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Review Article

The Role of NMDA Receptors in the Development of Brain Resistance through Pre- and Postconditioning

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ABSTRACT: Brain tolerance or resistance can be achieved by interventions before and after injury through potential toxic agents used in low stimulus or dose. For brain diseases, the neuroprotection paradigm desires an attenuation of the resulting motor, cognitive, emotional, or memory deficits following the insult. Preconditioning is a well-established experimental and clinical translational strategy with great beneficial effects, but limited applications. NMDA receptors have been reported as protagonists in the adjacent cellular mechanisms contributing to the development of brain tolerance. Postconditioning has recently emerged as a new neuroprotective strategy, which has shown interesting results when applied immediately, i.e. several hours to days, after a stroke event. Investigations using chemical postconditioning are still incipient, but nevertheless represent an interesting and promising clinical strategy. In the present review pre- and postconditioning are discussed as neuroprotective paradigms and the focus of our attention lies on the participation of NMDA receptors proteins in the processes related to neuroprotection.

Key words: N-methyl-D-aspartate receptors, preconditioning, postconditioning, neuroprotection

Brain Tolerance

Brain tolerance represents the transient resistance of the cerebral tissue to a lethal insult, which is established by preconditioning with a mild insult of short duration [1]. The term preconditioning was introduced by Janoff [2] and describes the tolerance response of an organism or tissue as the result of protective mechanisms towards potentially recurrent challenges. In fact, any stimulus able to generate damage to an organism or tissue can, when applied below the damage threshold, activate endogenous protective mechanisms, which may mitigate the impact of subsequent stimuli, which are above the damage threshold [3]. The general principle of preconditioning is thus a state of cellular protection, resulting from the exposure to sublethal insults that confer a significant tolerance to subsequent lethal insults [3,4].

The concept of preconditioning was first used to describe the tolerance towards ischemia in myocardial cells [5]. Ischemic preconditioning in the brain was described for neuroprotection promoted by a brief ischemic episode with respect to subsequent lethal ischemic events in several regions of the brain, e.g. the CA1 and CA3 areas in the hippocampus [6]. Further studies showed that brain tolerance towards lethal injury may be achieved after chemical, electrical or anoxic stimuli [1, 7-9]. It is hardly surprising that preconditioning attracted substantial attention as a novel therapeutic approach for neuroprotection, which could potentially also provide an improved mechanistic understanding of brain tolerance.

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Neuroprotection Paradigm

The process of neuroprotection disrupts or prevents a cascade, which occurs during the pathological processes of cell injury [10,11]. Excitotoxicity refers to toxicity caused by an increased concentration of glutamate during the synaptic transmission, which leads to neuronal death [12]. As excitotoxicity is associated with increased extracellular levels of glutamate, glutamate receptor antagonists can be used as neuroprotective agents [13].

Besides the excessive activation of glutamate receptors, it has been suggested that dysfunctions of the release and/or transport of glutamate occurs in acute and chronic forms of neuropathology, e.g. cerebral ischemia [14,15], traumatic brain injury (TBI) [16-18], as well as in neurodegenerative diseases such as Parkinson's [19,20] and Alzheimer's disease [21]. The involvement of excitotoxicity has also been discussed in the context of some neuropsychiatric diseases, e.g. bipolar disorder [22], schizophrenia [23], and depression [24].

Excessive stimulation of glutamate receptors can provoke various deleterious effects, such as a massively increased influx of Ca^{2+} or the release of nitric oxide (NO) [25]. It has been shown that the Ca^{2+} influx through the N-methyl-D-aspartate (NMDA) receptor is essential for glutamatergic excitotoxicity [26].

