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Sodium/calcium exchanger as main effector of endogenous neuroprotection elicited by ischemic tolerance

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ABSTRACT

The ischemic tolerance (IT) paradigm represents a fundamental cell response to certain types or injury able to render an organ more "tolerant" to a subsequent, stronger, insult. During the 16th century, the toxicologist Paracelsus described for the first time the possibility that a noxious event might determine a state of tolerance. This finding was summarized in one of his most important mentions: "*The dose makes the poison*". In more recent years, ischemic tolerance in the brain was first described in 1991, when it was demonstrated by Kirino and collaborators that two minutes of subthreshold brain ischemia in gerbils produced tolerance against global brain ischemia.

Based on the time in which the conditioning stimulus is applied, it is possible to define ***preconditioning***, ***perconditioning*** and ***postconditioning***, when the subthreshold insult is applied before, during or after the ischemic event, respectively. Furthermore, depending on the temporal delay from the ischemic event, two different modalities are distinguished: rapid or delayed preconditioning and postconditioning. Finally, the circumstance in which the conditioning stimulus is applied on an organ distant from the brain is referred as ***remote conditioning***.

Over the years the "*conditioning*" paradigm has been applied to several brain disorders and a number of molecular mechanisms taking part to these protective processes have been described. The mechanisms are usually classified in three distinct categories identified as *triggers*, *mediators* and *effectors*. As concerns the putative effectors, it has been hypothesized that brain cells appear to have the ability to adapt to hypoxia by reducing their energy demand through modulation of ion channels and transporters, which delays anoxic depolarization. The purpose of the present review is to summarize the role played by plasmamembrane proteins able to control ionic homeostasis in mediating protection elicited by brain conditioning, particular attention will be deserved to the role played by **Na^+/Ca^{2+} exchanger**.

1. Introduction

The brain is constantly subjected to the influence of intrinsic and extrinsic stimuli. During stressful and pathological conditions brain is able to activate endogenous neuroprotective strategies able to preserve its integrity. Among these neuroprotective strategies, preconditioning has been widely studied in the last decades for the opportunity to identify protective pathways to be translated into druggable targets. Preconditioning (PC) represents a strategy in which an injurious stimulus applied at a sub-toxic level before a longer harmful ischemia [1–3] exerts a remarkable neuroprotection, thus establishing a state of tolerance to anoxic conditions [4]. It is well-known that PC protection can be activated into two different phases: "rapid or acute preconditioning" [5] (Fig. 1). While "rapid preconditioning" starts 3–5 min after the preconditioning stimulus and ends up 1 h later; "delayed preconditioning" [5], begins 2–3 days after preconditioning and ends up 1 week later [2]. Rapid preconditioning induces a transient and less strong neuroprotection than delayed preconditioning, in addition, rapid preconditioning is not associated with de novo protein synthesis [2,6]. Conversely, delayed preconditioning is associated with longer molecular changes [7], including the de-novo synthesis of proteins, the induction of transcription factors, the activation of anti-apoptotic and

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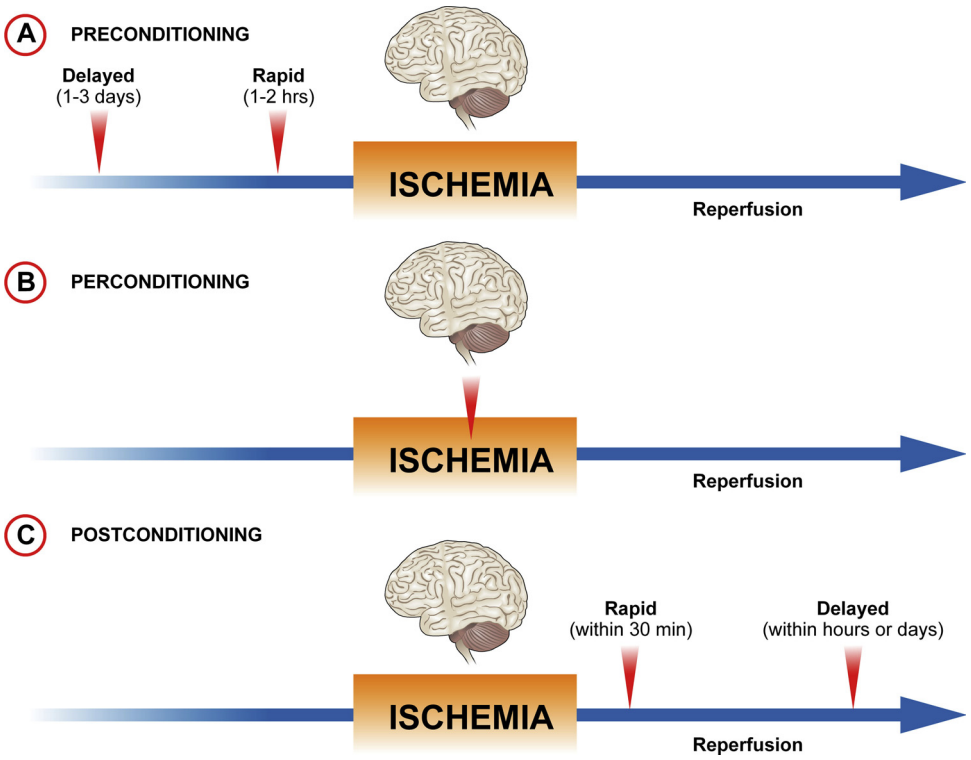

