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"Mechanisms of preconditioning in an *in vitro* model of cerebral ischemia"

Candidato

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Research is art
Research is gambling
Research is a game, a very serious game
Research is hard work
Research is innovation
Research is inventiveness
Research is life
Research is love
Research is passion
Research is fun… and I love having fun

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PART III

1. ISCHEMIC TOLERANCE

1.1 Ischemic preconditioning

Many studies *in vivo* and *in vitro* have demonstrated that neurons exposed to brief periods of sublethal anoxia develop resistance to subsequently more prolonged and lethal anoxic insults (Kitagawa et al., 1991; Kitagawa et al., 1990; Gidday et al., 1994) (Fig.1). This phenomenon, known as anoxic preconditioning (APC), was first described in the myocardium (Murry et al., 1986; Meldrum et al., 1997) and only recently in the brain (Kirino et al., 1991; Kitagawa et al., 1991; Meller et al., 2005). Consequently, over the last three decades, many efforts have been addressed to identify the molecular mechanisms involved in this phenomenon in order to open up therapeutic avenues for the treatment of cerebral ischemia.

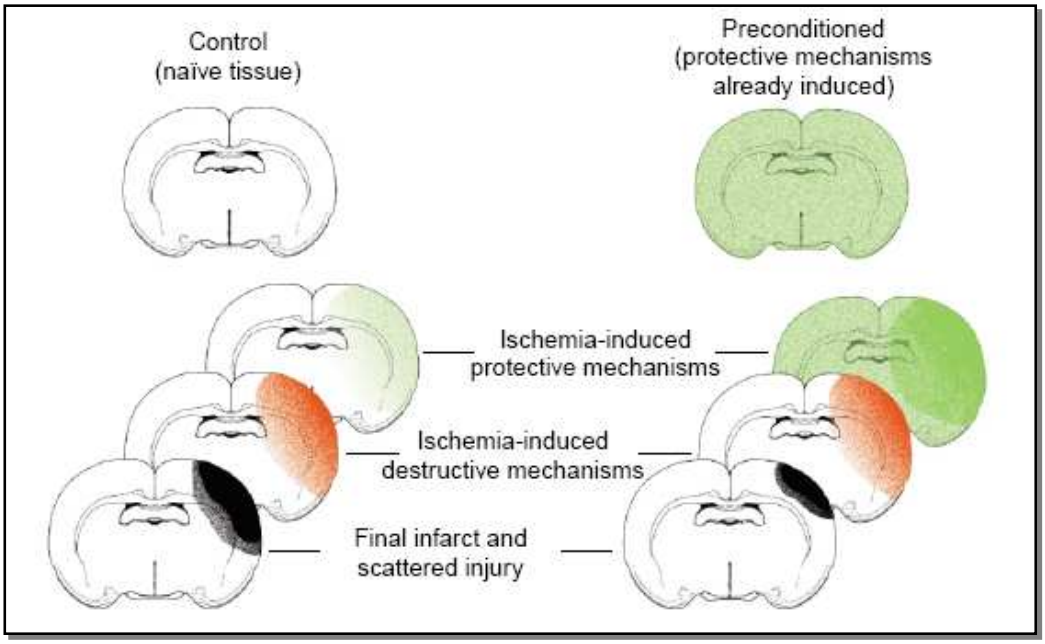


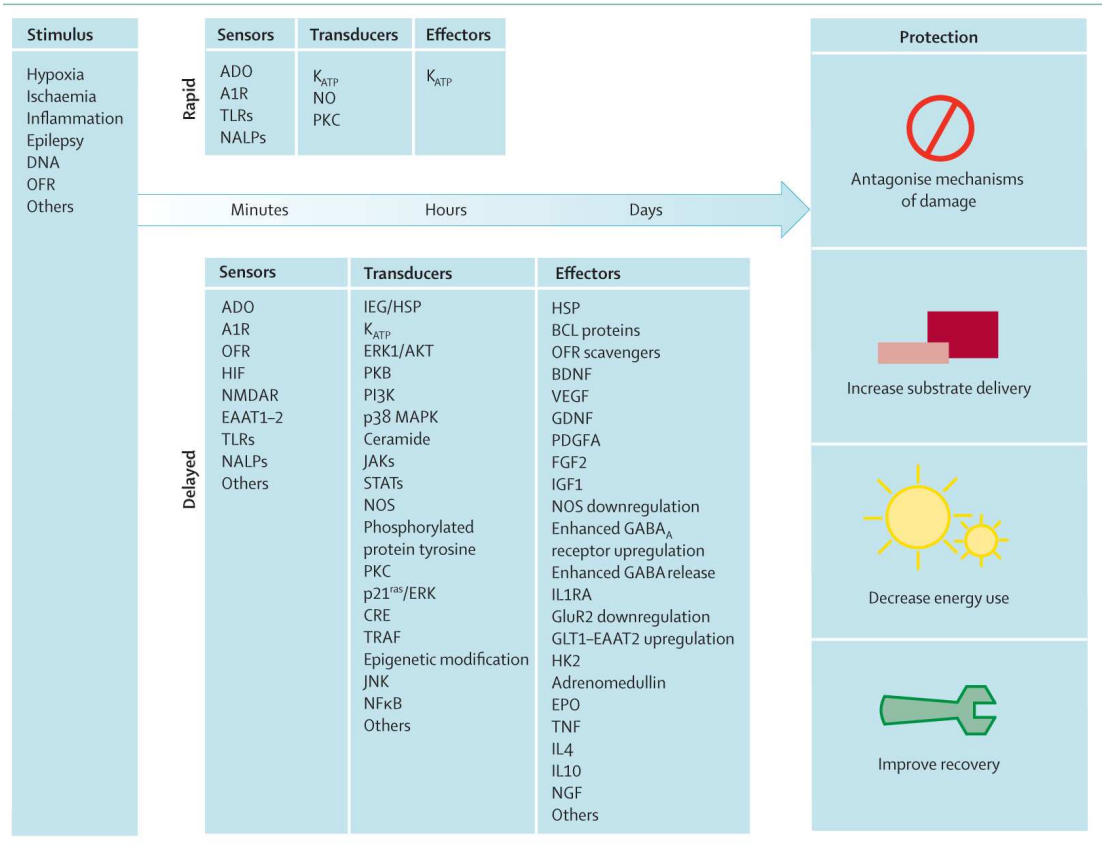
Fig.1

Dirnagl et al., *TINS* **26:** 248, 2003

Fig. 1 Ischemic tolerance is an evolutionary conserved cellular defense program in which exposure to a subtoxic insult (the preconditioning stimulus) results in resistance to a subsequent lethal stressor.

1.1.1 Ischemic preconditioning stimuli

Differences in the intensity, duration, and/or frequency of a particular stress stimulus determine whether that stimulus is too weak to elicit any response, or sufficient magnitude to serve as a preconditioning trigger, or too robust and therefore harmful (Dirnagl et al., 2003) (Fig.2). It is generally accepted that preconditioning requires small doses of an otherwise harmful stimulus to induce protection against subsequent injurious challenge (Dahl et al., 1964). Several distinct preconditioning stimuli can induce tolerance to ischemic brain injury; among them are non injurious ischemia, cortical spreading depression, brief episode of seizure, exposure to anaesthetic inhalants, and low doses of endotoxin, hyperthermia or heat shock (Garnier et al., 2003).



Dirnagl et al., *Lancet Neurol* 2009; **8**: 398–412 **Fig.2**

Fig. 2 Signalling cascades of preconditioning. Various stimuli lead to protection via modules of sensors, transducers, and effectors. Adapted from Dirnagl et al, with permission from Elsevier.

The existence of multiple, diverse preconditioning stimuli able to provide protection against an entirely different type of injury, constitutes the well-known phenomenon of 'cross-tolerance' (Schurr et al., 1986). Accordingly, one stressor can promote cross-tolerance to another, or the same stressors that elicit tolerance in the brain can elicit tolerance in other organs. Many exogenously delivered chemical preconditioning agents, such as inflammatory cytokines and metabolic inhibitors, can also induce ischemic tolerance, raising the possibility that in the future it will be possible to activate pharmacologically these distal pathways in the human brain.

Moreover, because inducers and mechanisms of tolerance might have similar features, the induction of tolerance in one organ can, via neural or paracrine mechanisms, spread to other organs. This phenomenon, known as 'remote preconditioning' or 'preconditioning at a distance', has been recently described both in the brain and in the myocardium after hind limb ischemia or intrarenal occlusion of the aorta, respectively. Differences in the intensity, duration and/or frequency of a particular stimulus potentially able to induce protection determine whether that stimulus is too weak to elicit a response, of sufficient intensity to serve as a preconditioning trigger, or too robust to be harmful.

In general, it is widely accepted that immediate acquisition of protein-synthesis-independent tolerance is mediated by post-translational modification and that the effective duration is brief. Conversely, there is general agreement that delayed induction of ischemic tolerance requires new protein synthesis and is sustained for a time interval ranging from a few days to a few weeks. In the brain, the time course of ischemic tolerance apparently follows the delayed pattern, suggesting that synthesis of active proteins may be necessary for full development of ischemic tolerance (Endres et al., 2003). Once induced, the ischemic tolerance is believed to last for a few days and to diminished gradually a few weeks after acquisition (Ding et al., 2005).

a) Sensors and transducers

To induce tolerance, the preconditioning stimulus must be recognized by molecular sensors as a sign of something potentially much more severe to come. So far,

numerous types of sensor have been identified, including neurotransmitter, neuromodulator, cytokine and toll-like receptors (Kariko et al., 2004), as well as ion channels and redox-sensitive enzymes (see the figure below). In turn, these sensors activate transduction pathways that initiate the adaptive response.

Although dependent in part on the nature of the preconditioning stimulus, members of these transduction pathways for which there is strong general support include mitogen-activated protein kinases (MAPKs) and their phosphorylated Ras, Raf, MEK and ERK subfamilies (Gonzalez-Zulueta et al., 2000; Jones et al., 2004), mitochondrial ATP-sensitive K+ (KATP) channels (Heurteaux et al., 1995; Yoshida et al., 2004), Akt (also known as protein kinase B) (Yano et al., 2001; Wick et al., 2002) and the protein kinase C-ε isoform (Raval et al., 2003).

The possibility that the nitric oxide-based adaptive response to hypoxia in Drosophila is evolutionarily conserved, suggests that this multifunctional modulator might be a logical choice as an autocrine and paracrine mediator of preconditioning stress. Indeed, pharmacological and genetic evidence supporting the involvement of nitric oxide (derived from the endothelial (Gidday et al., 1999), neuronal (Atochin et al., 2003) and inducible isoforms of nitric oxide synthase (NOS) in the transduction process is continuing to mount.

Given the redox sensitivity of many kinases and transcription factors, reactive oxygen species might also serve as transducers.

Adenosine, another prototypical paracrine mediator and ‘retaliatory metabolite’, the production of which is linked to ATP degradation, seems integral to tolerance induction in some models (Yoshida et al., 2004; Nakamura et al., 2004).

Finally, caspases might be essential induction catalysts, given that cyclic AMP responsive element-binding protein (CREB), the p50 and p65 subunits of nuclear factor-κB (NF-κB), and protein kinase C and other kinases are caspase substrates (McLaughlin et al., 2003). Notably, some of the aforementioned molecular transducers and signalling intermediates also serve as post-ischemic effectors of the ischemia-tolerant phenotype (Dirnagl et al., 2003).

Preconditioning-activated signalling pathways converge to induce post-translational modifications of existing proteins and/or to activate transcription factors

that drive the genomic response. Several transcription factors are known to be sensitive to regulation by hypoxia/ischemia and probably participate in this response, including activating protein 1 (AP1), CREB, NF-κB (Ravati et al., 2001; Blondeau et al., 2001), early growth response 1 and the redox-regulated transcriptional activator SP1 (Fig. 11).

However, the hypoxia-inducible factor (HIF) isoforms have garnered the most experimental support so far with respect to mediating the transactivation of adaptive, pro-survival genes, particularly those involved in glucose metabolism and angiogenesis (Liu et al., 2005). Transcriptional regulation of the genome by HIF is similar in Drosophila, C. elegans and mammals. In mammals, the HIF1α isoform is hydroxylated during normoxia by an oxygen- and iron-dependent prolyl hydroxylase, allowing interaction between HIF1α and an ubiquitin ligase that targets HIF1α for degradation by the proteasome pathway. However, hypoxia renders the hydroxylase enzyme non functional, and HIF1α, no longer able to associate with the ligase in its non hydroxylated form, then enters the nucleus, dimerizes with HIF1β, and promotes the transcription of genes that enhance hypoxic resistance. Prolyl hydroxylase inhibitors, such as deferroxamine, cobalt chloride and other 'HIF-mimetics' are therefore attracting attention as potential preconditioning therapeutics.

The HIF2α isoform is regulated in a similar way. It exists primarily in endothelial cells and, although it seems to be important in embryonic vasculogenesis, it does not seem to be induced in response to hypoxia in the neonate brain (Bernaudin et al., 2002) as it is in the adult brain (Wiesener et al., 2003). Details regarding transcriptional regulation by HIF2α are still unclear; although it may co-transactivate some genes with HIF1α, through the influence of kinases and other regulators, HIF2α might be more active on the promoters of endothelial cell-specific survival genes (Ralph et al., 2004) associated with angiogenesis, vascular remodelling, and endothelial cell homeostasis.

b) Effectors and genomic reprogramming

The induction of ischemic tolerance is accompanied by substantial change in gene expression, suggesting that preconditioning stimulates a fundamental genomic reprogramming of cells that confers cytoprotection and survival (Stenzel-Poore et al., 2007). The genomic response after ischemic preconditioning is a signature of the

complex interplay of multiple signalling pathways, highly specialized in different cell types of the brain seem to refine the cellular and systemic response to combat the noxious stimulus. Hundreds of genes are either upregulated or downregulated in response to ischemic preconditioning stimuli (Bernaudin et al., 2002; Tang et al., 2006). Changes in gene expression differ between harmful ischemia and ischemic preconditioning (Stenzel-Poore et al., 2003). Tolerance induced by ischemic preconditioning changes the expression of genes involved in the suppression of metabolic pathways, immune responses, ion-channel activity and it is regulated by transcription factors but also depends on epigenetic mechanisms such as DNA methylation and histone modification. In fact, inhibition of DNA methylation and increased histone acetylation have neuroprotective effects in experimental models of stroke (Endres et al., 2000). Ischemic preconditioning induces substantial changes in acetylation of the H3 and H4 histones, which are associated with neuroprotection (Yildrim et al., 2008). These changes seem to facilitate widespread changes in transcription and support the concept of genomic reprogramming by preconditioning. Pharmacological inhibition of histone deacteylases with trichostatin A leads to increased histone acetylation and has a neuroprotective effect (Endres et al., 2000). Epigenetic mechanisms of gene regulation might therefore provide another avenue of therapeutic neuroprotection (Stenzel-Poore et al., 2003). Primarily through recent oligonucleotide-based DNA microarray investigations and novel gene identification methods, the transcriptome of the ischemia-tolerant brain is gaining definition. Several themes are emerging from this work. First, genes representing many larger ‘families’ participate in the response — given the robustness of the protection in most tolerance models, this was not unexpected. Many share common promoter sequences that are responsive to preconditioning-activated transcription factors. On balance, genes functionally related to the cell cycle, metabolism, inflammation, excitotoxicity, ion homeostasis, signal transduction and so on are differentially expressed in response to preconditioning. Second, genes activated by preconditioning stimuli are often quite distinct from those associated with ischemia alone; similarly, the genomic expression pattern in response to ischemia is unique in a preconditioned animal, and differs considerably from the pattern activated by either preconditioning or ischemia in a non-preconditioned animal. Third,

although important and interesting from a cell survival standpoint, the genomic response is not simply one of activation of normally quiescent survival genes. Rather, gene repression also occurs, and, in fact, dominates the overall response to ischemia in a preconditioned brain. Finally, changes in gene transcription after preconditioning, or after ischemia in a preconditioned brain, have distinct temporal profiles. For example, following preconditioning, some genes are expressed or repressed within minutes or hours (adenosine A2a receptor and vascular endothelial growth factor (VEGF), whereas others are affected days later (β-actin, serine/threonine protein kinase, arachidonate 12-lipoxygenase, calretinin, the S100A5 calcium-binding protein, dihydropyrimidine dehydrogenase and the zinc transporter ZnT1 (Stenzel-Poore et al., 2003; Tang et al., 2006). At least three unique time windows of phenotypic, effector mechanism expression — that is, before, during and after ischemia — are worthy of closer examination. The first is the period of time that follows preconditioning but is prior to the lethal ischemic insult. At the cellular level, significant alterations in protein composition (such as elevations in the concentrations of various stress proteins, kinases and phosphatases, transcription factors, metabolic enzymes, transport and structural proteins, trophic factors and plasticity-related molecules, and cell cycle/apoptosis-related proteins) are underway or established prior to ischemia. These new protein signatures are representative of a tissue that is prepared to resist the threat of an impending ischemic event. The second window of effector mechanism expression relates to the period of ischemia itself, such that an identical insult is experienced as less severe in a preconditioned brain compared with a naive brain. In part, this occurs through increased inhibitory neurotransmitter levels, lower intra-ischemic levels of extracellular glutamate, reductions in calcium influx secondary to altered AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor subunit compositions, adenylation states, and desensitization status (Grabb et al., 2002; Dave et al., 2005).