The hyperactivation of the Ca^{2+}-permeable ionotropic glutamate receptor (iGluR) is selectively activated by NMDA. Therefore, the NMDA receptor has been considered responsible for the cell death induced by excitotoxicity [27]. The influx of Ca^{2+} can moreover lead to an activation of toxic cascades, including the activation of catabolic enzymes such as phospholipases, proteases or endonucleases (e.g. caspases and calpains) [28]. Still, most of the Ca^{2+} ions are sequestered by the mitochondria, resulting in metabolic acidosis, inhibition of oxidative phosphorylation, opening of permeability transition pores, bioenergetic collapse, and the formation of free radicals from the impairment of the mitochondrial electron transport chain [29-31].

NMDA receptors are heteromeric complexes consisting of four subunits, each one comprising a different isoform: GluN1, GluN2 (GluN2A–GluN2D) and GluN3 (GluN3A and GluN3B). The different subunit composition of NMDA receptors shows distinct brain distribution, properties and regulation. Due to the composition of these heteromeric subunits, NMDA receptors show heterogeneous functionality and pharmacological characteristics [32]. NMDA receptors consist predominantly of the GluN1 form, which is, in combination with the presence of at least one GluN2 isoform, essential for the functionality of the receptor [33,34]. Extrasynaptic NMDA receptors containing GluN2B have been linked to excitotoxicity, whereas synaptic NMDA receptors containing GluN2A have been associated with the trophic effects of these glutamate receptors, which are responsible for neuroprotection [35].

Several studies have demonstrated the involvement of NMDA receptors in the generation of endogenous neuroprotection in different models of preconditioning via the administration of various antagonists, such as MK-801 and ketamine [1, 7-9]. Despite the evidence resulting from different models, which implicate the activity of NMDA receptors in neuronal loss following ischemia, several clinical trials investigating distinct NMDA receptor antagonists failed to demonstrate positive effects against stroke events, presumably due to poor tolerance and/or efficacy [36]. Moreover, the complete inhibition of NMDA receptors has been shown to be ineffective in clinical trials [37]. On the other hand, the mild activation of NMDA receptors during preconditioning has been considered a more effective clinical strategy.

Preconditioning

Ischemic Preconditioning

For adults, cerebral ischemia is one of the most common causes of death and the main cause of disability. Its pathology is characterized by the interruption of cerebral blood flow, which results in a severe degeneration of neural cells and the loss of brain function [38,39]. In this context, the phenomenon of ischemic preconditioning, i.e. tissue exposure to a brief subtoxic insult, which results in an increased tolerance to a subsequent lethal ischemic event, has been extensively investigated as a neuroprotective strategy in experimental models of cerebral ischemia [3]. Studies have shown that preconditioning in ischemia [40], hypoxia [41] and hypothermia [42] models resulted in a protection against a subsequent ischemic injury in ischemia models using animals and neuron cultures [43,44].

One characteristic of ischemic injury, which is encountered especially in the hippocampus, is delayed cell death, which may be observed in the area affected by the infarct (ischemic core) resulting in a necrotic process [45-47]. Furthermore, apoptosis can occur after an ischemic event [48], triggered by a variety of noxious signals, including the production of reactive oxygen species (ROS), tumor necrosis factor (TNF), neurotrophins, deficiencies of growth factors, as well as the induction of p53 protein, and the release of cytochrome c by mitochondrial damage [49,50]. These events appear to occur in the penumbral region of the ischemic event, where cell integrity can still be preserved [51]. Parallel to the activation of a pathway of programmed cell death, the survival pathways activated in cells resistant to the ischemic injury should also be

considered. In these cells, survival may promote the induction of neurotrophic factors, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor (IGF), and heat shock proteins (HSP) [52-54]. These survival proteins, activated by the noxious signals of ischemia, are potentially linked to the generation of tissue tolerance.

Possible neurotransmitter systems involved in ischemic preconditioning include adenosine A_1 and NMDA-subtype glutamate receptors [55]. In agreement with this notion, a recent study showed that pre-incubation of hippocampal slices with MK-801 (an NMDA receptor antagonist) or 8-cyclopentyl-1,3dipropylxanthine (DPCPX, an A_1 receptor antagonist) reduced the tolerance to a second oxygen and glucose deprivation (OGD) event. These results suggest that the activation of NMDA or A_1 receptors induce ischemic preconditioning in mice [56].