Fig. 1. Diagram showing when to apply the sub-toxic stimulus in order to obtain (a) preconditioning, (b) perconditioning and (c) postconditioning.

antioxidant proteins [8,9]. The mechanisms through which endogenous protective phenomena, such as preconditioning, mediate their effects support an evolutionarily conserved endogenous response to decreased blood flow and oxygen limitation such as seen during hibernation [3].

2. Ionic homeostasis and brain conditioning

As concerns the putative effectors of brain conditioning neuroprotection, in 2008 Obrenovich hypothesized that brain cells appear to have the ability to adapt to hypoxia by reducing their energy demand through modulation of ion channels and transporters, which delays anoxic depolarization [10]. In fact, it has been demonstrated that preconditioning reprograms the response to ischaemic injury by triggering the change in the expression of a significant number of genes coding for proteins mainly involved in the maintenance of ionic homeostasis within CNS cells [11]. This assumption derives from studies carried out since 1990 firstly in a model of ischemic preconditioning in the heart and later on in the brain [12]. At that time, it was observed that during heart ischemia, ATP and phosphocreatine (PCr) declined, whereas intracellular hydrogen ion, Na^+, Ca^{2+} and Mg^{2+} concentrations all rise. If the ischemia is relatively short, thus representing a subthreshold insult, PCr, pH, $[Na^+]_i$, $[Mg^{2+}]_i$ and $[Ca^{2+}]_i$ are restored quickly on reperfusion and cell damage is reversible. Indeed, it is well established that a sustained rise in $[Na^+]_i$ and $[Ca^{2+}]_i$ during ischemia and/or lack of recovery during reperfusion is associated with irreversible cell injury. Interventions that reduce the rise in $[Na^+]_i$ and $[Ca^{2+}]_i$ during ischemia and reperfusion have been shown to reduce cell death [13]. Consequently, ion gradients are very sensitive to the impairment of energy metabolism [14]. During physiological conditions, in the brain are activated homeostatic mechanisms that maintain steady-state ion levels in the cerebrospinal fluid and CNS cells, in order to preserve relatively constant the ionic concentrations both inside and outside the membrane, with fluctuation in a small range. However, in different pathophysiological conditions such as during brain ischemia, due to energy depletion, this homeostatic balance is altered, thus determining important changes in ionic concentrations that are characterized by enhanced K^+ efflux and Na^+, Ca^{2+} and Cl^- influx [15–20].

Concerning ionic transporters mediating this noxious effect, Murry et al. reported that, after 40 min of heart ischemia, lactate production in preconditioned hearts was only 60 % compared to non-preconditioned heart. This reduced production of lactate would likely result in less intracellular acidification. The decreased intracellular acidification, in turn, would lessen the rise in $[Na^+]_i$ via Na^+-H^+ exchange and subsequently the rise in $[Ca^{2+}]_i$ via Na^+-Ca^{2+} exchange. Therefore, starting from the above mentioned studies in the heart, experiments conducted in models of cerebral ischemia and in transgenic animals have established that the pharmacological modulation of specific membrane transporters, involved in the control of Na^+, Ca^{2+} and K^+ homeostasis, might represent a promising therapeutic strategy not only to limit the magnitude of the damage, but also to counteract ischemia-induced neurodegeneration. Brain ischemic preconditioning is able to activate a series of cellular responses that may improve the metabolic efficiency of CNS cells subjected to this stressing stimulus thus representing the way through which the organs strengthen their ability to face a far more damaging insult [21]. Neurons subjected to preconditioning showed an inhibition of the deleterious increase in intracellular calcium concentration following post-ischemic anoxia and hypoglycemia, thus counteracting Ca^{2+} overload observed in stroke [22].

