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PERSPECTIVE

Neuroprotection induced by NMDA preconditioning as a strategy to understand brain tolerance mechanism

Excitotoxicity refers to toxicity caused by abnormal concentrations of glutamate in the synaptic cleft that may lead to neuronal death. Since its description, the phenomenon of glutamatergic excitotoxicity has been implicated in the physiopathology of a wide range of neurological and psychiatric disorders, from acute brain damage such as traumatic brain injury, ischemia as well as chronic conditions like epilepsy, depression and neurodegenerative pathologies such as Huntington's, Parkinson's and Alzheimer's diseases. Excessive stimulation of glutamatergic receptors, mainly N-methyl-D-aspartate (NMDA) receptors (NMDAR), can have numerous adverse effects on the cell viability, including increased nitric oxide release (NO), activation of proteases, increased production of reactive oxygen (ROS) and nitrogen (RNS) species and massive influx of calcium ions (Ca^{2+}), resulting in cell death. Thus, the use of strategies that modulate the excitotoxic cell damage represents a perspective for the treatment of diseases such as Parkinson's and Alzheimer's diseases, ischemia, traumatic brain injury (TBI) and seizures.

Besides the excitotoxic cascade following hyperactivation of NMDAR, this receptor is known to have a dual effect, which promotes neuronal survival or death depending on the level of activity and receptor composition. In fact, moderate activation of NMDAR has been shown to exert neuroprotective effects against posterior lethal insults. This phenomenon, known as NMDA preconditioning, may be achieved by subconvulsant doses of NMDA and it has been shown to protect neurons *in vitro* and *in vivo* against a wide range of acute injuries such as seizures, TBI and cerebral ischemia. Historically, preconditioning was firstly described by Janoff (1964) to explain the tolerance response of an organism to lethal stress induced by prior exposure to low doses of toxic agents or stimuli. Thus, the general principle of preconditioning is defined as obtaining a tissue protection state or the organism as a whole, by exposure to sublethal stimulus conferring thus tolerance to subsequent lethal damage. A series of recent studies have described a related phenomenon termed chemical preconditioning. Several substances interfering with cellular energy metabolism applied at subtoxic doses may provide protection against some lethal insults, such as the NMDA preconditioning.

Administration of subtoxic doses of NMDA, intraperitoneally (i.p.), is used as an *in vivo* model of chemical preconditioning against subsequent brain damage. One of the important points is the short time (therapeutic window) observed after NMDA preconditioning induction. For example, in the *in vivo* protocol it has been shown that protection is obtained 24 hours after NMDA administration and it remains for up to 48 hours. However, the protective effects of preconditioning are not observed within 1 hour or 72 hours after NMDA administration (Boeck et al., 2004). The onset of the therapeutic window may represent the time necessary to activate endogenous neuroprotective and repair mechanisms and its duration may be related to the return to basal levels of these mechanisms. It is conceivable that brain preconditioning may be related to the up-regulation of cellular defense and repair systems and down-regulation of injury-induced mechanisms.

Thus, considering the time-dependency (therapeutic window) of NMDA preconditioning, and in an attempt to better understand the molecular and cellular mechanisms related to the protection of the brain, we have performed a proteomic analysis of the hippocampus of mice subjected to NMDA preconditioning (do Amarale Silva Muller et al., 2013). 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 hours), proteins involved in protein processing (*e.g.*, aspartyl-tRNA synthetase, heat-shock protein of 70 kDa, 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 can be speculated that the resulting neuroprotection depends on protein synthesis, as well as on protein processing, increased cellular bioenergetics, and decreased extracellular glutamate levels. This suggests that the neuroprotective state induced by NMDA preconditioning may rely on the combination of an extensive set of endogenous stress responses expressed at the same time. As expected, therapeutic approaches that improve these single targets alone may be not as successful as those that may induce multiple neuroprotective mechanisms simultaneously.