Chemical Preconditioning

A large variety of chemical compounds has been proposed as chemical agents inducing preconditioning, resulting in cardio- and neuroprotective effects. Among them are anesthetics, ethanol, selective ligands to the (GABA)ergic (gamma amino butyric acid) system, opioid and glutamate receptors, and K^+ channel activators.

Volatile anesthetics have also been used to afford cardio- and neuroprotection. Isoflurane and xenon for example induce early and late neuroprotection [57, 58]. Xenon can inhibit NMDA receptors, with little effect on GABA-A and non-NMDA glutamate receptors. The xenon-mediated preconditioning mechanism is connected to the activation of the phosphatidylinositol-3 kinase (PI3K) signaling pathway, the preservation of mitochondria function, and the inhibition of Ca^{2+}-induced mitochondrial transition permeability pore (MTPP) openings [59].

Agonists of the delta opioid receptors have also been reported to induce chemical preconditioning. Studies of ischemia and reperfusion suggest a potential correlation between opioid agonism and a reduction of the infarct size in models of regional ischemia, similar to that observed in the ischemic preconditioning [60]. Other preconditioning models used a moderate administration of ethanol, in order to protect neurons in cultures against β-amyloid-induced toxicity. Ethanol preconditioning is associated with elevated levels of HSP70, HSP27, and phospho-HSP27 in neuron cultures [61].

Moreover, many studies have emphasized the importance of the activation of mitochondrial ATP-sensitive potassium channels (mK^+_{ATP}) in the development of both acute and delayed ischemic tolerance [62]. Chemical activation of these mK^+_{ATP} channels with e.g. diazoxide has been shown to be protective against ischemia-induced cell death via a modulation of apoptotic proteins, a suppression of Bax translocation, and an inhibition of the release of cytochrome c [63]. In addition, a depolarization of the mitochondria caused by the activator of the mitochondrial mK^+_{ATP} channels induced a protective effect by the attenuation of oxidative stress [64].

Large-conductance Ca^{2+} activated K^+ (BK_{Ca}) channels, which are activated by depolarization and increase the cytosolic Ca^{2+} concentration, play a regulatory role in physiological processes such as the neuronal excitability [65], and have also been recognized as a target in order to induce chemical preconditioning. The synthetic BK_{Ca} activator NS1619 was shown to induce depolarization of the mitochondria and increase ROS production, promoting acute and delayed preconditioning in cortical neuron cultures. However, the mechanism of neuroprotection seems to be independent from a direct activation of these K^+ channels [66, 67] and rely on caspase activation and the generation of ROS.

This review will focus on the activation of NMDA receptors as the main target for the induction of cellular tolerance by pre- or postconditioning.

NMDA preconditioning

Increased brain tolerance can be achieved by several induction mechanisms, e.g. by chemical, electrical or anoxic stimulus [3]. The role of NMDA receptors as a major factor in the induction of neuroprotection was clearly established by administering NMDA receptor antagonists such as MK-801 or ketamine [1, 7-9].

The dual function of NMDA, as a putative neuroprotective agent on one hand, and as source of excitotoxicity on the other, has been discussed with respect to its activity at synaptic and extrasynaptic sites [68]. As previously mentioned, the extrasynaptic NMDA receptors (GluN2B) play a crucial role in excitotoxicity, whereas the synaptic NMDA receptors (GluN2A) are responsible for neuroprotection [35].

NMDA-related neuroprotection has also been observed in neuronal cell cultures: subtoxic concentrations of NMDA are able to prevent neuronal death induced by glutamate, NMDA [69-71], or OGD [72, 73]. Neuroprotection resulting from stimulation of NMDA receptors relies on trophic effects, as the activation of the BDNF and neurotrophin signaling pathways protect neurons against glutamate excitotoxicity [74].

