC1,2 and p300 on NCX1-induced neuroprotection in neurons subjected to OGD\Rx, a model resembling brain ischemia, and to PC+OGD\Rx. The results of these experiments showed that HDAC1-2 inhibitor MS-275-induced neuroprotection is completely abolished in OGD\Rx by silencing of NCX1, thus further supporting the tight relationship existing between HDAC1-2 and NCX1 in the pathophysiological processes leading to stroke. These results are in accordance with Noh et al. (2012) showing that HDAC1 and 2 are recruited to the AMPA receptor subunit GluA2 promoter sequence, and that injection of pan-HDAC inhibitor thrichostatin A, an inhibitor of key components of the REST–corepressor complex, HDAC1 and HDAC2, administered to animals after an ischemic episode, ameliorates neuronal injury. In addition, p300 inhibitor C646-induced cell death is completely reverted in PC+OGD\Rx by NCX1 overexpression, thus suggesting a close relationship between the histone acetyltransferase p300 and NCX1 in the ischemic preconditioning. In light of all these findings, the development of drugs that epigenetically regulate NCX1, through the prevention of its down-regulation in stroke, or that increase its expression, as occured in PC+tMCAO, might be a new pharmacological strategy to ameliorate neuronal damage during brain ischemia.

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