Pathophysiological events occurring during postischemic reperfusion are also positively modulated in ischemic tolerance. Effector mechanisms acting during this time window broadly serve to stabilize the cell's energy and protein metabolism, ameliorate the actions of glutamate, reactive oxygen and nitrogen species and other injurious mediators, and reduce post-ischemic inflammation.

Tolerance mechanisms working at the level of endoplasmic reticulum function result in improved rates of recovery of neuronal protein synthesis; specifically, better preservation of the reinitiation and elongation steps of transcription, and increased levels of the chaperone glucose regulated protein 78 kDa (GRP78) are realized (Hayashi et al., 2003). In addition, post-ischemic protein aggregation, redistribution and ubiquitin-conjugation are reduced. The rate of repair of oxidative DNA damage is also enhanced. Overall, many facets of ischemic mitochondrial dysfunction, including changes in the redox activity of the respiratory chain components, oxidative phosphorylation deficits, calcium overload and the initiation of apoptosis, are abrogated in ischemic tolerance.

Compared with the naive brain, the preconditioned brain also shows post-ischemic increases in the Mn and CuZn isoforms of superoxide dismutase (Bordet et al., 2000; Garnier et al., 2001), glutathione peroxidase and glutathione reductase, uric acid, and haeme oxygenase-1, which enhance the tissue's free radical scavenging capabilities. Reductions in proinflammatory cytokine synthesis, and an upregulation of other feedback inhibitors of inflammation, including decoy receptors and intracellular signalling inhibitors, contribute to the promotion of an anti-inflammatory phenotype following ischemia.

1.1.2 Clinical use: challenges and opportunities

Ischemic brain injuries, resulting either from global or focal decreases in perfusion, are among the most common and important causes of disability and death worldwide. The consequences of global cerebral ischemia after cardiac arrest (and successful resuscitation), focal occlusions or disruption of brain vessels (ie, stroke, including subarachnoid haemorrhage and intraparenchymatous haemorrhage), and ischemic brain damage after cardiac or brain surgery affect many millions of people in the USA alone. Research into preconditioning aims at developing new therapeutic approaches to benefit these patients. Preconditioning is an attractive experimental strategy to identify endogenous protective or regenerative mechanisms that can be therapeutically induced or supplemented. On the other hand, preconditioning could be used as a therapeutic technique by inducing tolerance in individuals in whom ischemic events are anticipated,

such as high-risk surgical cohorts or patients with subarachnoid haemorrhage or transient ischemic attack. Many articles have reviewed various features of ischemic preconditioning, tolerance, and endogenous neuroprotection in the brain (Dirnagl and Meisel, 2008).

The options for inducing preconditioning and tolerance are not specific to the type of injury, which is important for the clinical adaptation of this technique. The table 1 gives an overview of the different types of preconditioning (cross, remote, immunological, pharmacological, anaesthetic, mimetic, and effectors).

	Principle	Example
Cross	Preconditioning stimulus is different from the noxious stimulus against which it protects	In the earliest description of the technique[20] the same stimulus was used to induce preconditioning and damage (trauma), whereas trauma was used by Janoff[21] to induce protection against sterile sepsis and bowel ischaemia
Remote	Preconditioning of one organ or system leads to protection of a different (remote) organ	The prototypical approach for remote preconditioning is the initiation of short ischaemic intervals to a limb to protect organs such as the heart[22,23] and brain.[24–26] Remote preconditioning might indicate a crosstalk between the brain and the rest of the body in response to stress. Randomised clinical trials have already shown the efficacy of this strategy for the heart.[27,28] Remote preconditioning is a particularly attractive strategy to protect organs that are highly susceptible to damage but that are difficult to target, such as the brain
Immunological	Preconditioning with an inflammatory stimulus	Low doses of the gram-negative bacterial cell wall component lipopolysaccharide can protect the heart[29,30] or brain.[31–34] Because the immune system is preconditioned, immunological preconditioning can be thought of as remote preconditioning, but other organs, such as the heart, are protected
Pharmacological	Pharmacological compounds that trigger the signalling cascades of preconditioning (without a physical stimulus)	One of the earliest examples of pharmacological preconditioning was the mild inhibition of the respiratory chain with nitropropionic acid or acetylsalicylic acid,[35–38] which induced cellular changes such as those seen with hypoxia. Another relevant example is the use of the iron chelator desferrioxamine,[39] which induces nuclear translocation of the transcription factor hypoxia-inducible factor 1, with consequent expression of a plethora of hypoxia-inducible genes, including erythropoietin, vascular endothelial growth factor, and hexokinase
Anaesthetic	Short application of any one of many different classes of anaesthetics can induce an ischaemia-protected state	Anaesthetic preconditioning is effective in the heart[40,41] and brain.[42–45] This protection might happen within minutes or might be delayed by many hours or days. For practical reasons, anaesthetic preconditioning is an appealing therapeutic option that has already been tested in randomised controlled trials for cardiac protection
Mimetics	Compounds that emulate the main danger signal can lead to preconditioning	Examples include inhibitors of mitochondrial respiration, stabilisers of hypoxia-inducible factor 1 (signal: hypoxia), or lipopolysaccharide (signal: infection)[46,47]
Effectors	The downstream mediators of protection	These effectors include proteins such as erythropoietin, vascular endothelial growth factor, and BCL family proteins. These proteins are attractive pharmacological candidates for preventing the consequences of anticipated ischaemia

Table: Types of preconditioning

Dirnagl et al., *Lancet Neurol 2009*, ***8*:** 398, 412. **Table 1**

Table 1 Type of preconditionings. Adapted from Dirnagl et al, with permission from Elsevier.

Preconditioning can protect the brain either almost immediately after stimulation (known as early, rapid, or classical preconditioning) or after a delay of 1 to 3 days to induce protein-synthesis-dependent protection (delayed preconditioning) (Ratan et al., 2007). Most stimuli can cause both early and delayed preconditioning, and most, but not

all, stimuli leave an unprotected time window between early and delayed preconditioning (Ren et al., 2008). Irrespective of the rapidity of onset, protection by preconditioning usually never lasts more than a few days. Of note, a recent study showed that a series of repetitive hypoxic preconditioning stimuli can induce neuroprotection in the retina that last many weeks. Such long-term tolerance might be associated with neuronal plasticity, including long-term potentiation, or long-lasting cellular memory associated with immune tolerance. Rapid preconditioning is appealing practically and clinically because this technique can be applied therapeutically in the same setting as procedures with high risks of complication, such as cardiac or brain surgery. Most of the experimental and clinical research in cardiology has thus focused on early preconditioning. Conversely, because protection conferred by delayed preconditioning seems to be more robust for the brain than that conferred early, delayed preconditioning has received more attention in neurology. However, although there are effective protocols for early preconditioning for the brain, there are few formal comparisons of early and delayed procedures for neuroprotection (Perez-Pinzon, 2004). The recently described event of postconditioning is commonly discussed in the context of preconditioning. Postconditioning is a form of therapeutic reperfusion by which an organ is intermittently reperfused, for example by a stuttering opening and closing of an experimentally (or clinically, as in angioplasty or in organ transplantation) occluded artery. Postconditioning is a new neuroprotective approach for lessening injury in focal ischemia and reperfusion (Zhao, 2007). The benefits of this procedure seem to be mediated, at least in part, by molecular pathways similar to those that control preconditioning (Scartabelli et al., 2008; Pignataro et al., 2008). As an experimental strategy postconditioning, like preconditioning, might lead to the discovery of endogenous mechanisms of protection and repair. However, postconditioning is unlikely to be of clinical relevance as a therapeutic strategy for ischaemic brain damage (Dirnagl et al.; 2009).

GLUTAMATE AND GLUTAMATE RECEPTORS

2.1 Glutamate synthesis, release and uptake

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS). In addition to its immediate impact as an excitatory amino acid, it has a role in long-term neuronal potentiation, as a proposed molecular substrate for learning and memory (Attwell, 2000; Meldrum, 2000; Tapiero et al., 2002; Tzschentke, 2002). Glutamate may also be a potent neurotoxin, and glutamate excitotoxicity has been implicated in the pathogenesis of many devastating human neurological diseases such as stroke, amyotrophic lateral sclerosis (ALS) and epilepsy (Smith, 2000). It is found throughout the mammalian brain and participates in many metabolic pathways (Attwell, 2000; Petroff, 2002).

Glutamine and α-ketoglutarate are thought to be the major precursors of glutamate, which is subsequently packaged into vesicles for future release into the synaptic cleft (Tapiero et al., 2002) (Fig. 3).

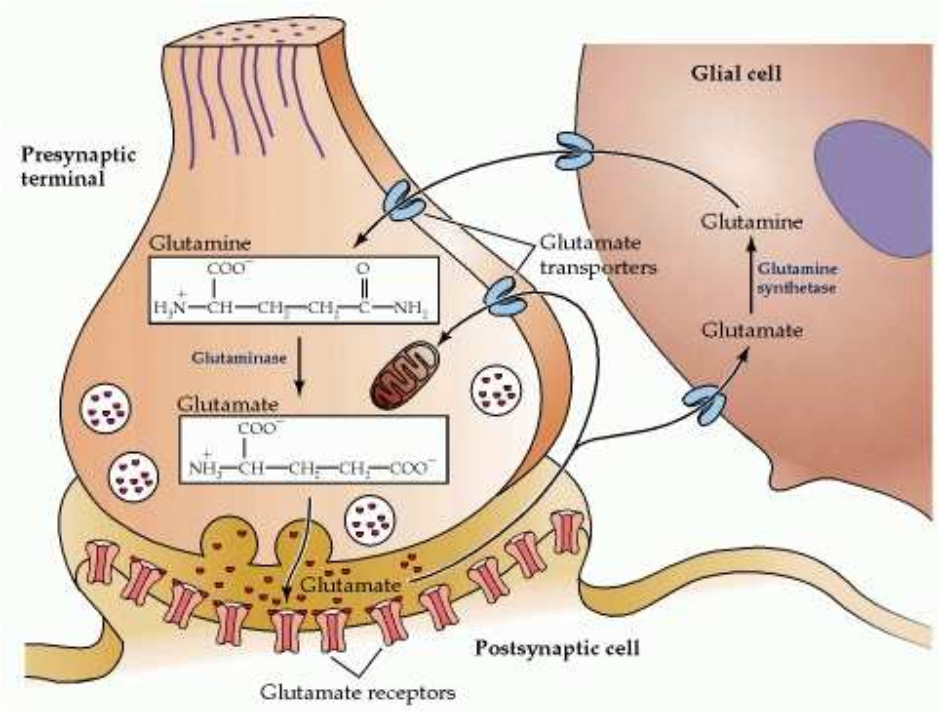


Fig. 3

Fig. 3 Synthesis and metabolism of glutamate.

Glutamine is taken up into the pre-synaptic terminal via an active, Na^+-dependent uptake protein (Anderson & Swanson, 2000; Daikhin & Yudkoff, 2000). It is then transported to mitochondria, where it is converted via phosphate activated glutaminase to glutamate and ammonia. α-ketoglutarate is also actively taken up into the pre-synaptic terminal, where it is transaminated into glutamate (Daikhin & Yudkoff, 2000). The glutamate in the terminal is then actively taken up into vesicles for future release. Upon release into the cleft, the glutamate either (i) is bound to pre- and post-synaptic receptors, (ii) is actively taken back up via a glutamate transporter and repackaged, (iii) diffuses away from the cleft, or (iv) is internalised by glial glutamate transporters (Anderson & Swanson, 2000; Attwell, 2000; Daikhin & Yudkoff, 2000) (Fig. 3).

Five different mammalian glutamate transporters have been cloned (Attwell, 2000; Masson et al., 1999; Meldrum, 2000). Apart from cells in the retina and cerebellum, which express high levels of tissue-specific transporters, the transporters expressed most commonly throughout the brain are GLT-1/EAAT2 in glial cells and EAAC1/EAAT3 in neurons (Attwell, 2000; Masson et al., 1999; Smith, 2000). Once in glial cells, the glutamate is metabolised via glutamine synthase into glutamine or metabolised into α-ketoglutarate by either glutamate oxaloacetate transaminase or glutamate dehydrogenase (Anderson & Swanson, 2000; Meldrum et al., 1999). This glutamine and α-ketoglutarate are then actively transported out of the glial cells and back into the pre-synaptic terminals for subsequent re-synthesis of glutamate (Meldrum et al., 1999). The extracellular concentration of glutamate is normally very low (≈1 μM) (Anderson & Swanson, 2000; Attwell, 2000) (see figure above).

Glutamate is released from vesicles in pre-synaptic terminals by a Ca^{2+}-dependent mechanism that involves voltage-dependent calcium channels (Anderson & Swanson, 2000; Meldrum, 2000). The glutamate concentration within the vesicle is thought to be approximately 100 mmol/L; release of a single vesicle produces an excitatory post-synaptic potential (EPSP) (Meldrum, 2000). Glutamate may also be ‘‘released’’ by reverse operation of the glutamate transporters (Anderson and Swanson, 2000). This will occur when the Na^+ and K^+ gradient across the membrane is reduced during cerebral ischemia (Meldrum, 2000).

The synaptic release of glutamate is controlled by a wide range of pre-synaptic receptors (Anderson & Swanson, 2000). These include not only the group II and group III metabotropic glutamate receptors but also cholinergic (nicotinic and muscarinic) receptors, adenosine (A1), κ-opioid, γ-aminobutyric acid $(GABA)_B$, cholecystokinin and neuropeptide Y (Y2) receptors (Anderson & Swanson, 2000; Meldrum, 2000).

Glutamate, like many other neurotransmitters, exerts its pleiotropic roles by means of multiple receptor proteins. Two main classes of glutamate receptors have been identified: ionotropic glutamate receptors (iGlus) and metabotropic glutamate receptors (mGlus), (Hollmann & Heinemann 1994; Nakanishi et al., 1998).

2.2 Ionotropic glutamate receptors (iGluRs)

Ionotropic glutamate receptors (iGluRs) are responsible for most fast excitatory signalling in the brain, and are thought to contribute to the synaptic plasticity that has been implicated in our ability to learn and form memories. In keeping with the physiological importance of iGluRs, their dysfunction is implicated in a range of neuropathologies, including epilepsy, stroke damage and the perception of pain (Dingledine et al., 1999).

Pharmacologically distinct subfamilies of iGluR have been identified, characterized by their affinities for the synthetic agonists N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainic acid (KA) (Hollmann et al., 1994).

2.2.1 NMDA receptors

The NMDAR channel is made up of a combination of three different subunits, NR1– 3. When activated, the NMDAR allows the influx of cations, though most notably calcium. Excessive intracellular calcium concentrations cause the activation of intracellular pathways leading to both physiological (i.e. learning and memory) and pathological processes (i.e. excitotoxic injury). The NMDAR exhibits a complex gating mechanism, requiring not only binding of various ligands but also cellular depolarisation. The traditional NMDAR is heterotetramer composed of two NR1

subunits and two NR2 subunits. The NR1 subunit is made up of ~938 amino acids and has eight splice variants. Together, two NR1 subunits form the ion channel proper and exhibit all the classical properties attributed to NMDARs, including glutamate activation, magnesium block, zinc inactivation, glycine activation, interactions with polyamines and pH sensitivity (Fig. 4).