The intraperitoneal administration of a subtoxic dose of NMDA has also been evaluated with respect to a chemical preconditioning model against several lethal posterior stimuli *in vivo*. This NMDA administration *in vivo* offers neuroprotection for murine pyramidal

hippocampal neurons against kainate-induced toxicity [75] and ischemia [76]. Our group has previously reported that NMDA preconditioning prevents seizures generated by intracerebroventricular administration of quinolinic acid (QA) in mice, where QA acts as an NMDA receptor agonist at the GluN2B subunit. Moreover, animals were protected from the necrotic cell death observed in the hippocampus as a result of the toxicity of QA [77, 78]. It is also noteworthy that subtoxic NMDA doses do not induce a hallmark parameter of apoptosis, i.e. DNA fragmentation in oligonucleosomes (Vandresen-Filho et al., unpublished observations).

The neuroprotective effect of NMDA is widely recognized, although the neural mechanisms involved in NMDA preconditioning are not completely understood. NMDA-mediated neuroprotection depends on the activation of A_1 receptors, because NMDA preconditioning could not be achieved when NMDA or A_1 receptors were blocked with selective antagonists [77]. However, blocking NMDA receptors with MK-801 neutralized even the neuroprotective effects against behavioral seizures and hippocampal cellular damage, which were promoted by NMDA preconditioning. The inhibition of A_1 receptors with the selective antagonist 8-cyclopentyl-1,3-dimethylxanthine (CPT) also eliminated any neuroprotection against seizures, but did not alter the hippocampal protection, which was promoted by NMDA preconditioning. It is possible that NMDA preconditioning may involve different signaling pathways: one depending on the activation of NMDA receptors, and another modulating the activation of adenosine receptors. We are currently investigating the role of adenosine receptors in the mechanism of NMDA preconditioning. Recent data from our laboratory show that NMDA preconditioning slightly increases the binding affinity of adenosine A_1 receptors in the hippocampus. Additionally, the activation of A_1 receptors after NMDA preconditioning precludes some of the behavioral and functional responses caused by the preconditioning (Constantino et al., unpublished data). An *in vitro* evaluation of the role of adenosine receptors in the mechanism of NMDA preconditioning in cerebellar granule neurons revealed that preconditioning facilitates a desensitization of the A_{2A} receptor response. The resulting cyclic AMP (cAMP) accumulation favors the activation of A_1 receptors [71] and contributes to NMDA-mediated preconditioning. The antagonistic effect of adenosine receptor activation is well understood and discussed elsewhere [79].

Our group has also investigated the intracellular signaling pathways involved in NMDA preconditioning. The inhibition of either protein kinase A (PKA) or PI3K pathway activation *in vivo* with selective inhibitors, completely eliminated any NMDA preconditioning against seizures induced by QA [80]. Additionally, the suppression of mitogen-activated protein kinase\kinase (MAPK-MEK) partially decreased the NMDA-mediated neuroprotection. Treatment with protein kinase C (PKC) or calcium-calmodulin dependent protein kinase II (CaMKII) inhibitors did not alter the NMDA-generated protection. Thus, important signaling pathways involved in cellular protection such as PKA, PI3K, and MAPK are used in order to provide NMDA-induced neuroprotection. The activation sequence of these signaling pathways, i.e. which enzymes are upstream or downstream in this protection cascade, still remains to be investigated.

NMDA preconditioning is a time-dependent approach to protection. In this *in vivo* protocol, protection is established 24 hours after NMDA administration, maintained up to 48 hours, and no longer observable after 72 hours [77]. Considering the time-dependency of NMDA preconditioning, and in an attempt to better understand the molecular and cellular mechanisms related to the protection of the brain, a proteomic analysis of the hippocampus of mice subjected to NMDA preconditioning was performed [81]. A differential expression of proteins involved in translation, processing, maintenance of energy homeostasis, and modulation of glutamatergic transmission was observed. Within the time-frame of possible neuroprotection after NMDA administration (24 h), proteins involved in protein processing (e.g. aspartyl-tRNA synthetase, HSP70) as well as proteins related to cellular bioenergetics (e.g. creatine kinase) were up-regulated. Simultaneously, a down-regulation of the vacuolar-type proton ATPase catalytic subunit was observed. This is the same protein, which is expressed in synaptic vesicles and is responsible for affording energy for neurotransmitter accumulation. Considering the mechanisms related to preconditioning, it might be speculated that the resulting neuroprotection depends - as previously shown - on protein synthesis [82], as well as on protein processing, increased cellular bioenergetics, and decreased extracellular glutamate levels.