3. NCX and brain ischemia

Na^+/Ca^{2+} exchanger, NCX, by regulating the homeostasis of Na^+ and Ca^{2+}, plays a key role in the evolution of ischemic neuronal damage [23]. Numerous studies have shown that, in the course of hypoxia in vitro and ischemia in vivo, NCX contributes to modulating the extent of neuronal damage. In particular, blocking NCX activity worsens cellular damage induced by ischemia [23–26]. Evidence that NCX is actually involved in the progression of ischemic damage has also been corroborated by experimental studies on brain tissue from ischemic rats which showed a variation of the expression of the three NCX isoforms at different time intervals after induction of brain ischemia [24,27]. From

these studies it emerged that among the three NCX isoforms only NCX1 and NCX3 are actually involved in the progression of ischemic damage. In details, the knocking-down of the NCX1 and NCX3 isoforms by antisense oligonucleotides selective for each isoform significantly worsens ischemic damage in an in vivo model of focal cerebral ischemia [24]. Further evidence for NCX involvement in ischemic damage derives from the demonstration that genetic deletion of the ncx3 gene in mice determines a dramatic enlargement of the ischemic lesion, mirrored by a worsening in neurological scores [25]. Furthermore, cortical neurons obtained from ncx3 -/- mice are more vulnerable to a hypoxic event and show basal levels of intracellular calcium greater than neurons obtained from wild type mice [25,28]. This concept has been further underlined by the results recently obtained with the new NCX activator named neurounina-1 [28]. In particular, neurounina-1 exerts a remarkable neuroprotective effect both in in vitro and in in vivo experimental models of cerebral ischemia likely by enhancing NCX activity. The effect of neurounina-1 on brain damage evolution has been recently confirmed also in a neonatal hypoxic-ischemic (HI) model of brain ischemia [29]. In particular, it has been demonstrated that a systemically administration of neurounina compound, until 7 h after HI induction, was able to reduce ischemic area in the ipsilateral hemisphere of HI mice compared to vehicle treated animals. In addition, it has been showed that chronically administered neuronunina was able to prevent the reduction of hippocampal neurons, evaluated one month later, thus exhibiting a time window potentially translatable in clinical practice. In accordance with morphological analysis it has observed that the systemic administration of neurounina prevented motor and memory deficit in adult mice subjected to neonatal hypoxic-ischemic injury [29]. Although the majority of the studies have focused mainly on the role of NCX1 and NCX3 in cerebral ischemia, for the observation of a constant downregulation in all ischemic regions (cores and peri-infarct regions), a mention must also be made on the role of NCX2. Indeed, this NCX isoform along with the previous ones is necessary to remove Ca^{2+} after ischemic insult working as Na^+ influx–Ca^{2+} efflux whereby the lack of NCX2 resulted in an increased infarct volume and increased neuronal loss in a model of transient focal cerebral ischemia [30]. Overall, the above described results support the idea that activation of NCX might contribute to the maintenance of Na^+ and Ca^{2+} homeostasis, thus preserving cell vitality in the penumbra region, where NCX would continue to operate in “forward mode”, mediating the extrusion of Ca^{2+} and the internalization of Na^+ [31,32,29]. In a complementary way, recent reports indicated that neuroprotective effects of NCX activity in stroke models might be related to its dampening of ischemia-induced sodium loading by operating in its “reverse mode” [33]. Therefore, it is possible to speculate that the activation of NCX would protect the hypoxic-ischemic brain by contributing to the maintenance of Na^+ and Ca^{2+} homeostasis in the ischemic core and in the penumbra [29]. Although the evidence regarding the protective role of NCX in cerebral ischemic pathology is quite robust, it is appropriate to report some opposite results [34]. In particular Jing Luo and colleagues evidenced a discrepancy about the non-noxious effects of silencing NCX1, NCX2 and NCX3 between an in vivo model of brain ischemia and in vitro neuronal cultures subjected to hypoxia. Authors affirm that this difference might be related to different factors, above all the dilution of the signal of NCX expression in the affected ipsilateral brains by sampling together mixed cell types and ischemic and nonischemic brain regions [35]. Furthermore, the potential protective effect described after brain ischemia in absence of NCX1 was observed only in conjuction with the genetic ablation of NKCC1, thus hypothesizing a concerted activity of these two plasmamebrane transporters [35]. These data suggest that NKCC1 in conjunction with NCX1 plays a role in reperfusion-induced brain injury after ischemia.