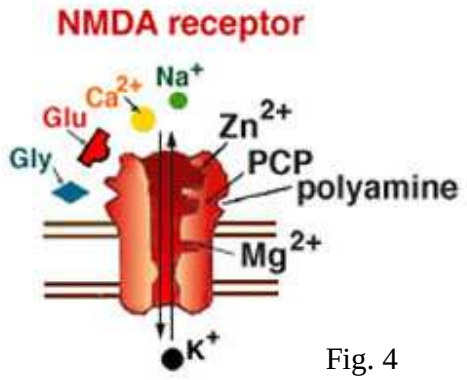


Fig. 4

In addition to glutamate binding, Mayer et al. (Mayer et al.; 1984) showed that depolarisation of the NMDAR-expressing neuron is necessary to electrostatically remove a magnesium ion normally blocking current entry at the level of the ion channel pore. Zinc can alternatively inhibit the NMDAR-mediated currents elicited by glutamate (Mayer et al.; 1989) (Fig. 5). Physiologically, magnesium is removed by the activation of other ionotropic glutamate channels (AMPA and kainate, to be discussed later). Glycine was shown by Johnson and Ascher (Johnson et al.; 1987) as a mandatory cofactor with glutamate necessary for NMDAR channel opening. The role of polyamines in NMDAR modulation is less clear and may participate in both cell growth and cell death. Spermine, a polyamine, can potentiate NMDAR currents at low concentrations, but also reduce currents in a voltage-dependent manner at higher concentrations (Rock, Macdonald; 1992). Spermine has also been shown to increase the frequency of channel opening and glycine affinity whilst acting as a voltage-gated NMDAR channel blocker when applied extracellularly (Benveniste and Mayer; 1993) To add to the complexity of the polyamine/NMDAR interaction, many of these effects

are dependent on the subunit constituents of the NMDAR channel (Williams, 1997) As such, the physiological significance of these interactions is still under investigation.

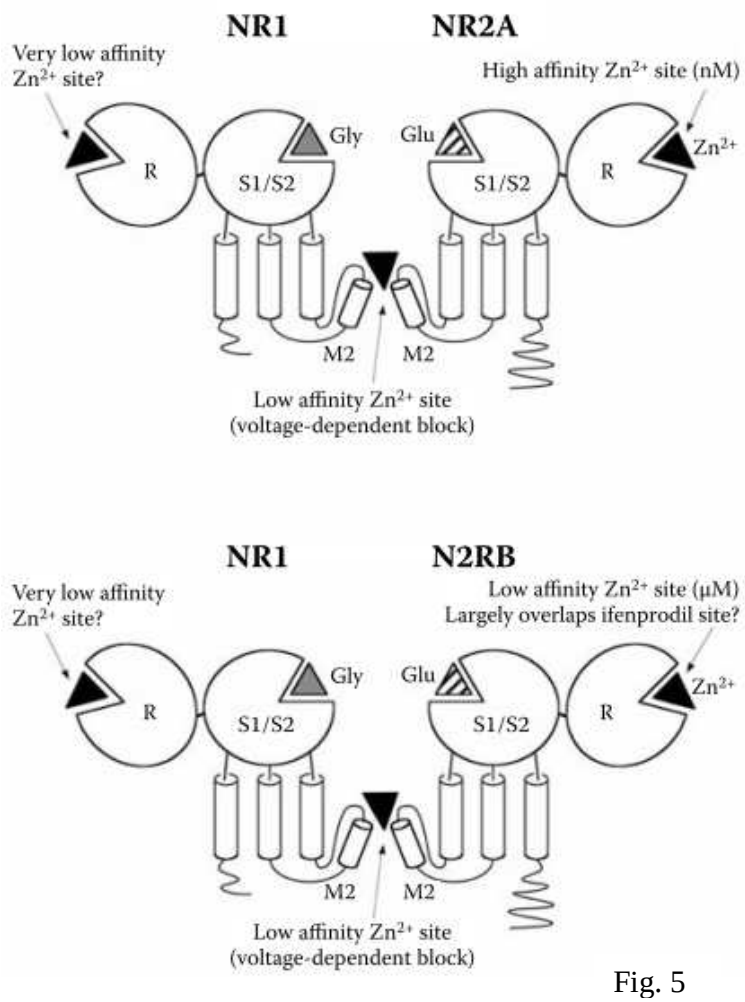


Fig. 5

Protons inhibit NMDAR current via a direct interaction with the NR1 subunit on a single lysine residue (Traynelis and Cull-Candy; 1990) which may be alleviated by spermidine and other polyamines (Ransom and Stec, 1988). Physiologically, alterations in pH common after acute neurological insults (such as stroke and traumatic brain injuries) can modulate NMDAR function, but also other acid-sensing ion channels (ASICs, to be discussed later). NR2 subunits have more of a regulatory and refining role in NMDAR function. Currently, four NR2 subtypes exist (NR2A–D), with NR2A widely distributed in the brain, NR2B expressed primarily in the forebrain, NR2C found predominantly in the cerebellum and NR2D localised to the thalamus (Buller et al.; 1994). Within the

NMDAR complex, NR2 subunits modulate the characteristics of the NR1 ion channel pore mentioned above, though recently, NR2 subunits have been ascribed another role in postsynaptic architecture. Specifically, NR2B subunits have been shown to bind and link postsynaptic proteins, creating specialised postsynaptic microenvironments (Sattler et al.; 1999). This close association is accomplished by postsynaptic densities comprising scaffolding proteins which allow the spatial approximation of intracellular enzymes (e.g. neuronal nitric oxide synthase) with ionic second messengers (e.g. Ca2+ influx from NMDARs). The physiological effect of this spatial relation can be dramatic and has been shown in animal studies to reduce histological damage as well as neurological dysfunction after stroke (Aarts et al.; 2002). Recent studies have shown a third subunit associated with the NMDA receptor gene family (Chatterton et al.; 2002). The NR3 subunit is expressed in two isoforms: NR3A, which is expressed throughout the CNS, and NR3B, which is expressed primarily in motor neurons. Preliminary evidence suggests that NR1/NR3A and NR1/NR3B complexes are not activated by NMDA or glutamate, but rather elicit an excitatory Ca2+-impermeant response via glycine (Lau and Tymianski ; 2010).

2.2.2 AMPA receptors

The AMPA and kainate classes of glutamate receptors belong to the same superfamily as the NMDARs and share approximately 25% homology (Fig. 6).

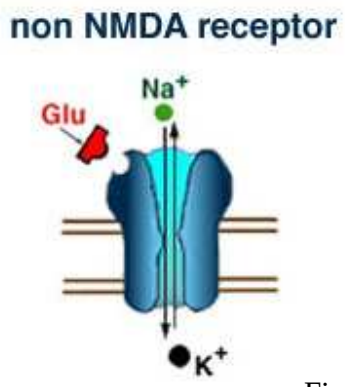


Fig. 6

AMPA receptors (AMPARs) are made up of a combination of four subunits (GluR1–4) and require only glutamate application for activation (Fig. 7).

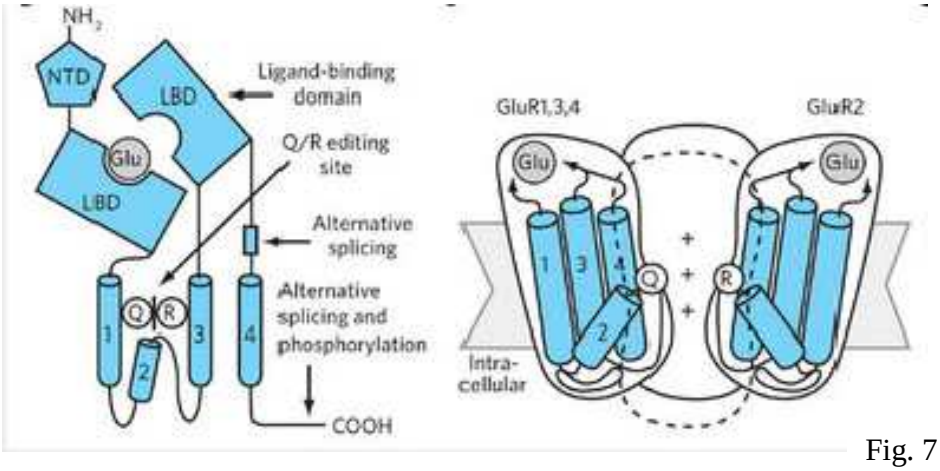


Fig. 7

The specificity of cation influx of AMPARs is variable, however, and is governed primarily by subunit composition. GluR1, GluR3 and GluR4 all display strong inwardly rectifying current–voltage and calcium permeability, whereas the GluR2 subunit removes calcium permeability (Burnashev et al. 1995; Geiger et al, 1995; Hollmann et al, 1991).

Physiologically, AMPARs are thought to regulate the fast excitation required to remove the magnesium block of nearby NMDARs. Kainate receptors are made up of subunits from GluR5–7 (also known as GluK5–7) and KA1–2 (also known as GluK1–2). The properties of kainate channels are similar to AMPARs in that they allow ion flux directly following glutamate application, though they are mostly impermeant to calcium ions. Although AMPARs are localised mostly in the postsynaptic membrane, studies have shown that kainate receptors may be localised in both the presynapse (Chittajallu et al; 1996) and post-synapse (Castillo et al, 1997; Vignes and Collingridge, 1997). Some studies have shown that the application of kainate can stimulate glutamate release (Schmitz et al.; 2001), whereas others have shown that kainate application inhibits presynaptic glutamate release (Frerking et al.; 2001). Postsynaptically, kainate channels serve a similar purpose as AMPARs in alleviating magnesium block in NMDARs (Lau and Tymianski; 2010).

2.3.1 Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluRs) are single peptide seven-transmembrane spanning proteins linked to intracellular G-proteins (Fig. 8). It was originally believed that all

metabotropic glutamate receptors used G-proteins as a transduction molecule, though recent evidence suggests that G-protein-independent signalling can occur (Heuss et a.; 1999). Currently, eight different mGluRs (mGluR1–8) are known and are classified into three groups (groups I, II and III) based on sequence homology and their intracellular effects.

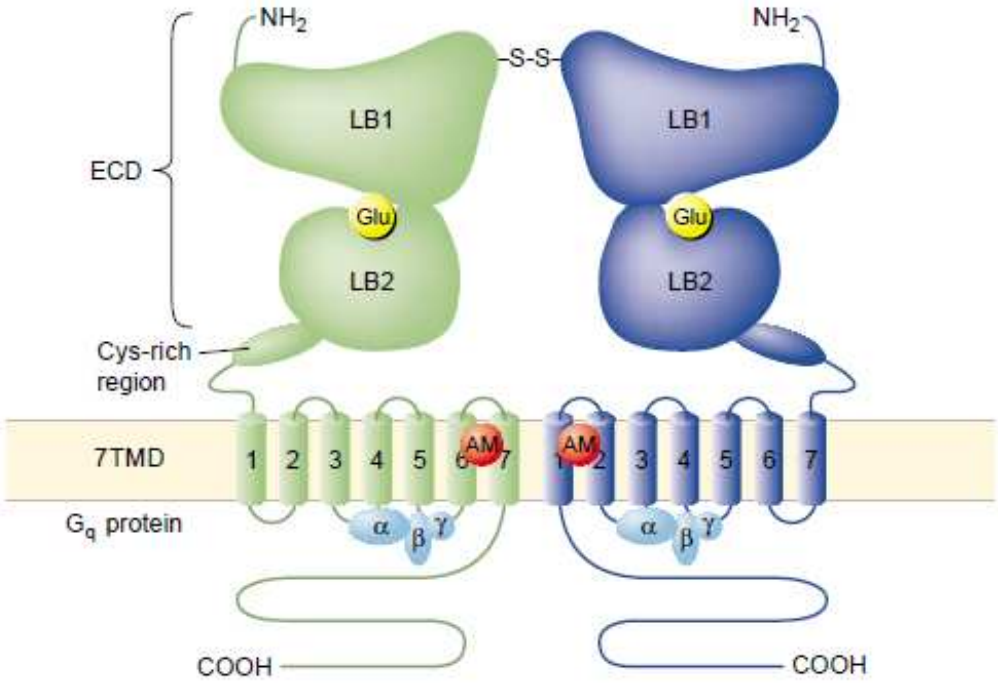


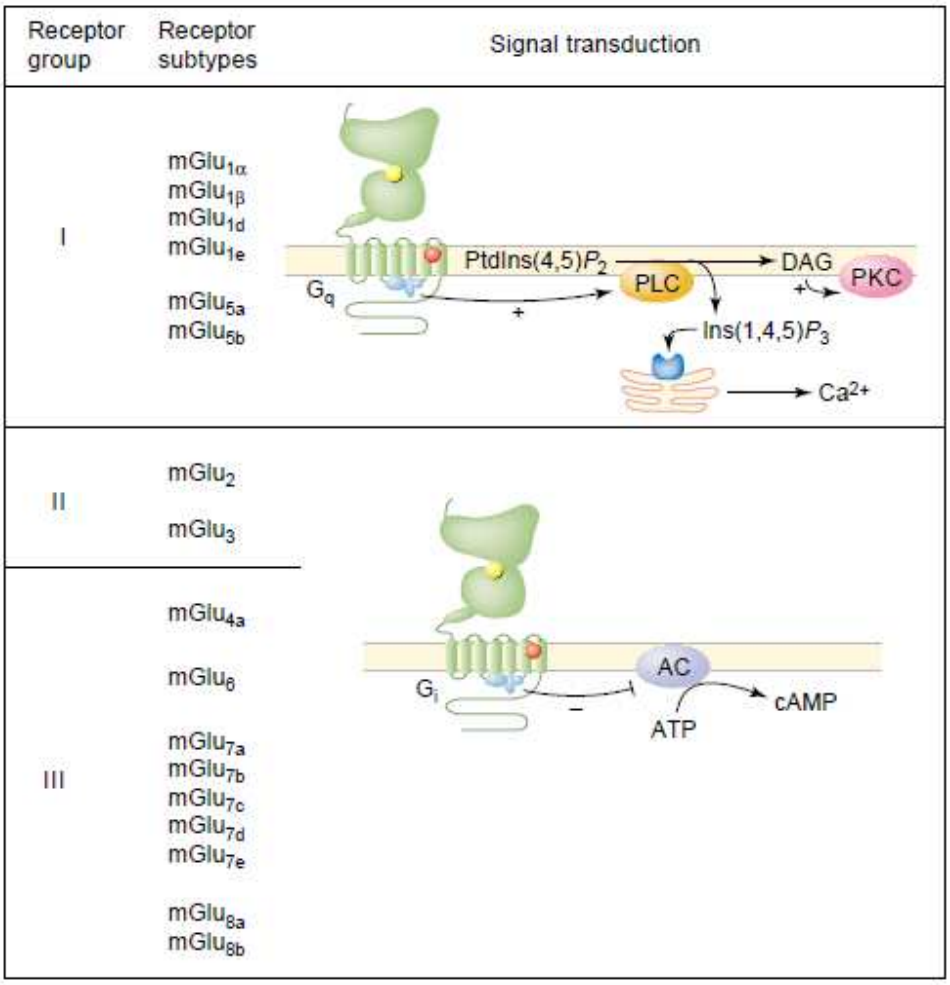
Fig. 8

Pellegrini-Giampietro D.E. *Trends Pharmacol Sci* 2003;**24** (9):461-70.

Fig. 8. Schematic representation of a homodimeric group I mGlu receptor stabilized by a disulfide bond. The large N-terminal extracellular domain (ECD) of each protomer consists of two globular ligand binding (LB1 and LB2) lobes linked by a hinge region where the agonist glutamate (Glu) binds. Agonists are thought to stabilize an active closed conformation by making contacts with residues of both lobes, whereas competitive antagonists bind at the same site as agonists but are unable to stabilize the active conformation. The ECD is connected to the seven-transmembrane domain (7TMD) by a cysteine-rich region. Gq proteins are associated with intracellular loops 2 and 3, whereas allosteric modulators (AMs) interact with TMD 6 and 7 (the coexisting tridimensional interaction with TMD 3 is not shown).

a) Signal transduction mechanisms of group I mGlu receptors

Group I mGlu receptors include mGlu1 and mGlu5 receptors and are coupled to phospholipase C (PLC) (Fig. 9).



Pellegrini-Giampietro D.E. *Trends Pharmacol Sci* 2003;**24** (9):461-70.