Regarding cellular bioenergetics, the modulation of oxidative stress resulting from an imbalance between ROS production and depletion may also be involved in the protective mechanism of preconditioning. The concept that preconditioning induced by ischemia could be related to an initial oxidative stress event was supported by the observation of an increased activity of antioxidant enzymes, e.g. catalase and superoxide dismutase (SOD) in the hippocampus and striatum. However, the increased enzyme activity was not necessarily accompanied by a complete inhibition of neurodegeneration [83]. By using antioxidants [84], it was possible to show that initial oxidative stress could be responsible for triggering preconditioning, whereas antioxidant enzymes did not

function as end-effectors in such neuroprotection. When we were evaluating antioxidant glutathione levels and the activity of glutathione related enzymes in mice models of NMDA preconditioning *in vivo*, we observed that the glutathione metabolism might not interfere directly with the tolerance level induced by the NMDA preconditioning [85].

Since Ca^{2+} ions permeate the NMDA receptors and increase neuronal excitability, it is very important to remember that the mild activation of NMDA receptors (probably mainly at the synaptic and not the extrasynaptic sites) does not reach the threshold level of toxicity. However, the mechanisms of neuroprotection or excitotoxicity caused by NMDA seem to differ only with regards to intensity and site of action. Accordingly, preconditioning doses of NMDA promote neuroprotection by enhancing neuronal excitability levels [86]. Using an *in vivo* model of NMDA preconditioning [77], we assessed the electroencephalographic responses of the hippocampus and cerebral cortex of mice with respect to a subconvulsant dose of NMDA and a convulsant dose of QA [87]. With these experiments, we confirmed that 50% of mice were protected against QA-induced seizures after NMDA preconditioning [77,85]. Although the electroencephalographic results did not allow us to deduce a behavioral generalization of the seizures, they showed that NMDA preconditioning induced spike-wave discharges. Moreover, the same 50% of the mice, which were protected against behavioral seizures, exhibited an increased number of spike-wave discharges relative to the mice, which experienced seizures. Therefore, we concluded that we recorded an increased electroencephalographic excitability, when NMDA preconditioning afforded neuroprotection. In addition, we observed a negative correlation between the number of NMDA-induced spike-wake discharges and the severity of QA-induced seizures, which we evaluated with the help of the Vandresen-Filho scale of QA-induced seizures [87]. Accordingly, it can be argued that the increasing excitability induced by NMDA preconditioning results in an increased protection against behavioral seizures.

In this context, it is worth mentioning a recent study, which aimed to evaluate the energy metabolism in the brain of NMDA preconditioned mice. Besides the widely reviewed QA-induced seizures, NMDA preconditioning was also tested against an *in vivo* model, where mice were subjected to TBI. There, NMDA preconditioning prevented a gait distortion of mice suffering from a mild TBI and improved the affected locomotor parameters such as coordination, balance, and sensorimotoric activity [88]. Mice subjected to NMDA preconditioning and subsequent TBI showed elevated activity levels for the mitochondria as the master organelle in preconditioning-triggered neuroprotection. A significant increase in mitochondrial complex II was observed in preconditioned mice and in those subjected to trauma [31]. Again, a similar response for the triggering mechanism of NMDA-mediated neuroprotection and for the event-inducing neurotoxicity was observed.