Besides the excessive activation of glutamate receptors, it has been suggested that dysfunction of the release and/or transport of glutamate occurs in acute and chronic forms of neuropathology, *e.g.* cerebral ischemia, TBI. In this vein, it would be expected that the neuroprotective effects of NMDA preconditioning may also be dependent on factors that modulate glutamatergic transmission. Among these factors, adenosine is an endogenous modulator of glutamatergic synapses activity that may exert control on neuronal excitation through inhibition of glutamate release *via* adenosine A1 receptors activation. Our group has shown that NMDA-mediated neuroprotection depends on the activation of adenosine A1 receptors, since NMDA preconditioning could not be achieved when NMDA or A1 receptors were blocked with selective antagonists (Boeck et al., 2004). The blockade of A1 receptors with the antagonist 8-cyclopentyl-1,3-dimethylxanthine (CPT) also eliminated any neuroprotection against seizures induced by quinolinic acid (QA), 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 binding affinity of adenosine A1 receptors was slightly increased in membrane preparations of hippocampus from preconditioned mice. Additionally, activation of A1 receptors after NMDA preconditioning precludes some of the behavioral and functional responses caused by preconditioning (Constantino et al., 2015). 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 A2A receptor response, favoring the activation of A1 receptors and contributes to NMDA-mediated preconditioning.

Importantly, NMDA preconditioning may lead to protective effects at a functional level. It has been demonstrated in a model of TBI that NMDA preconditioned mice presented improvement in locomotion parameters such as coordination, balance and the sensory-motor activity and these mice did not show distortion of gait (Costa et al., 2010).

Additionally, in our laboratory, we have been focusing in the

underlying mechanisms of NMDA preconditioning and the effect of preconditioning against seizures induced by QA. Actually, it is known that QA causes seizures through action of NMDA receptors, particularly those containing GluN2B subunits. Thus, activation of NMDA receptors by QA causes excitotoxicity increasing the intracellular Ca^{2+} levels, promoting mitochondrial dysfunction with adenosine-5′-triphosphate (ATP) exhaustion and excessive intracellular ROS and RNS production, resulting in lipid peroxidation and protein carbonylation. We have also demonstrated that NMDA preconditioning can prevent seizures and neural death in the hippocampus after intracerebroventricular (i.c.v.) QA infusion. However, electroencephalographic (EEG) analysis demonstrated that NMDA preconditioning promotes spike-wave discharges, but it does not display behavioral manifestation of seizures (Vandresen-Filho et al., 2013). This observation suggests that an increased electrical activity after NMDA administration might be the trigger for achievement of a protective state. It is also noteworthy that subtoxic NMDA doses do not induce a hallmark parameter of apoptosis, *i.e.*, DNA fragmentation in oligonucleosomes.

The search for the intracellular signaling pathways involved in NMDA preconditioning induction is also important in order to identify the molecular mechanisms of precondtioning. The blockade of either protein kinase A (PKA) or phosphatitylinositol-3 kinase (PI3K) pathway activation *in vivo* with selective inhibitors completely eliminated NMDA preconditioning protective effect against seizures induced by QA (de Araujo Herculano et al., 2011). 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 NMDA-generated protection. The activation sequence of these signaling pathways, *i.e.*, which enzymes are upstream or downstream in this protection cascade, still remains to be investigated.

The modulation of oxidative stress has also been suggested to be involved on the protective mechanisms of preconditioning. Evaluation of the antioxidant glutathione levels and activity of glutathione-related enzymes in mice subjected to NMDA preconditioning *in vivo*, showed that glutathione metabolism might not directly interfere with the tolerance level induced by NMDA preconditioning (Vandresen-Filho et al., 2007). Additionally, it was assessed the effect of NMDA preconditioning on calcium homeostasis and on glutamate transport after infusion of QA. NMDA preconditioning regulates extracellular glutamate clearance in association with the maintenance of intracellular calcium homeostasis, thus protecting mice against seizures induced by QA (Vandresen-Filho et al., 2015).

Another important factor related to brain preconditioning through moderate NMDAR activation is the receptor composition. The extrasynaptic receptors containing GluN2B subunit are involved in excitotoxic processes, while the synaptic receptors containing the GluN2A subunit are linked to the trophic effects of glutamatergic receptors that are responsible for neuroprotection (Vizi et al., 2013). Considering the dual effects of NMDAR, differential modulation of NMDAR containing GluN2A or GluN2B subunits may represent a potential mechanism of achievement of the endogenous tolerance state during preconditioning.

Finally, NMDA preconditioning induces a time-dependent neuroprotection that may rely in a variety of cellular modifications occurring simultaneously. These alterations involve modulation of ionic channels, antioxidant defenses, bioenergetics and modulation of glutamatergic transmission. The knowledge of the mechanisms involved in this neuroprotective state may provide a greater understanding of the induction of endogenous protective pathways and it may be a powerful tool in the development of new preventive strategies against neurological disorders.

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