Fig. 9

Fig. 5. Classification of metabotropic glutamate (mGlu) receptors. The features of each mGlu receptor family group are shown. Greek letters are used for mGlu1a and mGlu1b splice variants, according to their original designation. Splice variants mGlu1c and mGlu4b are not included because data from different laboratories strongly suggest that they do not exist as full-length transcripts but probably represent recombination artifacts (F. Ferraguti, pers. commun.). Group I mGlu receptors are coupled to phospholipase C (PLC) via Gq proteins: their stimulation promotes the breakdown of phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P2] into inositol (1,4,5)-trisphosphate [Ins(1,4,5)P3] and diacylglycerol (DAG). Ins(1,4,5)P3 releases Ca^{2+} from intracellular stores, whereas DAG activates protein kinase C (PKC). Group II and III mGlu receptors are negatively coupled via Gi/o proteins to adenylyl cyclase (AC): their stimulation inhibits the formation of cAMP from its precursor ATP.

PLC cleaves phosphatidylinositol-4,5-bisphosphate into inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), which release Ca^{2+} from intracellular stores and activates PKC, respectively. Mobilization of intracellular Ca^{2+} in response to mGlu1 receptor activation occurs through both the IP3 receptor and the ryanodine receptor present on the surface of the endoplasmic reticulum (del Río et al., 1999; Fagni et al., 2000). It should be highlighted that activation of mGlu1 receptors can also increase intracellular Ca^{2+} concentrations through the opening of voltage sensitive calcium channels (VSCCs) and nonselective cation channels. In addition to the classical activation of PLC and release of Ca^{2+} from intracellular stores, stimulation of mGlu1 and mGlu5 receptors can trigger a variety of signaling cascades and modulate the activity of ion and ligand-gated channels through functional coupling with other transduction pathways such as adenylyl cyclase, phospholipase A2, phospholipase D (Pellegrini-Giampietro et al., 2003), tyrosine kinase and mitogen-activate protein kinase.

Activation of mGlu1 and mGlu5 receptors might thus promote multiple processes that are known to participate in the pathological cascade leading to post-ischemic neuronal death, including: (i) an increase in neuronal excitability caused by the activation of inward cationic currents or the reduction of K^+ conductances; (ii) a rise in cytosolic free Ca^{2+} via a facilitatory coupling between ryanodine receptors and L-type Ca^{2+} channels or direct Ca^{2+} influx from the extracellular space through NMDA receptors and L-type channels (Fagni et al., 2000); (iii) an enhancement of the release of glutamate that correlates with the neurotoxic effects of group I mGlu receptor agonists; (iv) a potentiation of NMDA and AMPA receptor responses that has been observed in a large number of brain areas; and (v) activation of the mitogen activated protein kinase pathway via PKC (Calabresi et al., 2001).

b) Signal trasduction mechanisms og group II/III mGlu receptor

Group II mGluRs include mGluR2s and mGluR3s. These mGluRs cause a decrease in adenylyl cyclase signalling, resulting in downstream inhibition of voltage-dependent calcium channels (Chavis et al., 1994; Tanabe et al., 1993). These receptors are found at

both the presynapse and the post-synapse (Ohishi et al.; 1994). Since presynaptic calcium is integral to neurotransmission, group II mGluRs modulate neurotransmission via their action on voltagegated calcium channels. Group III mGluRs include mGluR4s, mGluR6s, mGluR7s and mGluR8s. These mGluRs have similar properties to the group II mGluRs and are also associated with a decrease in adenylyl cyclase signalling, resulting in downstream inhibition of voltagedependent calcium channels (Tanabe et al., 1993). These mGluRs are also found in both the presynaptic and postsynaptic terminals (Bradley et al.; 1996) and similar to group II receptors, these mGluRs modulate neurotransmission by functioning as autoreceptors and modulating calcium channel influxes. With respect to excitotoxicity, the group I family of mGluRs is associated with the post-synapse and appear to potentiate NMDAR-mediated Ca2+ influx (Bruno et al; 1995). The remaining metabotropic glutamate receptor heterodimers comprising mGluR2,3 and mGluR4,6,7,8 subunits are linked to the inhibition of cAMP formation (see the figure above). These receptors are primarily found in the pre-synapse and reduce Ca2+ influx via NMDARs (Bruno et al.; 1995). The above evidence shows that alterations in the amino acid sequences of glutamate receptor subunits could alter calcium permeability or other properties which could lead to worsening excitotoxicity. However, whilst there are currently no known channelopathies attributable to glutamate receptor mutations directly, some recent evidence has suggested that mutations in postsynaptic proteins bound to AMPARs may exert a channelopathy-like effect in epilepsy (Fukata et al.; 2006). Improved knowledge of the pharmacology and distribution of glutamate receptors may also lead to the development of improved receptor antagonists for the treatment of neurologic disease. Finally, the linkage of ionotropic receptors to intracellular enzymes may provide alternative targets for pharmacological neuroprotection following excitotoxic insults (Lau and Tymianski; 2010).

3. Membrane Associate Guanylate Kinases (MAGUK) proteins

3.1 Structure and function of membrane associated guanylate kinase (MAGUK) proteins

Neurotransmitter receptors at excitatory synapses are clustered at the postsynaptic density (PSD), an electrondense structure located beneath the postsynaptic membrane (Fig. 10).

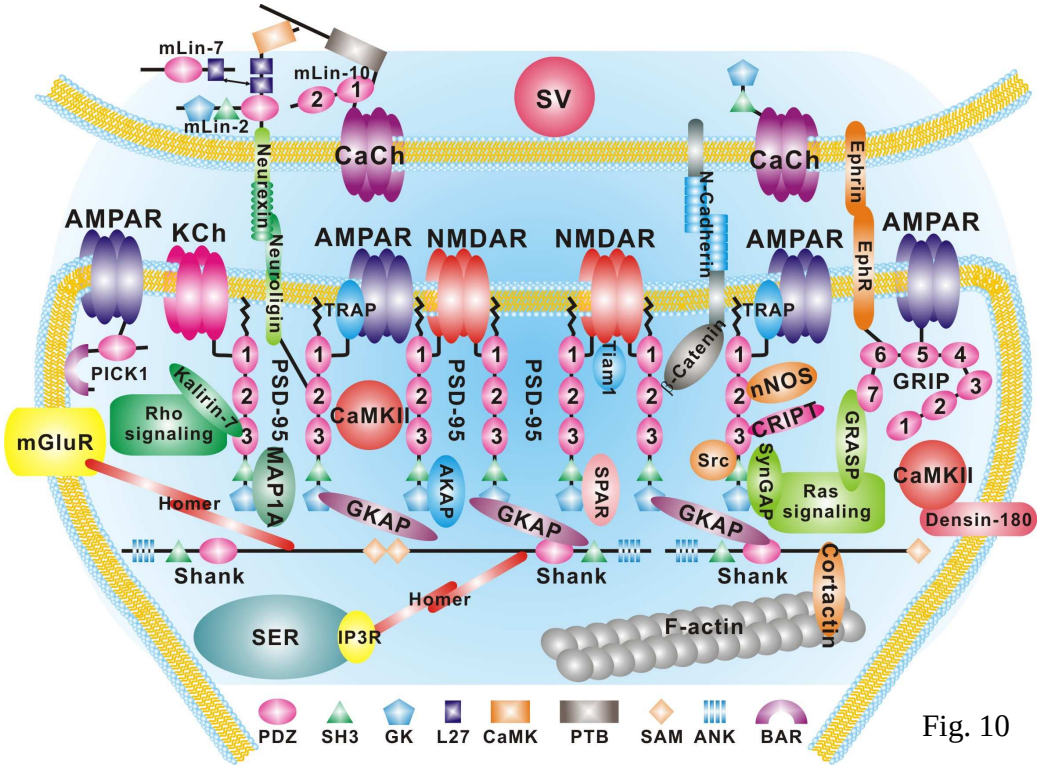


Fig. 10

Fig. 10 Neurotransmitter receptors at excitatory synapses clustered at the postsynaptic density (PSD) and scaffold proteins.

The PSD also contains scaffolding proteins, the prototypical one being PSD-95 that is a member of the neuronal MAGUK family of proteins. The PSD-95-like subfamily of MAGUKs (PSDMAGUKs) includes PSD-93 (also known as Chapsyn-110), SAP-102 and SAP-97 (Figure 1b,c). PSD-MAGUKs share a common domain structure organization with three N-terminal PDZ domains, a Src homology 3 (SH3) domain and a C-terminal catalytically inactive guanylate kinase (GK) domain. Despite these similarities in domain structure, PSD-MAGUKs are particularly distinct in their N-terminal amino acid sequences. Furthermore, each PSD-MAGUK has several isoforms,

which differ in their N-terminal amino acid sequences and domain structure organizations. For example, whereas the N-terminal region of the a-isoforms of PSD-95 and SAP-97 contain two cysteines that undergo palmitoylation, the bisoforms contain instead N-terminal L27 domains. Although PSD-MAGUKs are widely expressed throughout the central nervous system, the relative abundance of each PSD-MAGUK in specific brain regions varies with development. In the hippocampus, for example, SAP-102 expression is high in late embryonic development and early postnatal life, and decreases with age. By contrast, the expression of PSD-95 and PSD-93 is low during early postnatal life but increases substantially by one month of age (Sans. et al. 2000).

Each member of MAGUK protein family is distributed differently in the brain cell compartments. PSD-95 and PSD-93 are highly enriched in the PSD, especially due to their high palmitoylation degree (El-Husseini et al., 2000). SAP102 and SAP97 are found in dendrites and axons and are abundant in the cytoplasm as well as at synapses. Although they show similar specificities of protein interaction *in vitro,* MAGUK family members interact with different (but overlapping) sets of proteins *in vivo*. The COOH-terminal cytoplasmic tails of NR2 subunits of NMDA receptor directly interact, at least *in vitro*, with PDZ domains of all members of the MAGUK family (Kim and Sheng, 2004). In particular, the last three amino acids of the carboxyl termini of NR2A and NR2B subunits have a C-terminal consensus motif threonine/serine-Xvaline (T/SXV, where X is any amino acid) that has been demonstrated to be responsible for efficient binding to PDZ domains of MAGUK protein members such as PSD-95 (Niethammer et al., 1996). PSD-95, in turn, binds to the amino terminal of neuronal nitric oxide synthase (nNOS), a Ca2+-activated form of nitric oxide synthase (NOS), through its PDZ domain. Therefore, PSD-95 may concentrate nNOS near the NMDA receptor at postsynaptic sites in neurons (Christopherson et al., 1999) thereby connecting NMDA receptors to specific signal transduction pathways (Kennedy, 1998). On the other hand, the interaction with SAP97 appears to be more relevant for processing alpha amino- 3-hydroxy-5-methyl-4- isoxazole propionic acid (AMPA) receptor subunits (Rumbaugh et al., 2003; Mauceri et al., 2004) and NR2A subunits of NMDA receptors (Mauceri et al., 2007), whereas SAP102 is crucial for driving NR2B complexes to spines (Sans et al., 2003). Of relevance, modifications of MAGUK protein function in the glutamatergic

synapse have been recently described in several neurological disorders such as Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, Ischemia, Schizophrenia and neuropathic pain. In particular, modifications of MAGUK proteins interactions with NMDA receptors regulatory subunits are a common event in several neurodegenerative disorders (Gardoni et al., 2006; Aarts et al., 2002). Thus, a better knowledge and understanding of MAGUK structure and function as well as of the molecular events regulating MAGUK-mediated interactions in the glutamatergic synapse could lead to the identification of new targets for pharmaceutical intervention in neurodegenerative disorders.

3.2 Modulation of PSD-95 interaction with NR2B subunit as a possible stroke treatment

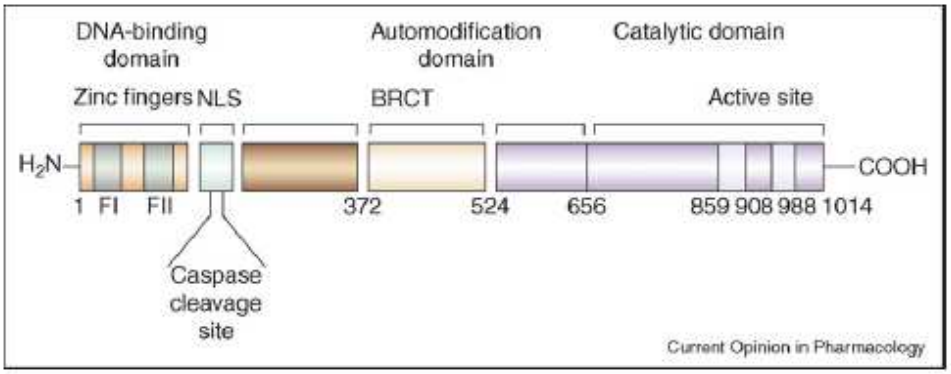
Neuronal injury caused by cerebral ischemia is believed to be mediated by excessive activation of glutamate receptors. Most of the studies focused on ischemia-induced changes in NMDA receptor complex in the postsynaptic density structure because of its temporal and spatial proximity to the initial events, which occur after an ischemic challenge. Changes in the composition and morphology of forebrain postsynaptic density have been reported to occur after an ischemic challenge. In addition, molecular interactions involving PSD-95 are modified by an ischemic challenge, in the most vulnerable CA1 region of the hippocampus. Ischemia also resulted in a decrease in the size of protein complexes containing PSD-95, but had only a small effect on the size distribution of complexes containing the NMDA receptor, indicating that molecular interactions involving PSD-95 and the NMDA receptor are modified by an ischemic challenge (Takagi et al., 2000). In addition, transient cerebral ischemia increases tyrosine phosphorylation of NMDA receptor subunits NR2A and NR2B, but has no effect on the amounts of individual NMDA receptor subunits in the postsynaptic density. The ischemia-induced increase in the interaction of NR2A and NR2B with the SH2 domains of Src and Fyn suggests a possible mechanism for the recruitment of signalling proteins to the synapse and may contribute to altered signal transduction in the post-ischemic hippocampus. Of great relevance, recent studies suggested to treat stroke

transducing neurons with peptides able to disrupt the interaction of NMDA receptor NR2B subunits with the postsynaptic density protein PSD-95 (Aarts et al., 2002). This procedure dissociated NMDA receptors from downstream neurotoxic signalling without blocking synaptic activity or calcium influx and protected cultured neurons from excitotoxicity, reducing focal ischemic brain damage in rats, and improved their neurological function.

4. Poly (ADP-ribose) polymerase and Poly (ADP-rybosil)ation

4.1 The PARP superfamily

Poly(ADP-ribose) polymerase-1 (PARP-1) also known as poly(ADP-ribose) synthetase and poly(ADP-ribose) transferase, is a nuclear enzyme present in eukaryotes. PARP-1 is a 116-kDa protein consisting of three main domains: the N-terminal DNA binding domain containing two zinc fingers, the automodification domain, and the C-terminal catalytic domain (Mazen et al., 1989; de Murcia and Menissier de Murcia, 1994; de Murcia et al., 1994; Schreiber et al., 1995; Smith, 2001) (Fig. 11). The primary structure of the enzyme is highly conserved in eukaryotes (human and mouse enzyme have 92% homology at the level of amino acid sequence) with the catalytic domain showing the highest degree of homology between different species; the catalytic domain contains the so-called PARP signature sequence, a 50-amino acid block showing 100% homology between vertebrates (Fig. 12).



Moroni F., Current Opinion in Pharmacology 2008, 8:96–103

Fig. 11

Fig.11 Schematic representation of PARP-1 domains. First, the amino (N)-terminal DNA-binding domain contains two zinc fingers, which are responsible for DNA binding and DNA-damage-induced PARP activation. NLS is a nuclear-localization signal. Second, the automodification domain is a central regulating segment with a breast cancer-susceptibility protein-carboxy (C) terminus (BRCT) motif, which is common in many DNA repair and cell cycle proteins. Third, the C-terminal domain contains the NAD-binding region and the catalytic center of PARP

PARP-2 (62 kDa) is the second member of the family and was discovered as a result of the presence of residual 10– 15% PARP activity in fibroblast derived from PARP-1- deficient mice. Although PARP-2 DNA-binding domain consists of only 64 amino acids and lacks any obvious similarity with the corresponding PARP-1 domain,

the catalytic and automodification domains display significant similarity. PARP-2, similarly to PARP-1, is activated by DNA-strand interruptions and contributes to the maintenance of genomic stability. The two proteins probably display redundant functions because Parp-1 and Parp-2 double gene disruption is lethal to embryos.