It seems therefore feasible to conclude that the increased excitability, the induction of mild oxidative stress, the modulation of bioenergetics, the ionic homeostasis, and the modulation of glutamatergic transmission within non-excitotoxic levels comprise the underlying mechanisms for NMDA-mediated preconditioning.

Postconditioning

The clinical approach of preconditioning has obvious limitations, e.g. the inability to predict the onset of the injury. However, it has been indicated that cell mechanisms evoked by preconditioning can be reproduced after the injury. Postconditioning is a neuroprotective strategy that has been studied well for ischemic events, where the reperfusion period is controlled or reduced [89]. Pharmacological postconditioning, as a protection strategy against delayed neuronal death, has been intensively studied in recent years [90-93]. Protective cell pathways activated by postconditioning are - at least in part - identical to those activated in preconditioning. However, postconditioning has the advantage of being able to be applied after the insult. Several studies discuss basic approaches to postconditioning and show two main methods of how this can be achieved: i) rapid postconditioning, where the interruption of reperfusion occurs between minutes and hours after the injury [94,95], and ii) delayed postconditioning, where treatment is applied between hours and days after the incident [96,97]. Neuroprotective signaling pathways, triggered by different conditioning strategies in the brain share some common mediators, e.g. inflammatory cytokines, NO, and the activation of anti-apoptotic proteins. Recently, a growing number of reports have contributed to the understanding of the underlying mechanisms of conditioning effects. This knowledge will permit the development of translational strategies for the clinical practice in order to induce brain resistance.

Ischemic Postconditioning

Protection arising from postconditioning was initially studied within the context of myocardial damage induced by ischemia [98] and this knowledge has subsequently been transferred to the field of protection against damage following cerebral ischemia. Rapid ischemic postconditioning can be achieved in rats by three cycles

of brief obstruction (10 s) of the bilateral common carotid artery (CCA) followed by reperfusion (30 s), combined with a permanent occlusion of the distal middle cerebral artery (dMCA) [94]. It was observed that postconditioning reduced the size of the infarcts in the cerebral cortex two days after ischemia. This means that postconditioning could be induced by repetitive series of brief interruptions of the reperfusion applied after ischemia, conferring neuroprotection probably by an attenuation of the reperfusion-induced injury. Following these findings, several other groups have focused their attention on postconditioning as a viable strategy for the repair of damage resulting from stroke incidents in animal models and in clinical studies. Mechanical postconditioning, induced by four cycles of occlusion-reperfusion (1 min/1 min) via the inflation and deflation of a balloon, reduced the size of infarcts resulting from microvascular obstruction in human patients suffering from acute ST-elevation myocardial infarction (STEMI) [99]. More promising results were obtained for patients sustaining acute myocardial infarcts [100]. These results indicate that postconditioning could potentially be a safe method to induce neuroprotection, although preclinical and clinical trials remain necessary in order to confirm this hypothesis efficiently.

Brain injury following ischemia can be effectively attenuated in animal models when ischemic postconditioning is applied. Ischemic postconditioning ameliorates brain edema and decreases the blood-brain-barrier (BBB) leakage induced by focal cerebral ischemia (occlusion of the middle cerebral artery) [97]. Since glutamate and ROS play a critical role in ischemic damage, their depletion is pivotal for neuroprotection. In fact, ischemic postconditioning increases the levels of glutamine synthetase in the hippocampus of rats. These elevated glutamine synthetase levels contribute to neuroprotection by rapidly converting glutamate to glutamine in the glia, resulting in decreased extracellular glutamate levels after the injury [93]. Concomitantly, the contents of glutamate transporter 1 (GLT-1) were increased following ischemic postconditioning, contributing to the clearance of glutamate [101]. Moreover, the reduction of protein oxidation is accompanied by an increase in SOD (MnSOD and CuZnSOD) and catalase activity levels, which in turn decrease intracellular ROS concentrations [102]. Endothelial nitric oxide synthase (eNOS) plays an important role in ischemic pre- and postconditioning, since the generation of NO is crucial for the vascular functioning and homeostasis [103,104].