3.1. NCX in astroglia

All three NCX isoforms are present in astrocytes at the level of distal processes surrounding synapses [68,69]. Among them, NCX1 isoform transcripts are the most highly represented in astrocytes [70]. Recent papers demonstrated the importance of NCX in the regulation of astrocytes excitability throught Na^+ and Ca^{2+} transmembrane movements [71,72]. It is reported that NCX in astrocytes can operate in its reverse mode in response to small increase in $[Na^+]i$ and/or depolarization [73]. In effect, the largest Na^+ increase following in vitro ischemia is observed during the reoxygenation phase; this Na^+ load causes reversal of NCX and cellular Ca^{2+} influx eventually resulting in mitochondrial Ca^{2+} accumulation. This mechanism in turn causes the opening of the mitochondrial permeability transition pore and cell death [74]. Furthermore, Ca^{2+} entry through NCX causing the elevation of cytoplasmic $[Ca^{2+}]i$ leads to an induced Ca^{2+}-dependent exocytotic release of glutamate from astrocytes [73].

3.2. NCX in microglia

In 2009 Boscia and collaborators demonstrated that Na+-dependent Ca2+ influx through NCX operating in the reverse mode is necessary for microglial activation under ischemic conditions. In particular, immunoreactivity signal for NCX1 was found progressively increased in microglia/macrophages invading the infarct core at 3 and 7 days after pMCAO. Interestingly, in BV2 microglial cells exposed to OGD, NCX1 silencing completely prevented the intracellular Ca^{2+} rise triggered by hypoxia, further demonstrating that enhanced NCX, operating in the reverse mode, is responsible for the increase in $[Ca^{2+}]i$ observed following OGD-Reoxygenation in microglia [75].

4. Preconditioning and Na^+/Ca^{2+} exchange

In accordance to findings indicating a fundamental role for NCX in stroke progression, recent studies have reported that NCX1 and NCX3 play a neuroprotective role during ischemic preconditioning (HPC). In fact, an increased expression of these two isoforms was observed during preconditioning and a prevention of the neuroprotective effect induced by preconditioning was reported in animals lacking NCX1 or NCX3 [4].

The protective function of NCX1 and NCX3 is certainly linked to their ability to restore the dysregulation of the intracellular homeostasis of sodium $[Na^+]_i$ and calcium $[Ca^{2+}]_I$ occurring after an ischemic event.

Regarding the transcriptional factors involved in the control of the gene regulation of the NCX isoforms during preconditioning, a key role seems to be played by the Hypoxic Inducible Factor, HIF. Interestingly, in human pluripotent stem cells (PSCs) it has been demonstrated that hypoxic preconditioning and oxidative stress induced anti-apoptotic mechanisms through the stabilization of the transcription factor HIF-2α, which inhibits p53 and increased Bcl-2 expression [36,37]. While, in mesenchymal cultured stem cells (MSCs) hypoxic preconditioning promoted HIF-1α stabilization, which is normally degraded by HIF prolyl-4-hydroxylases in normoxia conditions [37,38]. HIF-1α regulates the activity of glycolytic enzymes and inhibits the pyruvate dehydrogenase, these events induce the shift of metabolism from oxidative phosphorylation to glycolysis, producing ROS decrease [37]. Therefore HIF-1α is a necessary component of the protective mechanisms involved in HPC. Indeed, during hypoxic condition, HIF-1α translocates from the cytoplasm to the nucleus, where it dimerizes with HIF-1β, generating the heterodimeric complex (HIF-1α and HIF-1β), able to bind to hypoxia response element (HRE) in the 3′ enhancer of the gene for erythropoietin, finally inducing the increase of expression of Epo gene [39,40]. The increase of Epo expression stimulates the rapid expansion of erythroid progenitors. Conversely, during normoxic conditions, HIF-1α is rapidly degraded by prolyl-hydroxylase. The Epo acts in bone marrow hematopoietic framework by stimulating the colony forming unit-erythroid (CFU-E), reducing apoptosis and increasing the frequency of mitosis (faster differentiation in proerythroblast). It is well known that in humans and murine brain, during both fetal development