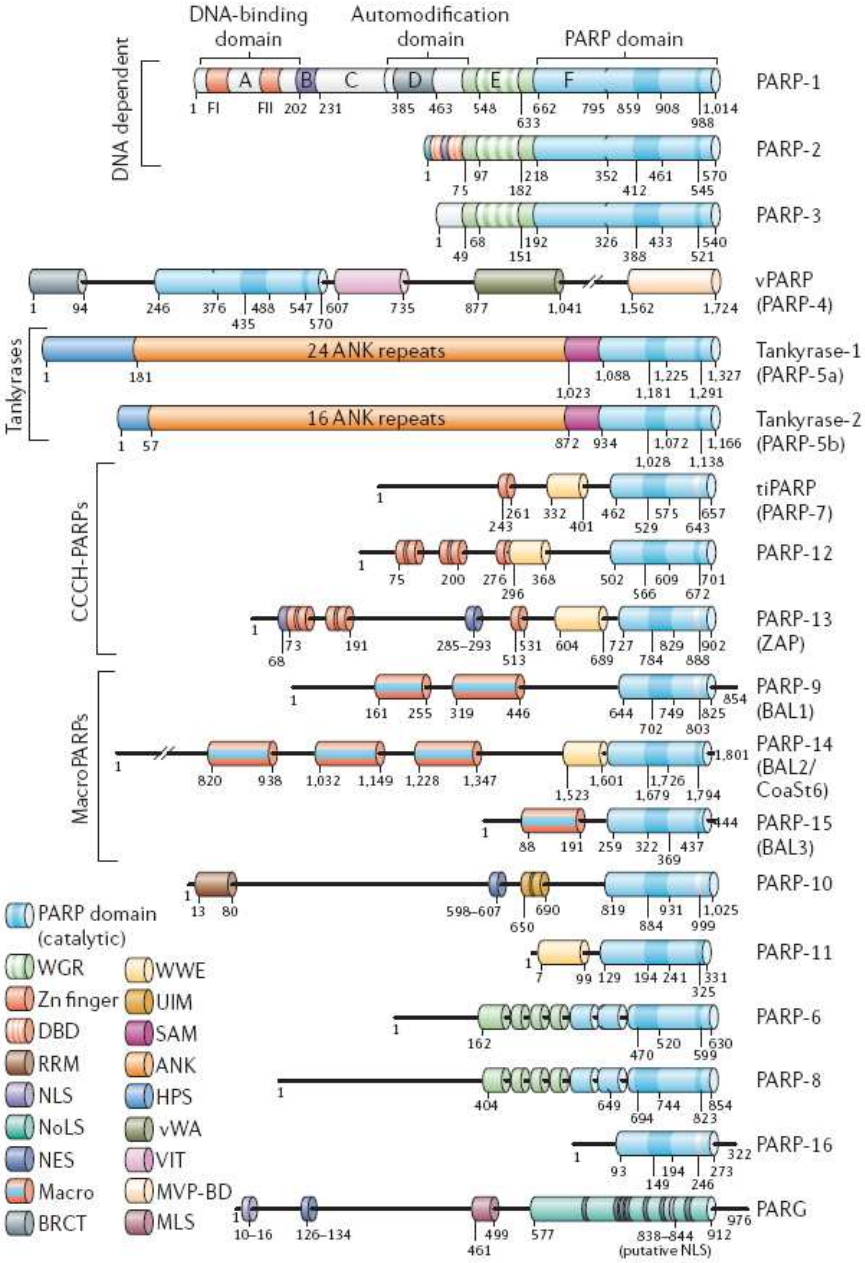
PARP-3 (60 kDa) has a molecular structure rather similar to PARP-2; it is mostly localized in the centrosome and its function is related to cell cycle transition [7]. The enzyme is almost absent in the brain.

PARP-4 (193 kDa) is the largest member of the family and was originally identified as a component of the vaults (hence vPARP). These are large ribonucleoprotein particles (three times the dimension of a ribosome) located in cell cytoplasm and are named after their shape reminiscent the vaulting in a cathedral. It has been proposed that these particles are involved in cellulartransport and are associated with multidrug resistance to anticancer drugs.

PARP-5 (142 kDa) was only recently identified and because it is a partner of the human telomeric protein TRF1 was named Tankyrase 1 (TRF1-interacting, ankyrin-related ADP-ribose polymerase) and its main function is probably related to the maintenance of telomere length. Experiments with siRNA showed that the enzyme contributes in the assembly of bipolar spindles: a decrease of its expression causes mitotic arrest with the appearance of abnormal mitosis.

PARP-10 is a partner of the proto-oncoprotein c-Myc, a key transcriptional regulator that controls cell proliferation. PARP-10 contains an RNA recognition motif (RRM) and a Gly-rich domain, both of which have also been found mediating RNA binding in a c-Myc nucleolar partner protein-nucleolin. PARP-10 shuttles between the cytoplasm and the nucleus and accumulates within the nucleolus where it acquires a CDK2-dependent phosphorylation during late-G1–S phase and during prometaphase to cytokinesis39. PARP-10 is a potent inhibitor of the cell transformation that is mediated by c-Myc in the presence of Ha-Ras, but its PARP activity is not required8. PARP-10 could counteract some nucleolar c-Myc functions that promote cellular transformation. Furthermore, PARP-10 poly(ADP-ribosyl)ates histone H2A, which indicates that it has a role in chromatin regulation.

No known domains have been found in PARP-6, PARP-8, PARP-11 and PARP-16 so far (except a WWE domain in PARP-11), which makes it difficult to speculate on their possible functions (Moroni F; 2008).



Schreiber et al., (2006) Nat Rev Mol Cell Biol. 7(7):517-28. Fig. 12

Fig. 12 The PARP superfamily. The domain architecture of the 17 members of the poly(ADP-ribose) polymerase (PARP) superfamily and of poly(ADP-ribose) glycohydrolase (PARG). Protein domains that are illustrated by coloured boxes were defined according to the Pfam 19.0 or CCD v2.06 (National Center for Biotechnology Information) databases.

4.2 Function of PARPs

PARP is a family of nuclear enzymes involved firstly in DNA repair operating as sensor of DNA damage and signalling molecule binding to both single- and double-stranded DNA breaks. Upon binding to damaged DNA mainly through the second zinc-finger domain, PARP-1 forms homodimers and catalyzes the cleavage of NAD^+ into nicotinamide and ADP-ribose and then uses the latter to synthesize branched nucleic acid-like polymers poly(ADP-ribose) covalently attached to nuclear acceptor proteins (de Murcia et al., 1994; de Murcia and Menissier de Murcia, 1994; Lindahl et al., 1995; Schreiber et al., 1995; Burkle, 2001; Smith, 2001) (Fig. 13).

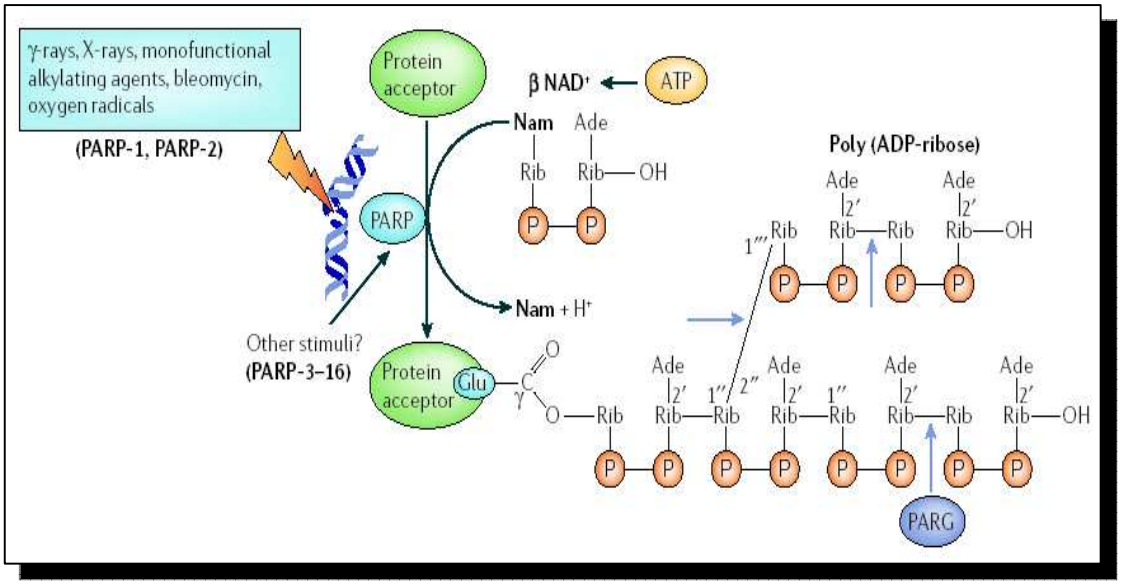


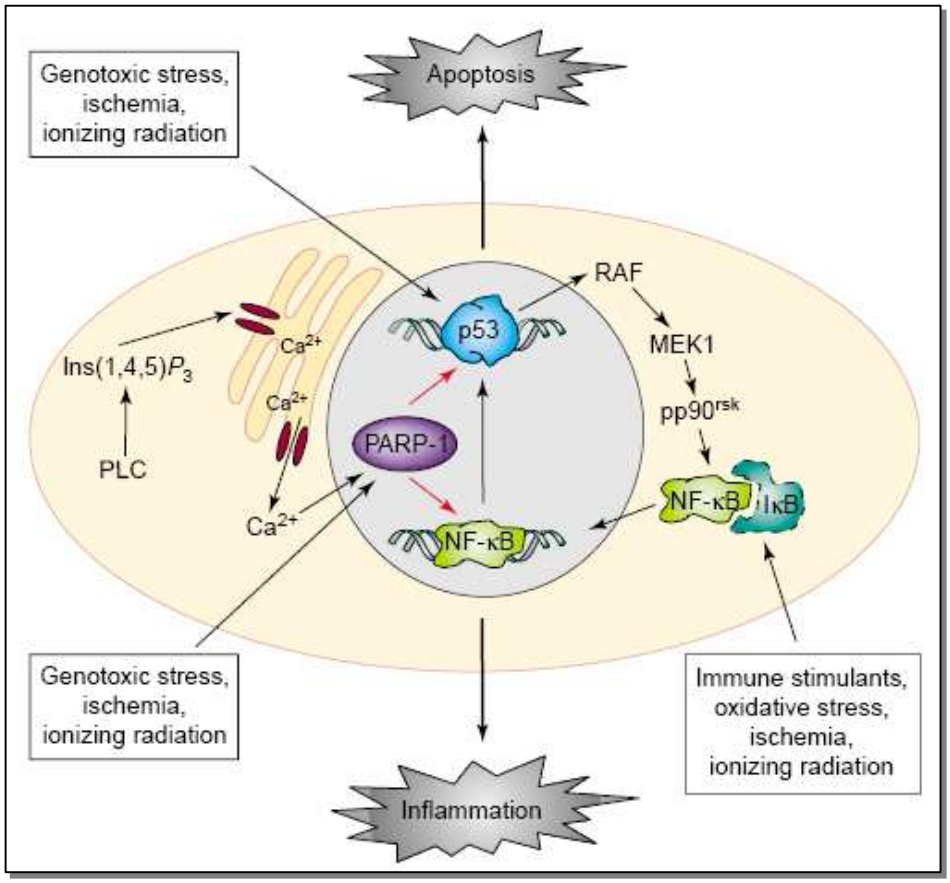
Fig. 13 Metabolism of poly(ADP-ribose) during DNA damage and repair. Fig. 13

The size of the branched polymer varies from a few to 200 ADPribose units. Because of its high negative charge, the covalently attached ADP-ribose polymer dramatically affects the function of target proteins. Poly-ADP ribosylation is a dynamic process, indicated by the short (~1 min) *in vivo* half-life of the polymer (Whitacre et al., 1995). Two enzymes—poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosyl protein lyase—are involved in the catabolism of poly(ADP-ribose), with PARG cleaving ribose-ribose bonds of both linear and branched portions of poly(ADP-ribose) and the lyase removing the protein proximal ADP-ribose monomer (Davidovic et al., 2001). The

regulation of PARP-1 activity is established through different mechanisms. The best characterized mechanism is the down-regulation of enzyme activity through auto-poly-ADP-ribosylation (Kawaichi et al., 1981). Furthermore, nicotinamide, the smaller cleavage product of NAD_, also exerts inhibitory effect on PARP-1, allowing negative feedback regulation. Recently, the purines hypoxanthine, inosine, and adenosine also were identified as another class of endogenous PARP inhibitors (Virag and Szabo, 2001). Phosphorylation of PARP by protein kinase C also results in enzyme inhibition (Tanaka et al., 1987; Bauer et al., 1992). The abundance of PARP may also change under certain conditions, suggesting a transcriptional or posttranscriptional regulation (Bergeron et al., 1997; Tramontano et al., 2000; Doucet-Chabeaud et al., 2001). The physiological or pathophysiological relevance of this pathway is poorly understood at present. The biological role of poly(ADP-ribose) is complex and involves main functions:

I) PARP-1 has been implicated in ***DNA repair*** and maintenance of genomic integrity (de Murcia and Menissier de Murcia, 1994; de Murcia et al., 1994,1997; Schreiber et al., 1995; Chatterjee et al., 1999b; Shall and de Murcia, 2000). This "guardian angel" function is indicated by delayed DNA base-excision repair and by a high frequency of sister chromatid exchange in PARP-1-deficient cells exposed to ionizing radiation or treated with alkylating agents (de Murcia et al., 1997). High levels of ionizing radiation and alkylating agents elicit higher lethality in PARP-1- deficient mice when compared with wild-type ones (de Murcia et al., 1997).

II) PARP-1 also regulates the expression of various proteins at the ***transcriptional level***. Of special importance is the regulation by PARP-1 of the production of inflammatory mediators such as the inducible nitricoxide synthase (iNOS) (Hauschildt et al., 1992; Le Page et al., 1998; Szabo et al., 1998c; Oliver et al., 1999), intercellular adhesion molecule 1 (ICAM-1) (Zingarelli et al., 1998; Szabo et al., 2001b), and major histocompatibility complex class II (Otsuka et al., 1991). NF-kB is a key transcription factor in the regulation of this set of proteins, and PARP has been shown to act as a coactivator in the NF-kB-mediated transcription (Oliver et al., 1999) (Fig. 14).



A. Chiarugi, TIPS 23:122, 2002

Fig. 14

Fig. 14 Schematic representation of the 'transcriptional hypothesis'. The balance between survival and death signals is finely tuned at the level of transcription. Several lines of evidence demonstrate an intricate relationship between the transcription factors p53 and nuclear factor κB (NF-κB) in both induction and execution of apoptosis. In particular, NF-κB activation by the RAF–MEK1–$pp90^{rsk}$ pathway seems essential in the p53-triggered death program. NF-κB also *trans*-activates p53, leading to the initiation of an apoptotic, vicious cycle. Intriguingly, poly(ADP-ribose) polymerase 1 (PARP-1) promotes both p53 and NF-κB activation. Therefore, the Ca^{2+}-activated or DNA-damage-triggered poly(ADP-ribosyl)ation might regulate cell death as a key modulator of the deadly p53–NF-κB system. NF-κB activation is also central in the inflammatory response. Thus, it is conceivable that the pro-inflammatory effects of PARP-1 might be attributed to the facilitation of NF-κB-driven transcription. Abbreviations: Ins(1,4,5)P_3, inositol (1,4,5)-triphosphate; MEK, mitogen-activated protein kinase kinase; PLC, phospholipase C.

Poly(ADP-ribosylation) of histones may also contribute to the transcription-promoting effect of PARP-1, because poly(ADP-ribose) confers negative charge to histones, leading to electrostatic repulsion between histones and DNA. Thus, poly(ADP-ribosylation) can loosen the chromatin structure and can thereby make genes more

accessible for the transcriptional machinery. Nuclear receptor-mediated transcription is regulated by PARP-1 in a different manner: PARP-1 seems to suppress nuclear receptor-mediated transcription (Miyamoto et al., 1999).