Matrix metalloproteinases-9 (MMP-9) degrades extracellular matrix components contributing to BBB leakage [105] and the co-expression of MMP-9 and activated-caspase-3 following ischemic stroke events was recently observed for humans. These proteins were co-expressed in the nuclear compartment of glial and neuronal cells at perilesional areas from post-mortem cortical tissue fragments in aged humans [106]. Ischemic postconditioning in rats was reported to reduce the expression of MMP-9, attenuating the focal cerebral ischemia-induced reduction of laminin and fibronectin expression, thus preserving the BBB integrity after injury [107].

Intracellular signaling activated by ischemic postconditioning includes the inhibition of MTPP openings due to an increased influx of Ca^{2+} (during ischemia and reperfusion), a depletion of ATP during ischemia, and the formation of ROS [108,109]. Mitochondrial integrity is closely related to the production of ATP, which in turn is increased by the opening of mK^{+}_{ATP} channels in the brain. This induces a depolarization potential on the mitochondrial membrane and thus promotes an increase of the electron transport chain activity [110]. Postconditioning administered to humans suffering from transient limb ischemia reduced the endothelial injury after ischemia via a mechanism involving mK^{+}_{ATP} channels [111]. In rat models, delayed remote limb ischemic postconditioning was reported to be neuroprotective when mK^{+}_{ATP} channels were activated [112]. Members of the Bcl-2 (anti- and proapoptotic) protein family are located in the outer mitochondria membranes and they control the activation of downstream caspase-9 and -3 enzymes, which represent critical intracellular factors in the mitochondria-mediated apoptosis pathway [113]. Ischemic postconditioning elevates the content of anti-apoptotic Bcl-2 proteins, decreases the content of proapoptotic Bax proteins and down-regulates the proteins caspase-3, -6 and -9 in the hippocampus of rats, which were subjected to early global brain ischemia mitigating cell death by apoptosis [114,115]. Another important neuroprotective pathway associated with ischemic postconditioning involves increased phosphorylation of PI3K/Akt [95] and its activity [116]. Besides PI3K/Akt, other proteins are involved: postconditioning inhibits the cleavage of δPKC and enhances phospho-εPKC levels. Moreover, it reduces phosphorylation of MAPK pathways including c-jun N-terminal kinase (JNK) and extracellular signal-regulated kinase 1/2 (ERK1/2) [116,117]. Altogether, the signaling of mK^{+}_{ATP} channels, as well as Akt and εPKC proteins contribute to the inhibition of the MTPP openings and provide a neuroprotective effect after ischemic postconditioning.

In cortical neuron cultures, postconditioning may be induced via hypoxia (0.1% O_2) 14 hours after OGD. Protective effects arise through angiogenesis proteins such as hypoxia-inducible factor-1 (HIF-1) and its target genes, erythropoietin and adrenomedullin [118].

Hypobaric hypoxia also improves neuronal survival efficiently *in vivo*. However, only delayed postconditioning provides an emotional behavioral recovery (plus-maze task) and an associated increase in corticosterone hormonal levels [119].

In summary, studies reporting the beneficial effects of ischemic postconditioning indicate that postconditioning shares the cellular mechanisms activated when neuroprotection is achieved by preconditioning.