and adult life, a local production of Epo occurs [41,42]. Epo binds to its specific receptor, Erythropoietin receptor (EpoR), expressed on the surface of the membranes. EpoR is a transmembrane glycoprotein, that is a component of cytokine type I receptor superfamily; it is expressed on erythroid cells but also on the different brain cells, such as glial cells [43], neurons [44], endothelial cells [45], internal capsule and midbrain of different brain regions, such as hippocampus, cortex [46]. The crucial role of HIF-1α in the neuroprotective mechanisms mediated by HPC was also investigated by Sheldon et al., that demonstrated a worsening in brain damage occurring in HIF-1α knockout mice subjected to hypoxic-ischemic injury [47]. Therefore, the ablation of HIF-1α determined the repression of the neuroprotective effect triggered by HPC [47]. In a similar manner, the ablation of HIF-1α gene prevented the neuroprotective effect of ischemic preconditioning in ischemic rats [47,48]. Notably, Valsecchi et al. investigated the relationship between HIF-1 and NCX1 protein, in in *vitro* and in *vivo* model of ischemia. In this study the authors demonstrated that the NCX1 protein can be included in the family of those genes activated by the transcription factor HIF-1. They observed a significant HIF-1 translocation into the nucleus and great upregulation of HIF-1 induced by OGD or CoCl2 exposure and by brain ischemic preconditioning, with a consecutive increase of NCX1 transcript and protein, which, in turn, was accompanied by a relevant neuroprotective effect [48]. It was proven that the silencing of HIF-1 abolished NCX1 protein upregulation in brain rats subjected to ischemic preconditioning, determining a partially increase of infarct volume in these animals [48]. These results demonstrated that ncx1 gene is a novel HIF-1 target and that HIF-1 exerts its pro- survival role also through NCX1 up-regulation during brain preconditioning through the interaction between HIF-1 and NCX1 promoter [48].

In 2015, the mechanism of epigenetic regulation of sodium/calcium exchanger isoform 1 (NCX1), mediated by two functional protein complexes REST/Sp3/HDAC1/HDAC2 and HIF-1/Sp1/p300 was elucidated [49,50]. In particular, whereas the former downregulates NCX1 expression during brain ischemia, the latter upregulates it during preconditioning. Notably, the development of drugs that epigenetically regulate NCX1 by preventing its downregulation in stroke might be a new pharmacological avenue to ameliorate neuronal damage during brain ischemia [49,50].

The pivotal role played by NCX during preconditioning has been further highlighted in other papers. In particular, Sisalli et al., demonstrated in primary cortical neurons, that IPC increased NCX1 and NCX3 protein expression 48 h after OGD plus reoxygenation induction and this effect was mediated by NO production and PI3K/Akt activation. More in detail, it has been reported that the upregulation of NCX1 and NCX3 activity mediated the endoplasmatic reticulum refilling and mitochondrial calcium extrusion, thus preventing intracellular calcium dysregulation induced by OGD [51]. In preconditioned brain was also observed an increase of AKT phosphorylation (pAKT) protein levels [52]. Moreover, by confocal immunofluorescence analysis it was observed that the temporoparietal brain cortex of preconditioned rats showed a greater increase in the expression levels of p-AKT, NCX1 and NCX3 when compared with the same brain region of rats subjected only to 100 tMCAO. This finding was confirmed by the close relationship existing between NCX1, NCX3 and p-AKT found in co-localization experiments, in which the greater expression of these three proteins occurred in the same brain cells; this was the proof of the existence of a causal link between them. Overall, these evidences have supported the importance of p-AKT in mediating the beneficial effects of preconditioning and suggested that NCX1 and NCX3 are indeed two additional mediators downstream of p-AKT involved in the neuroprotective process of ischemic preconditioning.

It is interesting to note that the activation of the abovementioned mechanisms, mediated by HIF-1 and by pAKT seems to be of long duration, since the upregulation of NCX1 and NCX3 was still present even after 72 h from the induction of preconditioning, thus indicating NCX1 that NCX3 as two possible effectors of delayed preconditioning [48].