III) PARP-1 regulates ***replication and differentiation***. The involvement of PARP-1 in the regulation of replication is supported by observations that poly(ADPribose) metabolism is accelerated in the nuclei of proliferating cells (Tanuma et al., 1978; Kanai et al., 1981; Leduc et al., 1988; Bakondi et al., 2002a). Furthermore, PARP-1 is part of the multiprotein replication complex (MRC) (Simbulan-Rosenthal et al., 1996), indicated by copurification of PARP-1 with key components of MRC (Simbulan-Rosenthal et al., 1996; Dantzer et al., 1998). Moreover, several replication factors and centromere proteins have been shown to serve as substrates for PARP (Simbulan-Rosenthal et al., 1996; Saxena et al., 2002). Another mechanism by which PARP may regulate nuclear processes is poly(ADP-ribosylation) of histones facilitating the assembly and deposition of histone complexes on DNA during replication (Boulikas, 1990).

IV) Poly(ADP ribosylation) has been implicated in the ***regulation of telomerase*** activity. The overexpression of tankyrase-1, a recently discovered PARP enzyme, in telomerase-positive human cells resulted in a gradual and progressive elongation of telomeres (Smith and de Lange, 2000). Besides tankyrase-1, PARP-1 has also been implicated in the maintenance of telomere length. Genetic ablation of PARP-1 has been shown to result in telomere shortening (d'Adda di Fagagna et al., 1999) but others found no difference in telomere length of PARP-proficient and –deficient cells (Samper et al., 2001).

V) PARP-1 activation has been proposed to represent a cell-elimination pathway (Berger et al., 1983, Schraufstatter et al., 1986b; Sims and Benjamin, 1987; Schreiber et al., 1995; Kleczkowska and Althaus, 1996) through which severely damaged cells are removed from tissues. PARP-1-mediated cell death occurs in the form of necrosis (Schreiber et al., 1995; Virag et al., 1998a,b), which is probably the least desirable form of cell death. During necrotic cell death, the cellular content is released into the tissue, exposing neighboring cells to potentially harmful attacks by proteases and other released factors. This scenario is best exemplified by cells that have been exposed to DNA-

damaging stimuli. Mild genotoxic noxa cause PARP activation that facilitates DNA repair and cell survival. Severe DNA damage, however, causes overactivation of PAR,P resulting in the depletion of NAD_ and ATP and consequently in necrotic cell death.

VI) Poly(ADP-ribose) polymer has been identified recently as an emergency source of energy used by the base-excision machinery to synthesize ATP (Maruta et al., 1997; Oei and Ziegler, 2000).

VII) Similarly to ubiquitination, poly(ADP-ribose) may also serve as a signal for protein degradation in oxidatively injured cells (Ullrich and Grune, 2001; Ullrich et al., 2001a). Hydrogen-peroxide treatment of K562 cells caused a PARP-1-dependent up-regulation of 20S proteosome activity. During this process, the proteosome becomes poly(ADP-ribosylated), resulting in the enhanced degradation of poly(ADP-ribosylated) histones (Ullrich and Grune, 2001). Immunoprecipitation experiments demonstrated a protein-protein interaction of the functionally active PARP with the proteasome in correlation with the proteasome activity (Ullrich et al., 2001a).

VIII) In addition to PARP-catalyzed covalent poly-(ADP-ribosylation), poly(ADP-ribose) polymers can noncovalently bind to specific (ADP-ribose)n binding motifs in proteins, such as histones, XRCC1, p53, and DNA polymerase _, and thereby modify their function (Althaus et al., 1993; Pleschke et al., 2000). Such (ADPribose) polymers can be formed during the catabolism of poly(ADP-ribose) by poly(ADP-ribose) glycohydrolase (Davidovic et al., 2001).

In the **IX** and final function, poly(ADP-ribosylation) may also be involved in the regulation of cytoskeletal organization. A recent study reported morphological alterations in *Drosophila* overexpressing PARP-1 (Uchida et al., 2001). The overexpression of PARP-1 disrupted the organization of cytoskeletal F-actin, resulting in aberrant cell and tissue morphology. Furthermore, heat-induced PARP expression disrupts the organization of cytoskeletal F-actin in embryos and tissue polarity in adult flies. Whether these morphological alterations are indeed related to PARP-1 function or, alternatively, whether PARP-1 overexpression interferes with the function of cytoplasmic PARP enzymes remains to be seen.

4.3 Poly(ADP-ribosyl)ation and stroke

It has now clearly established that neuronal death following stroke is mainly due to a cascade of events originating from massive release of the excitatory neurotransmitter glutamate. Subsequent over-activation of neuronal glutamate receptors prompts de-regulation of ionic fluxes through the plasma membrane with persistent intracellular Ca^{2+} accumulation in neurons. This in turn causes a prolonged and non-specific activation of numerous enzymes such as proteases, lipases and nucleases which results in excitotoxic neuronal death (Lipton et al.; 1994). Ischemic neuronal death is mediated, at least in part, by augmentation of nitric oxide (NO) caused by NMDA receptor-dependent activation of neuronal NO synthase (nNOS) activity. PARP-1 hyperactivity is causative in post-ischemic brain damage. In the intricate cascade of events that leads to ischemic neurodegeneration, PARP-1 is thought to be localized downstream to DNA damage induced by reactive oxygen and nitrogen species. Several lines of evidence support this assumption. For instance, using the comet assay to evaluate single and double strand DNA breaks, it has been demonstrated that NMDA receptor antagonists reduce DNA ruptures and ischemic brain injury, whereas PARP-1 inhibitors decrease infarct volumes without reducing the extent of DNA damage (Giovannelli et al.; 2002). Furthermore, PARP-1 activation is significantly reduced in the ischemic brain of mice deficient in nNOS (Endres et al.; 1998), and PAR formation is diminished in the ischemic cortex of rats receiving the nNOS inhibitor 7-nitroindazole (Tokime et al.; 1998). However, PARP-1-null mice are less sensitive to ischemia than those nNOS−/− (Skaper et al.; 2003), suggesting that mechanisms in addition to NO concur to PARP- 1 activation. Hyperactivation of PARP-1 in the ischemic brain is not a single, time-limited event in the complex and prolonged process of ischemic neuronal death. Indeed, a vicious cycle triggered by intraneuronal Ca^{2+} accumulation leads to multiple waves of reactive oxygen species (ROS) formation in the ischemic brain tissue (Pellegrini-Giampietro et al.; 2003), thereby eliciting prolonged DNA damage and sustained PAR formation. The latter can also be due to self-amplifying positive forward cycles of ischemia-induced immune cell recruitment and ROS production (Szabo et al.; 1997), and/or to direct Ca2+-dependent PARP-1 activation (Homburg et al.; 2000). Consistent with PARP-1 recruitment in ischemic brain damage, enzymatic activity of PARP-1 in

gerbils subjected to transient (5 min) global ischemia increases in the injured tissue 4.3- and 1.7-fold at 1 and 24 h of reperfusion, respectively (Nagayama et al.; 2000). Prolonging duration of ischemia (10 min) leads to significant increases of PARP-1 activity up to the seventh day of reperfusion (Strosznajder et al.; 2003). In a focal and transient model of brain ischemia [2 h middle cerebral artery occlusion (MCAo) and 2 h reperfusion] in mice, PAR formation is highly increased in the nuclei of cells of cerebral cortex compared to those present in the contralateral one. Of note, polymer formation is drastically decreased both in the ischemic and contralateral cortex of PARP-1−/− mice subjected to MCAo (Eliasson et al.; 1997). A parallel study in mice reports that following 2 h MCAo increased PAR formation in the ischemic cortex occurs as early as 5 min after reperfusion in cells showing swelling and nuclear disruption (Endres et al.; 1997) PAR accumulation, however, is not evident at later times (3–6 h) of reperfusion or after milder ischemic insult (1 h MCAo) (Endres et al.; 1997). PAR formation has also been investigated in a permanent model of MCAo in rats. Authors report that PAR immunoreactivity increases in the ischemic core and penumbra 2–8 h after ischemia, returning to basal levels 16 h post-ischemia. Importantly, PAR immunoreactive cells of the ischemic cortex show the classical morphology of pyramidal neurons (Tokime et al.; 1998).. Similarly, the ischemic cortex of rats subjected to distal MCAo and transient bilateral common carotid artery occlusion have a □3-fold increase of PAR immunoreactive cells 10 min after 1.5 h ischemia. Remarkably, PAR formation also occurs in neural cells of the infarcted human brain at 18–24 post-insult and rapidly declines, thereafter. A second wave of poly(ADP-ribosyl)ation is due to PAR-positive macrophages which start infiltrating the ischemic human brain 3 days after the ischemic injury (Love et al.; 2000). In a subsequent study in humans it is reported that brain ischemia causes accumulation of PAR in the ischemic core and penumbra mostly during the first 2 days after cardiac arrest. Remarkably, double immunostaining for PAR and the neuronal marker MAP2 indicates that the majority of PAR-positive cells are neurons (Love et al.; 1999). It has also been reported that brain ischemia alters PARP- 1 expression levels. For example, Love and colleagues show that expression of PARP-1 increases in the infarcted tissue of the human brain 18–24 h post-insult and, similarly to PAR, declines thereafter (Love et al.; 2000). Accordingly, a classic study on PARP-1

and excitotoxic neuronal death reports that PARP-1 protein as well as its mRNA levels increase in the nucleus of cultured cerebellar granular cells upon exposure to neurotoxic concentrations of glutamate (Cosi et al.; 1994). Indeed, the group of Sharp reports an increase of PARP-1 mRNA in the dentate gyrus of gerbil brains 4 h after 10 min of global ischemia and return to basal levels 8 h after ischemia (Liu J et al.; 2000). Conversely, Nagayama and associates (Nagayama et al.; 2000) as well as Strosznajder and colleagues (Strosznajder et al.; 2003) show that PARP-1 mRNA and protein do not increase in the gerbil hippocampus in the same ischemia model. These results taken together unambiguously establish a central role of cerebral ischemia in altering the homeostasis of poly(ADP-ribosyl)ation in neurons of different species including humans.

5. Histone deacetylases and Histone acetyl transferases

The histones are highly alkaline proteins found in eukaryotic cell nuclei, which package and order the DNA into structural units called nucleosomes. They are the chief protein components of chromatin, act as spools around which DNA winds, and play a role in gene regulation. Histones "are highly conserved and can be grouped into five major classes: H1/H5, H2A, H2B, H3, and H4". These are organized into two super-classes as follows:

- core histones – H2A, H2B, H3 and H4
- linker histones – H1 and H5

Two of each of the core histones assemble to form one octameric nucleosome core (Fig. 15).

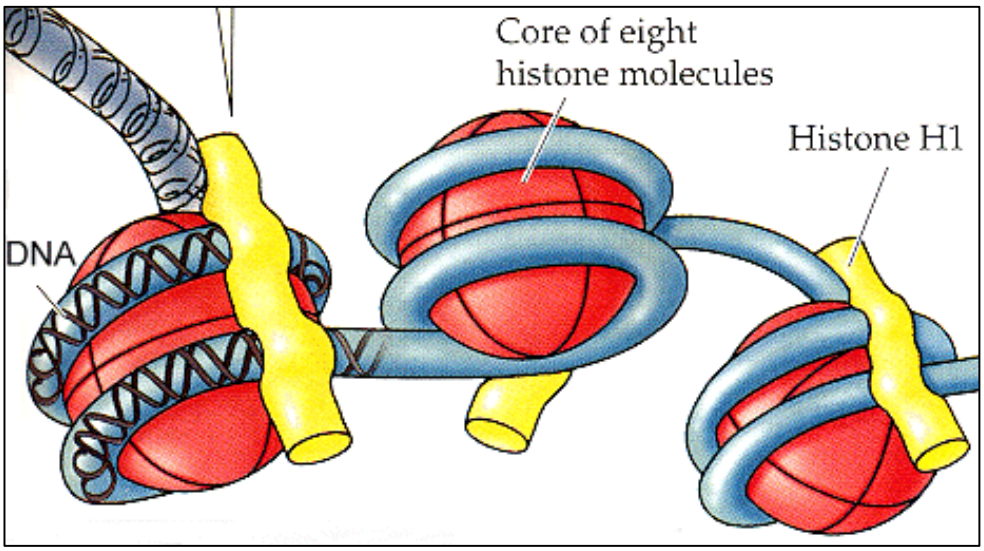


Fig. 15 Core histones assemble to form one octameric nucleosome core around which the **DNA** is rolled up. Fig. 15

The assembled histones and DNA is called chromatin. The nucleosome core is formed of two H2A-H2B dimers and a H3-H4 tetramer, forming two nearly symmetrical halves by tertiary structure. The 4 'core' histones (H2A, H2B, H3 and H4) are relatively similar in structure and are highly conserved through evolution. Histones are subject to post translational modification by enzymes primarily on their N-terminal tails, but also in their globular domains. Such modifications include methylation, citrullination, acetylation, phosphorylation, SUMOylation, ubiquitination, and ADP-ribosylation. This

affects their function of gene regulation. In particular, protein acetylation is dynamic and maintained by two classes of functionally antagonistic enzymes: the protein acetylases and the deacetylases (Kouzarides; 2007) (Fig. 16). Levels of histone acetylation depend on the activities of histone acetylases (HATs) and HDACs, which add or remove acetyl groups from protein substrates, respectively (Sun et al.; 2003).

Although transcriptional regulation is highly complex and dynamic, in general an increase in histone acetylation causes remodelling of chromatin from a tightly packed configuration to a loosely packed configuration, which subsequently leads to transcriptional activation. Conversely, a decrease in histone acetylation may cause chromatin structure to condense and result in transcriptional silencing. So, upregulation of transcription can be achieved in cells either by stimulation of HAT or by inhibition of HDAC activities, and the opposite is true for transcriptional downregulation. In addition to modification of histones, other (nonhistone) cellular proteins are substrates for HDACs, and these proteins mediate diverse biological functions via transcriptional-dependent as well as independent mechanisms (Zhang. et al., 2003*;* Bolden et al., 2006). In fact, phylogenetic analysis of bacterial HDAC relatives suggests that evolutionary development of modern HDACs preceded the evolution of histone proteins, and raises the possibility that the primary activity of some HDACs is directed against non-histone substrates (Gregoretti et al.; 2004).

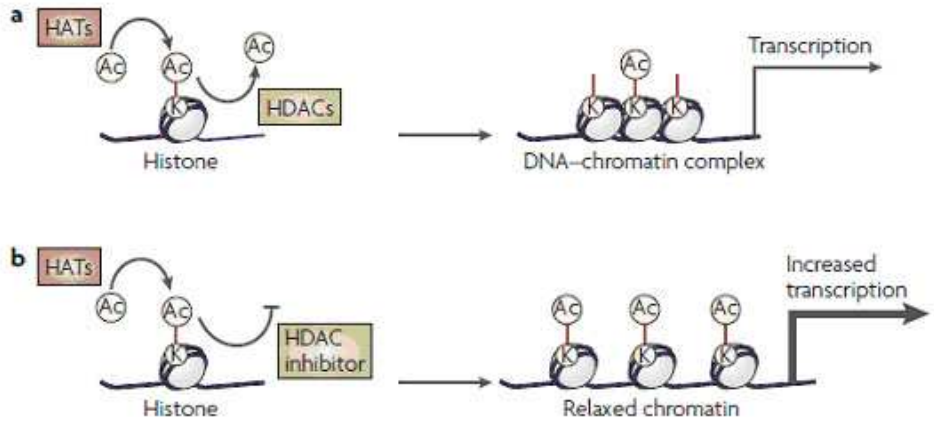


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Fig. 16 a) Levels of histone acetylation at specific lysine (K) residues determinet by concurrent reaction of acetylation and deacetylation mediated by histone acetylases (HAT) and histone deacetylases (HDACs) b) HDAC inhibitors block the hitone acetylation level s, leading to an increased acetylation, chromatin modification to relax conformation and transcription upregulation.

5.1 Structure–function analysis of human HDACs

The superfamily of HDACs consists of five main subtypes: classes I, IIa and IIb, and IV, and the structurally distinct class III (Gregoretti et al., 2004; Butler et al., 2006) (Fig. 17).

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; Wang et al., 2004). 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 (Fig. below). 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).