Chemical Postconditioning

After understanding neuroprotection induced by ischemic or hypoxic postconditioning, the next challenge for the clinical translation is the control of brain resistance after injury using pharmacological approaches. Until now, only few studies have investigated the effects of pharmacological postconditioning (with the exception of anesthetics) with respect to neuroprotective effects. The anesthetics isoflurane and sevoflurane can be used as postconditioning agents at early reperfusion stages of strokes *in vivo* or OGD *in vitro* [120,121]. Isoflurane postconditioning reduces brain infarcts and attenuates the neurological damage in rats after cerebral ischemia [120]. In rat models and in cortical neuron cultures, anesthetics provide protection via activation of the PI3K/Akt pathway [122,123] and increased expression of HIF-1α and the inducible NOS (iNOS) gene [124,125]. Isoflurane postconditioning moreover involves an inhibition of CaMKII [126]. CaMKII is regulated by the complex Ca^{2+}/calmodulin, which is highly expressed in the brain and further enriched at excitatory synapses and their postsynaptic densities (PSDs). CaMKIIα can interact with a variety of proteins in the PSD, including proteins at the NMDA receptor complex. Interestingly, CaMKIIα exhibits a higher binding affinity towards GluN2B relative to GluN1 [127]. McMurtey and Zuo [126] suggested isoflurane postconditioning-induced neuroprotection to involve an inhibition of NMDA receptors, i.e. the exact opposite mechanism shown for the preconditioning, in which the activation of NMDA receptors is crucial to neuroprotection [7].

In fact, the PSD protein complex is pivotal for basic glutamate transmission and the generation of synaptic potentiation. Postconditioning with the sedative propofol inhibits the internalization of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, containing GluA2 subunits, which is indicative of an active participation of AMPA receptors during postconditioning-induced brain resistance. This was supported by the observed reduced infarct volume and improved spatial memory after ischemia/reperfusion injuries [128]. However, ischemic postconditioning also attenuates the binding of the PSD-95 protein to kainate receptors containing GluK2 subunits [129]. The participation of iGluRs in neuroprotection induced by postconditioning is still unclear and requires further elucidation.

In contrast, glutamate receptor ligands potentially induce postconditioning against hippocampal ischemia. Low doses of the group I metabotropic glutamate receptor (mGlu1 and mGlu5) agonist 3,5-dihydroxyphenylglycine (DHPG) were reported to protect organotypic hippocampal slices from OGD-induced cell damage in a mode dependent on Akt activation [90]. Also, low doses of kainate administrated 48 hours after ischemia resulted in recovered spine density and prevented long-term potentiation (LTP) impairment [92].

Our group is currently investigating the potential neuroprotection in mice induced by a low dose of NMDA applied after mild TBI. Preliminary data show that NMDA postconditioning attenuates recognition memory deficits 48 hours after a mild TBI, and hippocampal cell death 96 hours after the trauma (Bavaresco et al., unpublished data). NMDA is more effective when administered 15 minutes after TBI, rather than 1 hour later, indicating that NMDA receptors can be a target of neuroprotection in accordance with the postconditioning paradigm.

From the Laboratory to the Clinical stage

The studies discussed in this review report *in vitro* and *in vivo* approaches, i.e. modifications in cell or organotypic cultures as well as in rodents. All of these investigated pre- and postconditioning as neuroprotective strategies. Moreover, most of the studies aim to unravel the mechanisms operative in these neuroprotective approaches, which can be considered either as preventive or as neuroprotective rescue strategies. From a translational point of view, preconditioning may find clinical applications in prophylactic situations, e.g. inducing neuroprotection in patients, who are undergoing brain surgery or suffer from subarachnoid hemorrhages and are exposed to an elevated risk of immediate brain injury. In this scenario, chemical preconditioning represents an excellent therapeutic approach compared to other forms of protection such as surgical preconditioning, since those procedures can potentially be harmful.

On the other hand, postconditioning is also an interesting therapeutic approach, since the window of application lies after the diagnosis of a stroke or TBI. There is consensus in the scientific literature that the intervention against cerebral damage can be better controlled using pharmacological strategies. Still, little is known about chemical postconditioning, its mechanisms and effects. The pursuit of basic research into chemical

preconditioning will therefore furnish a better comprehension of the underlying mechanisms and signaling pathways associated with the development of tissue tolerance, and ultimately provide the foundation for future clinical applications. Accordingly, there is hardly any doubt that further in-depth investigations are required in order to reveal the mechanisms by which pharmacological agents induce neuroprotection after cerebral injury, so that patients can be treated more efficiently in the future.

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