Neuronal calcium buffering during ischemic conditioning seems widely recognized as the way by which brain can counteract the deleterious effects of a stroke not only directly through NCX, but also through other players. Among proteins involved in this phenomenon, Ca^{2+}-ATPase (PMCA) and Store operated-calcium entry (SOCE) play a central role. The plasma membrane Ca^{2+}-ATPase (PMCA) is a transport protein that functions removing calcium from the cell, cooperating with Na^{+}/Ca^{2+} exchanger, and represents the only high affinity Ca^{2+} transporting system on the plasmamembrane. Differently from other plasma membrane buffering systems, such as NCX and ATPases, plasma membrane Ca^{2+}-ATPase may export intracellular Ca^{2+} even with relatively lower transport capacity during prolonged membrane hyperpolarization and during conditions of increased intracellular Na^{+} concentrations, as occurred under ischemic conditions. Ohta and collegues observed that ischemia-tolerant CA1 neurons, in a model of bilateral common carotid arteries occlusion in gerbils characterized by a pre-exposure to 2-min ischemia, export more efficiently a larger amount of Ca^{2+} in response to stroke [21,53,22]. Recently, Secondo and coworkers demonstrated that the disturbance of Ca^{2+} homeostasis in the endoplasmic reticulum (ER) represents a common denominator of neuronal cell injury in many neurological disorders, including cerebral ischemia. Upon depletion of Ca^{2+} store, STIM senses Ca^{2+} level reduction and migrates from ER-like sites to the PM where it activates Orai [54]. Conversely, by restoring ER Ca^{2+} homeostasis in ER and in intracellular calcium storage, organelles could be a new potential strategy for neuroprotection. Ischemic preconditioning, performed in rats as modeled above, by increasing ORAI1 and STIM-1, which cooperate with each other to form SOCE, mediated Ca^{2+} refilling into ER and it has demonstrated to exert a neurobeneficial effect on stroke not only by refilling ER calcium but also by suppressing the upregulation of ER stress markers ad GRP78 and caspase-3 [55].

Furthermore, Boscia et al., analyzed, by using both in vitro and in vivo models of adult ischemia, whether the sodium/calcium exchanger NCX1 and calretinin, a Ca^{2+}-binding protein, cooperate to confer neurons greater resistance to degeneration. They demonstrated that the neuroprotective effect of calretinin was correlated to NCX1 expression in striatal interneurons. Indeed, the silencing of calretinin in brain cells prevented the neuroprotection mediated by ischemic preconditioning and reduced the expression of NCX1 protein (Boscia et al., 2019).

Regarding NCX3 in preconditioning its role has been further characterized and it has been linked to the post-translational mechanism known as sumoylation. Small ubiquitin-like modifier (SUMO) conjugation, or sumoylation, is a post-translational modification of various proteins similar to ubiquination, and has been noted in stress conditions including anoxia, hypothermia, and hypoxia. Changes in sumolyation patterns have been reported after brain ischemia, where it is thought to be possibly protective. Indeed, SUMO1 conjugation does increase at various times points following induced ischemia via transient middle cerebral artery occlusion (tMCAO) (at 5 and 24 h), after preconditioning (at 3, 5, 24, and 72 h) and when preconditioning was combined with tMCAO (at 5 h). Using immunohistochemical stains, it has been demonstrated a colocalization between NCX and SUMO1 to the neuronal cell bodies in the primary cortical neurons, with a probable sumoylation site in the NCX f-loop of the antiporter. Interestingly, SUMO1 knockdown induced in ischemic rats prevented the upregulation of NCX3 occurring after preconditioning and displayed a significant increase in ischemic volume. Interestingly, in the torpor phase of hibernating ground squirrels, which represents a preconditioning-like state, a massive conjugation of SUMO1 occurs that provides a protection to the oligemic conditions.

Collectively, these results show that SUMO1 plays a fundamental role in the neuroprotection elicited by ischemic preconditioning and that its protective role might be, at least in part, mediated by NCX3 sumoylation. Identifying targets for neuroprotection seems to be the next frontier in the world of stroke research. This takes us one step

closer to characterizing the mechanisms underlying the possible neuroprotectant effect of ischemic preconditioning, whereby targeting either sumoylation of NCX, or regulation of NCX itself, may lead to the development of better neuroprotectants.

Notably, beside stroke, NCX seems to play a relevant role also in other neurological disorders such as Amyotrophic Lateral Sclerosis [56]. Indeed, a sub-toxic acute exposure to the cycad neurotoxin beta-methylamino-L-alanine (L-BMAA) is able to delay ALS progression in SOD1 G93A mice, and NCX3 by handling the deregulation of ionic homeostasis occurring during ALS, takes part to this neuroprotective effect. In particular, it was shown that a sub-toxic dose of L-BMAA works as preconditioning stimulus and is able to delay ALS onset and to prolong ALS mice survival. Interestingly, preconditioning prevented NCX3 downregulation in SOD1 G93A mice spinal cord, leading to an increased number of motor neurons associated to a reduced astrogliosis, and reduced the denervation of neuromuscular junctions observed in SOD1 G93A mice. These protective effects were mitigated in ncx3+/- mice, thus further supporting the role of NCX3 in mediating L-BMAA-induced preconditioning [56].