Class IIa HDACs consist of four members — HDAC4, HDAC5, HDAC7 and HDAC9 — with distinct tissue specific patterns of expression, predominantly in muscle and heart (Martin et al., 2007; Majdzadeh et al.; 2008). These proteins contain extended amino-terminal domains of ~600 amino acids, which mediate interactions with HDAC3, myocyte enhancer factor 2 (MeF2), repressor complex NCoR2/SMRT and 14-3-3 proteins, followed by the Zn^{2+}-containing catalytic domain. MITR — an amino-terminal splice variant of HDAC9 lacking the catalytic domain — HDAC4 and HDAC5 interact with heterochromatin protein 1 (HP1). HP1 is an adaptor protein that recognizes histone methylated lysines and mediates transcriptional repression by recruiting histone methyltransferases (Zhang et al.; 2002). 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 (Wen et al.; 2000; 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 (*Lahm et al., 2007; Jones et al., 2008). This suggests that vertebrate class IIa HDACs may have evolved to maintain low basal activities on acetyl-lysines and to efficiently process restricted sets of specific, still undefined, natural substrates (Lahm et al., 2007). HDAC4, HDAC5, HDAC7 and MITR shuttle between the nucleus

and cytoplasm, whereas full-length HDAC9 is localized in the nucleus (Wang & Yang, 2001; Petrie et al.; 2003).

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 (Bertos et al.; 2001). HDAC6 functions in the cytoplasm where it deacetylates a-tubulin and alters microtubule stability (Hubbert et., 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; however it coprecipitates with the primarily cytosolic HDAC6 (Gao et al.; 2002). 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 (Michan & Sinclair 2007; Gan et al.; 2008). 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 et al., 2008; North et al.; 2005). 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 substrate54. Structurally, human SIRT1 is the closest analogue of yeast Sir2, which regulates cellular metabolism and ageing (Michan. & Sinclair 2007). 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, TAFI68, p300 and peroxisome proliferatoractivated receptor-g, coactivator 1a (PGC1a). SIRT2 is a cytosolic protein that deacetylates a-tubulin and microtubules, a function that is redundant with that of HDAC6 (North et al., 2005; Southwood et al.; 2007). Subsequently, it has been shown that SIRT2 also deacetylates K16 on H4 (Vaquero et al.; 2006) and localizes to neuronal nuclei. SIRT3, which shares the most structural similarity with SIRT2, 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 (Schwer et al., 2002; Hallows et al., 2006). Mitochondrial subcellular localization has also been suggested for SIRT5 (Michishita et al.; 2005), but the function of this protein is not yet known. 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 (Michishita et al.; 2008).

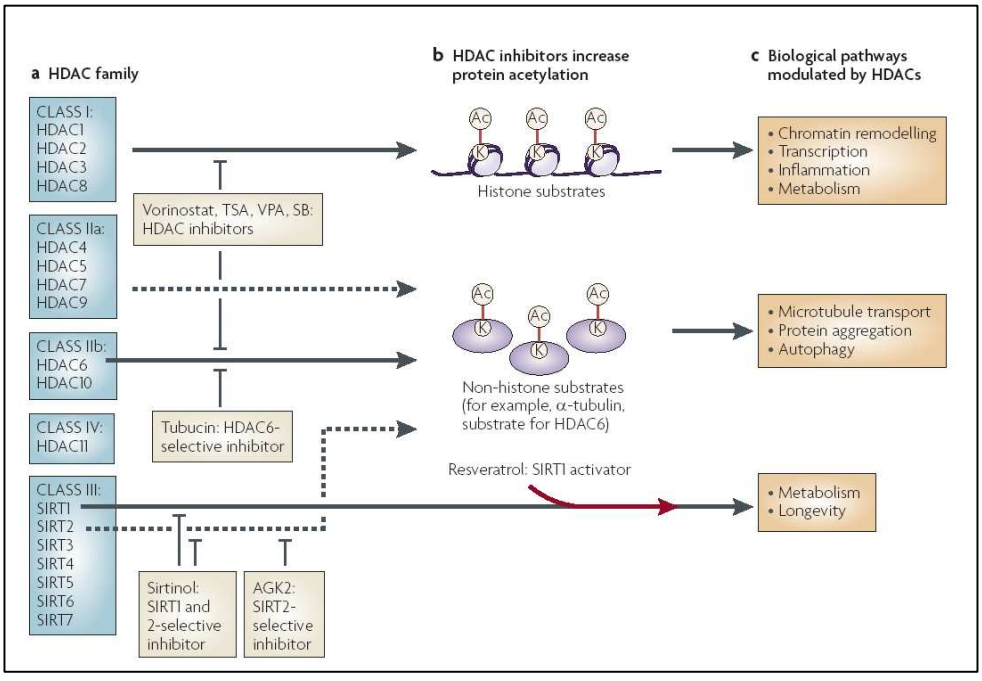

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Fig. 17

Fig. 17 Histone deactylase (HDAC) family (classes I–IV) and HDACs implicated as disease modifiers.

5.2 HDAC inhibitors and clinical use

The HAT–HDAC system, owing to their involvement in turnover of histone and transcription machinery regulation, is one of the ultimate regulatory switches of gene expression. In addition to transcriptional regulation, HAT–HDAC system is also postulated to modulate other chromatin-associated processes like replication, site-specific recombination and DNA repair, thereby playing a major role in modulating overall cellular fate. During normal conditions, protein concentration (availability) and enzymatic activity of HATs (like CBP and p300) and HDACs remain in a highly harmonized state of balance where adequate active molecules from either group are present to effectively regulate chromatin in a controlled manner (Fig. 18). Such equilibrium manifests neuronal homeostasis and is responsible for regulated gene expression leading to normal neurophysiological outputs like long-term potentiation, learning and memory. The maintenance of precise balance between HATs and HDACs as a prerequisite of neuronal survival in normal conditions. The loss of acetylation homeostasis alters significantly during neurodegenerative circumstances when histone acetylation level in neuron decreases globally, reflecting a malfunctioning acetylation apparatus (Rouaux et al.; 2003) (Fig. 18).

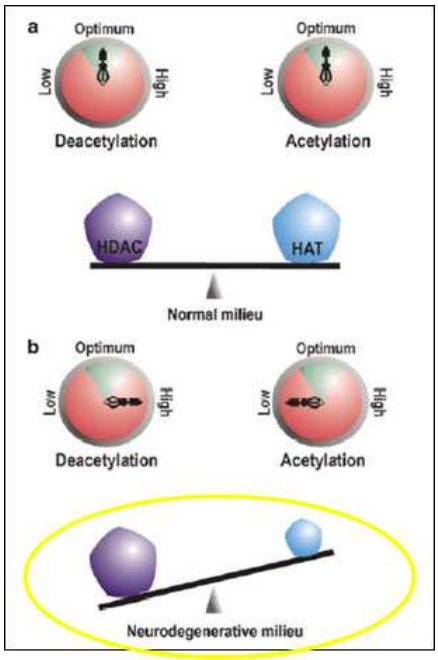


Fig. 18

Saha RN, Pahan K. (2006) Cell Death Differ. 13(4):539-50.

Fig. 18 Neuronal acetylation homeostasis. (**a**) In neurons under normal conditions, the dose and activity of HATs and HDACs are poised in a fine balance where they counteract each other to ensure physiological homeostasis. (**b**) During neurodegeneration, critical loss of HAT protein level ensue a rebated HAT dose and activity. This reclines the acetylation balance towards excessive deacetylation of target moieties

Recent years have witnessed an explosion in the development of new HDAC inhibitors (Carey and La Thangue; 2006). HDAC inhibitors can be classified into four main chemical families, the shortchain fatty acids (e.g. sodium butyrate, phenylbutyrate, and valproic acid), the hyroxamic acids (e.g. trichostatin A and suberoylanilide hydroxamic acid (SAHA)), the epoxyketones (e.g. trapoxin), and the benzamides. Of these, the most widely studied are sodium butyrate, phenylbutyrate, trichostatin A, and SAHA. The butyrates are known to cross the blood–brain barrier (Carey and La Thangue; 2006; Minucci et al.; 2006). The initial interest in these inhibitors came from studies linking HDACs to a wide variety of human cancers. HDAC inhibitors arrest growth, induce differentiation and, in somecases, apoptosis and have potent anitcancer activities, with remarkable tumor specificity (Carey and La Thangue; 2006; Minucci et al.; 2006). For this reason, inhibitors of class 1 and 2 HDACs are in phase I/II clinical trials for cancer therapy and potentially cancer prevention. In the nervous system, the anticonvulsant and mood-stabilizing drug valproic acid was identified as an inhibitor of HDAC1, thereby linking its antiepileptic effects to changes in histone acetylation. More recent work has revealed that inhibitors of class 1 and 2 HDACs represent novel therapeutic approaches to treat neurodegenerative disorders, depression and anxiety, and the cognitive deficits that accompanymany neurodevelopmental disorders.

Recent findings suggest that HDAC inhibitors can ameliorate deficits in synaptic plasticity, cognition, and stress-related behaviors in a wide range of neurologic and psychiatric disorders including Huntington’s disease, Parkinson’s disease, anxiety and mood disorders, Rubinstein- Taybi syndrome, and Rett syndrome. These agents may prove useful in the clinic for the treatment of the cognitive impairments that are central elements of many neurodevelopmental, neurological, and psychiatric disorders.

In particular the develope of novel treatment strategies for neuroprotection, rescue, and repair in order to ameliorate the deleterious consequences of stroke. Global ischemia can arise as a consequence of cerebrovascular accidents, cardiac arrest, cardiac surgery, profuse bleeding, near-drowning, and carbon monoxide poisoning. Brief ischemic insults cause selective, delayed death of hippocampal CA1 neurons, and severe cognitive deficits. The substantial delay between insult and cell death is consistent with a role for transcriptional changes. The transcriptional repressor RE1 silencing transcription factor

(REST, also called NRSF) is widely expressed during embryogenesis and plays a crucial role in terminal neuronal differentiation (Ballas and Mandel, 2005; Roopra et al.; 2001). In neural progenitors and non-neural cells, REST actively represses a large array of neural-specific genes important to synaptic plasticity including synaptic vesicle proteins, structural proteins, voltage-gated channels, and ligand-gated receptors, allowing non-neuronal transcripts to be expressed (Ballas and Mandel, 2005; Roopra et al.; 2001). As neurons differentiate, REST orchestrates a set of epigenetic modifications that distinguish neuronal from non-neuronal cells. In global ischemia, selectively vulnerable hippocampal neurons exhibit aberrant accumulation of REST/NRSF in the nucleus and REST-dependent silencing of target genes essential for neuronal function (Calderone et al.; 2003). A fundamental mechanism by which REST silences target genes is by recruitment of MeCP2 and corepressor complexes that promote histone H3 lysine 9 (H3K9) deacetylation and methylation. Recent findings that REST recruits CoREST, G9a, and MeCP2 to promoters of target genes and promotes epigenetic remodeling of target genes in selectively vulnerable hippocampal neurons implicates REST-dependent epigenetic remodeling in the pathogenesis of global ischemia (Formisano et al.; 2007). Dysregulation of REST and its target genes is implicated not only in global ischemia but also in the pathogenesis of Down's syndrome, AD Huntington's disease, epilepsy, and X-linked mental retardation (Ballas and Mandel, 2005; Roopra et al.; 2001). Two recent studies demonstrate a role for HDAC inhibitors in amelioration of neuronal death and cognitive deficits in postischemic neurons. A study of our laboratory, (Faraco et al., 2006) reports that the potent HDAC inhibitor SAHA administered intraperitoneally to mice at zero and six hours after induction of ischemic stroke by middle cerebral artery occlusion (MCAO) prevented H3 deacetylation, promoted expression of neuroprotective proteins Bcl-2 and Hsp70, and reduced infarct volume, indicating a neuroprotective action for SAHA (Faraco et al., 2006). In a second study, Moskowitz and colleagues observed aberrant DNA methylation in the brains of wild-type mice subjected to mild ischemic brain injury by the MCAO model; administration of the demethylating agent 5-aza-20-deoxycytidine and the HDAC inhibitor trichostatin A conferred stroke protection in wild-type mice subjected to mild, but not severe ischemic damage (Endres et al.;

2000). These findings underscore the therapeutic potential of HDAC inhibitors for therapeutic intervention in the neurodegeneration associated with stroke.

6. AIM OF THE STUDY

Ischemic tolerance is an evolutionary conserved cellular defense program in which exposure to a subtoxic preconditioning stimulus results in resistance to a subsequent lethal ischemic insult (Gidday, 2006; Steiger & Hanggi, 2007; Stenzel-Poore et al., 2007; O'Duffy et al., 2007; Obrenovitch, 2008; Dirnagl et al., 2009). The key element in this process is the preconditioning stimulus and its ability to regulate neuronal activity, involving a number of transductional and traslational pathways that generate an array of mechanisms in which specific transducers lead to neuroprotection by modifying the gene expression of vulnerable cells. The endogenous molecular mechanisms of increased neuronal resistance induced by preconditioning offer attractive targets for the development of therapeutic strategies, but these processes are still not clearly understood.

A number of possible molecular mediators of ischemic tolerance that determine either inhibition of programmed cell death or augmentation of programmed cell survival processes have been proposed. Particular attention among transducers has been devoted to ionotropic (iGlu) and metabotrobic glutamate (mGlu) receptors, which have been repeatedly demonstrated to be involved in the induction of tolerance to ischemia and excitotoxic insults. Recent studies have demonstrated that the mGlu 1/5 receptor agonist DHPG can be used as a preconditioning stimulus, being able to protect CA1 pyramidal cells in rat hippocampal slices from the deleterious effects of 30 min oxygen and glucose deprivation (OGD) (Blaabjerg et al., 2003; Werner et al., 2007). Moreover, low doses of NMDA in hippocampal neurons lead to neuroprotection activating diverse and multiple pathways (Grabb and Choi, 1999; Soriano et al., 2006).

The aim of this study was to investigate whether modifications of glutamate receptors may underlie the tolerant responses to excitotoxicity induced by preconditioning.

We know that modifications of MAGUK protein function in the glutamatergic synapse have been recently described in several neurological disorders such as Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, Ischemia, Schizophrenia and neuropathic pain. In particular, modifications of MAGUK proteins interactions with NMDA receptors regulatory subunits are a common event in several

neurodegenerative disorders (Gardoni et al., 2006; Aarts et al., 2002) but are also involved in the induction of ischemic tolerance (Du et al., 2009).

Moreover, the ability of preconditioning stimuli to regulate neuronal activity involves several translational pathways and generates an abounding system in which specific transducers lead to neuroprotection by modification of gene expression both in a rapid and delayed fashion (Dirnagl et al., 2003; Gidday, 2006) and shown to produce neuroprotection through various mechanisms, including moderate increases in the concentrations of intracellular calcium, the rapid release of brain-derived neurotrophic factor (BDNF), the formation of NO, the activation of the Ras/ extracellular signal-regulated kinase (Erk) and phosphatidylinositol 3 (PI3)-kinase/Akt pathways, and the increased activity of the transcription factor NFκB (Gidday et al., 1999; Shamloo et al., 1999; Gonzalez-Zulueta et al., 2000; Bickler and Fahlman, 2004). The induction of ischemic tolerance is accompanied by substantial change in gene expression, suggesting that preconditioning stimulates a fundamental genomic reprogramming of cells that confers cytoprotection and survival.

To this aim, we further characterized a DHPG preconditioning paradigm recently described in a previous report (Werner et al., 2007) and developed a new pharmacological preconditioning protocol using subtoxic concentrations of NMDA. In the present study, we examined the role of group I mGlu receptors and NMDA receptors in both the induction and the expression of ischemic tolerance. We used organotypic hippocampal slices exposed to either pharmacological preconditioning paradigms. In particular, we examined whether activation of mGlu1 or NMDA receptors was necessary to initiate the process leading to ischemic tolerance. To this aim, we exposed organotypic hippocampal slices to our preconditioning protocols and 24 h later to 30 min OGD, which promotes selective CA1 pyramidal cell death. In particular, we have examined two different aspects in the induction of ischemic tolerance: the modulation in the Post Synaptic Density and the changes in gene expression. For the first point, we investigated whether MAGUK proteins and glutamate receptor responses could be modified in rat organotypic hippocampal slice models of pharmacological preconditioning. In particular we have looked at glutamate receptors subunits (NR2A or

NR2B for NMDA receptors and GluR1 for AMPA receptors) and at MAGUK proteins, in particular PSD95.

For the second point, we choose to investigate the contribution of two classes of enzymes involved in gene modulation and early studied in our laboratory: PARP and HDACs. We know that both NMDA (Eliasson et al., 1997; Lo et al., 1998; Mandir et al., 2000) and DHPG (Meli et al. 2005) can stimulate the activity of PARP-1, and that the dynamic chromatin remodeling events in hippocampal nerurons are associated with NMDA receptor-mediated activation and histone modulation (Tian et al., 2009).