In light of these results, NCX1 and NCX3 emerge as new effectors of brain preconditioning and represent two potential druggable targets to be analyzed in the study of the molecular mechanisms involved in cerebral ischemia (Fig. 2). The design of molecules that can activate these proteins may represent a new therapeutic approach for the treatment of stroke.

5. Postconditioning and Na^+/Ca^{2+} exchange

The strategy of ischemic postconditioning (IPostC) has been developed from the concept of ischemic preconditioning and it consists in a neuroprotective strategy mediated by a controlled reperfusion, usually obtained by a series of brief reperfusion and re-occlusion applied after the ischemic episode [57]. The first study on postconditioning effects was carried out on a model of myocardial ischemia by Zhao et al. in 2003. In this study it has been demonstrated that dogs subjected to coronary ischemia for three hours and subsequent ischemic post-conditioning (IPostC)/reperfusion showed a decreased myocardial injury compared to those animals that underwent to ischemia/3 -hs-reperfusion only; IPostC was reached by applying in the reperfusion period three cycles of 30-seconds reperfusion and 30 s left anterior descending artery reocclusion [58]. Furthermore, many years later postconditioning strategy was performed in brain to investigate whether it could trigger neuroprotective mechanism after stroke [59–62].

The post ischemic neuroprotective approach seems to be more realizable, compared to preconditioning strategy, seen that that harmful anoxic event is not predictable. Therefore, much efforts have been spent to investigate the molecular mechanisms that contribute to post-conditioning induced neuroprotection.

Several studies hypothesized that postconditioning and pre-conditioning share some common pathways of neuroprotection. Indeed, the application of both pre and postconditioning does not guarantee an additive protective effect (Pignataro et al., 2008). As concern the role played by NCX, the peri-ischemic temporoparietal cortex of rat exposed to 100 min of MCAO, 10 min of reperfusion and a re-occlusion of 10 min of the MCA, showed an up-regulation of NCX3 in terms of mRNA and protein [63]. Interestingly, NCX3 silencing through siRNA molecules, prevented the neuroprotection induced by post-conditioning, indicating the most prominent role of this NCX isoform in this phenomenon. Furthermore, NCX3 up-regulation was mediated by AKT activation that occurred during post-conditioning. Indeed, NCX3 promoter can be activated by the transcription factor CREB, an AKT downstream player [64]. On the other hand, NCX1 and NCX2 did not

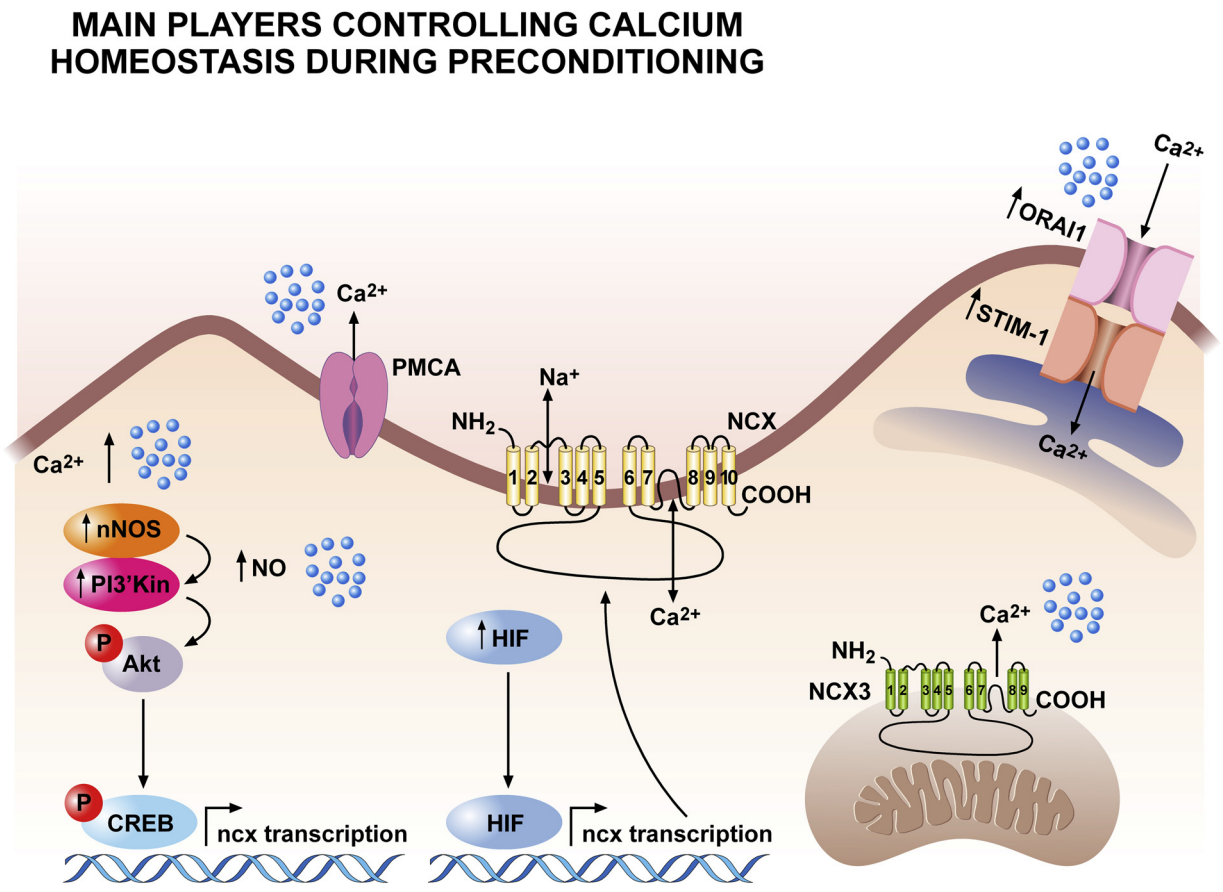

Fig. 2. Main players controlling calcium homeostasis through NCX and involved in brain conditioning protective effects.

show a relevant role in this process, probably due to their different sensitivity to ATP depletion: NCX1 and NCX2 isoform activity is reduced in absence of ATP, whereas NCX3 is still operative [65,66]. In addition, the cardioprotective effect observed after ischemic post-conditioning in the heart was prevented when blocking the reverse mode of operation of the sodium/calcium exchanger by KB-R7943 administration [67].

In summary, among the three NCX isoforms expressed in the CNS, NCX3 represents an additional new molecular effector involved in the neuroprotection exerted by ischemic postconditioning. In particular, in our experimental model of ischemic postconditioning, obtained by subjecting adult male rats to 10 min of subthreshold tMCAO applied 10 min after 100 min of tMCAO, we provided solid evidence showing that p-AKT is the mediator of this action since (1) p-AKT expression after postconditioning increased and timely mirrors that of NCX3; (2) NCX3 downregulation, induced by siRNA, reverts the neuroprotection induced by ischemic postconditioning; and (3) the selective p-AKT inhibition prevents NCX3 up-regulation thus reverting the post-conditioning-induced neuroprotection [63].

6. Conclusions

From results reviewed in the present paper NCXs emerge as solid effectors of preconditioning and postconditioning neuroprotection.

On the other hand, since the beginning of studies in the field, it has been hypothesized that brain cells are able to adapt to hypoxia by reducing their energy demand through modulation of ion channels and transporters, which delays anoxic depolarization. In particular, the role exerted by NCX in brain preconditioning has been extensively characterized and, over the years, a complex scenario converging on NCX regulation has been depicted. The picture of preconditioning-induced factors able to activate NCX includes: (1) transcription factors such as HIF1a, REST, SP1/SP3, DREAM; (2) survival factors, such as ERK and AKT; (3) post-translational modificators, such as SUMO; and (4) proteins contributing to Ca2+ homeostasis control such as ORAI/STIM and calretinin. The identification of these factors shed light on preconditioning as a strategy of neuroprotection and contribute to define new potential druggable targets to investigate in the attempt to develop new therapeutic strategies in stroke.

CRediT authorship contribution statement

G. Pignataro: Writing - original draft, Writing - review & editing, Supervision. **P. Brancaccio:** Writing - original draft, Writing - review & editing. **G. Laudati:** Writing - original draft, Writing - review & editing. **V. Valsecchi:** Writing - original draft, Writing - review & editing. **S. Anzilotti:** Writing - original draft. **A Casamassa:** Writing - original draft. **O. Cuomo:** Writing - original draft. **A. Vinciguerra:** Writing - original draft, Writing - review & editing.

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