In particular, experimental evidence of our laboratory strongly suggest that PARP inhibitors may reduce brain damage after stroke (Meli et al., 2003) and that pharmacological inhibition of histone deacetylases specifically alters gene expression and reduces ischemic injury in the mouse brain (Faraco et al., 2006).

7. MATERIALS AND METHODS

7.1 Materials

DHPG and staurosporine were purchased from Ascent Scientific (Weston-Super-Mare, UK). Glutamate, NMDA, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) and propidium iodide (PI) were purchased from Sigma (St Louis, MO, USA). Z-VAD-FMK (carbonbenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fuoromethylketone) was purchased from Promega (Madison, WI, USA). Thieno(2,3-c)isoquinolin-5-one (TIQ-A) was synthesized as described in Pellicciari et al. (2003), whereas *N*-(6-oxo-5,6-dihydro-phenanthridin-2-yl)-(*N*,*N*-dymethilamino)acetamide hydrochloride (PJ-34) was purchased from Alexis Biochemicals (Vinci, FI, Italy). Tissue culture reagents were obtained from Gibco-BRL (San Giuliano Milanese, MI, Italy) and Sigma (St Louis, MO, USA).

7.2 Preparation of rat organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared as previously reported (Pellegrini-Giampietro et al., 1999a,b). Briefly, hippocampi were removed from the brains of 8–10 days old Wistar rats (Harlan, MI, Italy), and transverse slices (420 µm) were prepared using a McIlwain tissue chopper in a sterile environment. Slices were first placed in Hanks' balanced salt solution (supplemented with 5 mg/ml glucose and 3.75 mg/ml amphotericin B) and then transferred onto 30 mm diameter semiporous membranes inserts (Millicell-CM; Millipore, Italy), which were placed in sixwell tissue culture plates containing 1.2 ml medium per well. The slices culture medium consisted of 50% Eagle's minimal essential medium, 25% heat-inactivated horse serum, 25% Hanks' balanced salt solution, 5 mg/ml glucose, 2 mM L-glutamine, and 3.75 mg/ml amphotericin B. Slices were maintained at 37 °C in an incubator in atmosphere of humidified air and 5% CO2 and culture medium was changed twice a week. Slices were kept in culture for 12–14 days and before experiments all slices were screened for viability by incubating them for 30 min with PI (5 mg/ml); slices displaying signs of neurodegeneration were discarded from the study.

7.3 Oxygen and glucose deprivation in rat organotypic hippocampal slices

Cultures were exposed to OGD as previously reported in detail (Pellegrini-Giampietro et al., 1999a,b). Briefly, the slices were subjected to OGD by exposing them to a serum- and glucose-free medium saturated with 95% N2 and 5% CO2. Following 30 min of incubation at 37 °C in an airtight anoxic chamber equipped with an oxygen gas controller (BioSpherix, New York, USA), the cultures were transferred to oxygenated serum-free medium (75% Eagle's minimal essential medium; 25% Hank's balanced salt solution; 2 mM L-glutamine; and 3.75 mg/ml amphotericin B) containing 5 mg/ml glucose and returned to the incubator under normoxic conditions until neuronal injury was evaluated 24 h later.

7.4 OGD and exposure to glutamate receptor agonists in rat organotypic hippocampal slices

Cultures were exposed to OGD as previously reported in detail (Pellegrini-Giampietro et al., 1999a; Pellegrini-Giampietro et al., 1999b). Briefly, the slices were subjected to OGD by exposing them to a serum- and glucose-free medium saturated with 95% N_2 and 5% CO_2. Following 30 min of incubation at 37°C in an airtight anoxic chamber equipped with an oxygen gas controller (BioSpherix, New York, USA), the cultures were transferred to oxygenated serum-free medium (75% Eagle's minimal essential medium; 25% Hank's balanced salt solution; 2 mM L-glutamine; and 3.75 μg/ml amphotericin B) containing 5 mg/ml glucose and returned to the incubator under normoxic conditions until neuronal injury was evaluated 24 h later.

Exposure to the ionotropic glutamate receptor agonist NMDA (0.1-100 μM for 15, 30 or 60 min) or to the group I metabotropic glutamate receptor (mGlu) agonist DHPG (0.1-300 μM for 30 min) was carried out in the incubator using serum-free medium as previously described (Pellegrini-Giampietro et al., 1999a). Hippocampal slices were then cultured for an additional 24 h in a fresh serum-free medium and then

evaluated for CA1 pyramidal cell injury. In order to achieve a maximal degree of neuronal injury, hippocampal slices were exposed for 24 h to 10 mM glutamate in the incubator using serum-free medium.

7.5 Assessment of CA1 pyramidal cell injury

Cell injury was assessed using the fluorescent dye PI (5 μg/ml), a highly polar compound which enters the cell only if the membrane is damaged and becomes fluorescent upon binding to DNA. PI was added to the medium at the end of the 24 h recovery period following OGD or exposure to NMDA or DHPG. Thirty minutes later, fluorescence was viewed using an inverted fluorescence microscope (Olympus IX-50; Solent Scientific, Segensworth, UK) equipped with a xenon-arc lamp, a low-power objective (4X) and a rhodamine filter. Images were digitized using a video image obtained by a CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) controlled by software (InCyt Im1^{TM}; Intracellular Imaging Inc., Cincinnati, OH, USA) and subsequently analyzed using the Image-Pro Plus morphometric analysis software (Media Cybernetics, Silver Spring, MD, USA). In order to quantify cell death, the CA1 hippocampal subfield was identified and encompassed in a frame using the drawing function in the image software (ImageJ; NIH, Bethesda, USA) and the optical density of PI fluorescence was detected. There was a linear correlation between CA1 PI fluorescence and the number of injured CA1 pyramidal cells as detected by morphological criteria (Pellegrini-Giampietro et al., 1999a).

7.6 Electrophysiological recordings in organotypic hippocampal slices

NMDA and DHPG preconditioning was performed as described in paragraph *7.4*. Whole-cell voltage-clamp recordings were performed and AMPA-stimulated inward currents were recorded in CA1 pyramidal cells from organotypic hippocampal slices preconditioned 24h before recordings. To this aim, a small rectangle of the membrane

surrounding an attached culture slice was excised with a scalpel blade, removed from the culture disch and placed into a recording bath submerged in 5%CO_2/95%0O_2 oxygenated aCSF containing (in mM): NaCl 130, KCl 3.5, $Na_2H_2PO_4$ 3, $NaHCO_3$, glucose, $MgCl_2$ 1.5 and $CaCl_2$ 1.5 at pH 7.4.

Recording microelectrodes were prepared from borosilicate glass (WPI Inc; Sarasota, FL) by a Narishige Instruments micropipette puller (Tujunga, CA) (resistance ranging from 4 to 6 M_) and filled with solution of the following composition (in micromolar concentrations): K-gluconate 142.5, potassium methylsulfate 20, NaCl 8, Hepes 10, EGTA 0.1, MgATP 2, and GTP 0.2. The pH of the internal solution was adjusted to 7.2 with KOH and the osmolarity was adjusted to 300 mOsm with H_2O and sucrose. After establishing a GIGA seal a whole-cell configuration was achieved by rupturing the membrane. Recordings were done using a Multiclamp preamplifier (Axon Instruments; Foster City, CA) and filtered at 5 kHz. Both the frequency and peak amplitude of detected events were analyzed (Fig. 7D).

The AMPA receptor blocker NBQX (10 μM) was added at the end of each experiment to verify that the spontaneous AMPA-stimulated inward currents were AMPA receptor-mediated.

Application of 1 μM TTX was used during the experimental recordings in a second set of experiments to verifye a possible presynaptic mechanism mediated by our preconditioning paradigms (Fig. 7E).

7.7 TIF (triton insoluble fraction) preparation

Cultured slices were homogenized in 0.32 M cold sucrose containing 1mM HEPES, 1 mM MgCl2, 1 mM NaHCO3, and 0.1 mM PMSF, pH 7.4 in the presence of a complete sets of proteases inhibitors (CompleteTM) and phosphatase inhibitors. The homogenized tissues were centrifuged at 1,000 g for 10 min. The resulting supernatant were centrifuged at 13,000 g for 15 min to obtain a fraction of mitochondria and synaptosomes. The pellet were resuspended in hypotonic buffer (in the presence of protease inhibitors) in a glass–glass potter and centrifuged at 140,000 g for 1 h. The

pellet was resuspended in 1 ml of buffer containing 150 mM KCl and 1% Triton X-100 and centrifuged at 140,000 g for 1 hr 4°C. The final pellet was homogenized by 10 strokes in a glass–glass potter in 20 mM HEPES. An equal volume of glycerol were added and stored at -80°C. This fraction is enriched in proteins composing the postsynaptic compartment and is referred to as the "Triton-Insoluble Fraction" (TIF).

7.8 Western blot analysis

Cultured slices were washed with cold 0.01 M phosphate-buffered saline, pH 7.4 and 4 slices/sample were gently transferred and dissolved in a tube containing 1% SDS. Total protein levels were quantified using the Pierce (Rockford, IL, USA) BCA (bicinchoninic acid) Protein Assay. Lysates (20 μg/lane of protein) were resolved by electrophoresis on a 4-20% SDS-polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA) and transferred onto nitrocellulose membranes. Blots were blocked for 1 h at room temperature in 20 mM Tris-buffered saline, pH 7.6 - 0.1% Tween 20 (TBS-T) containing 5% non-fat dry milk, and then incubated overnight at 4°C with monoclonal anti-PAR (10H) antibody (Alexis Biochemicals, Vinci, FI, Italy) or with polyclonal rabbit anti-PARP-1, anti- phospho ERK, antibody (Cell Signaling Technology, Beverly, MA, USA) diluted both 1:1000 in TBS-T containing either 5% non-fat dry milk (anti-PAR) or 5% bovine serum albumin (anti-PARP-1). Immunodetection was performed with secondary antibodies (1:2000 anti-mouse or anti-rabbit IgG from donkey, Amersham Biosciences, UK) conjugated to horseradish peroxidase in TBS-T containing 5% non-fat dry milk. Membranes were washed with TBS-T (3 X 15 min) and then reactive bands were detected using chemiluminescence (ECLplus; Euroclone, Padova, Italy). Quantitative analysis was performed using the QuantityOne analysis software (Bio-Rad, Hercules, CA, USA).

7.9 Measurement of NAD^+ and ATP contents in organotypic hippocampal slices

NAD^+ contents were quantified by means of an enzymatic cycling procedure as described by Shah et al. (1995), while ATP levels were measured using the ATPlite™ Luminescence ATP Detection Assay System (Perkin-Elmer, Waltham, MA, USA). Specifically, for NAD^+ contents, cultured slices were washed with cold 0.01 M phosphate-buffered saline, pH 7.4 and 2 slices/sample were gently transferred and dissolved in 100 µl of $HCLO_4$ 1N, sonicated and neutralized with 100 µl di KOH 1N. 200 µl bicine 100 ml/L (pH 8) were added and the samples were centrifuged at 14.000 g for 5 min. Then, 100 µl of homogenate were transferred onto white multiwell plates with 100 µl of buffer containing bicine, 23 µl/ml ethanol, 0.17 mg/ml MTT, 0.57 mg/ml fenazine etosolfate and 10 µg di alcool dehidrogenase and luminescence was measured using a luminescence counter.

7.10 Caspase 3 activity dosage

Caspase-3 and -7 activities were assessed using the Caspase-Glo® 3/7 Assay kit (Promega, Madison, WI, USA). Organotypic hippocampal slices were washed with cold 0.01 M phosphate-buffered saline, pH 7.4 and 2 slices/sample were gently transferred into an Eppendorf tube containing 110 µl of Caspase-Glo® 3/7 reagent and maintained at 22 °C for 1 h. Then, 100 µl of lysate were transferred onto black multiwell plates and luminescence was measured using a TopCount-NXT™ (Packard, Warrenville, Illinois, USA) microplate scintillation and luminescence counter.

7.11 PARP pure activity dosage

PARP activity assay ^{3}H-PAR was synthesized *in vitro* according to Banasik et al. (1992). Briefly, 0.05U of purified bovine PARP-1 was incubated at 37 1C in a final volume of 100mL 50mM Tris-HCl buffer (pH 7.4), containing 5mM MgCl2, 2mM DL-dithiothreitol, 10 mg sonicated DNA, 0.5mgmL_1 bovine albumin, 10 µM NAD^{+} and 1 µL ^{3}H-NAD (35.5nmol). Afterm 60 min, PARP-1 activity was blocked with 30 µM PJ34, 10 mM nicotinamide or SAHA 10 µM. After 60 min incubation at 37 1C, the reaction was stopped by adding 1mL of trichloroacetic acid (TCA) 10% (w/v). Then, the mixture was centrifuged (15min at 12 000 g at 4 1C), and the pellet containing ^{3}H-PAR was washed twice with water and resuspended in 0.1mM NaOH. Radioactivity was measured by scintillation counting (Tri-Carb 1900 TR; Packard, Meriden, CT, USA).

7.11 Statistical analysis

Statistical significance of differences between PI or DCF fluorescence intensities and Western blot optical densities was evaluated by performing analysis of variance (ANOVA) followed by the Tukey's w test for multiple comparisons. Differences were considered significant for $P < 0.05$.

8. RESULTS

PART I

8.1 Ischemic and pharmacological preconditioning in organotypic hippocampal slices

As previously reported (Pellegrini-Giampietro et al., 1999a; 1999b), maximal damage was achieved in this system by exposing organotypic hippocampal slices to 10 mM glutamate for 24 h (Fig. 1A). Exposure to OGD for periods ranging from 10 to 30 min led 24 h later to selective and time-dependent increases in the levels of PI fluorescence in the CA1 region (Fig. 19).

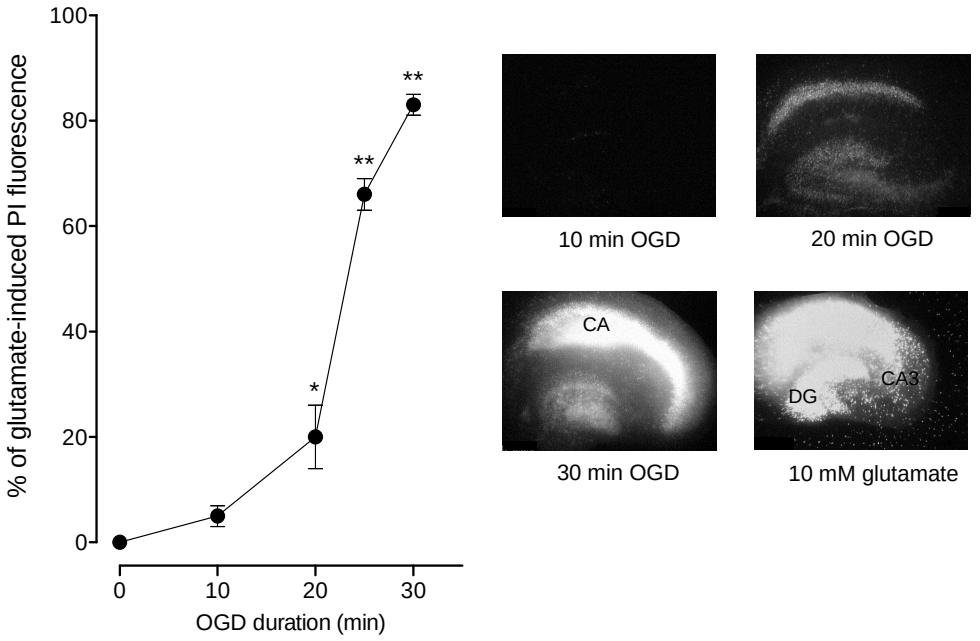


Fig. 19

Fig. 19 Sublethal, mild and severe OGD in organotypic hippocampal slices

CA1 injury was modest but significant when slices were exposed to 20 min OGD and increased to approximately 65% and 85% of the fluorescence intensity observed with 10 mM glutamate when slices were exposed, respectively, to 25 and 30 min OGD.

We have previously shown that exposure of organotypic hippocampal slices to iGlu and mGlu receptor agonists such as NMDA, α-amino-3-hydroxy-5-methyl-4-

isoxazole (AMPA) or DHPG at relatively large concentrations or for prolonged periods of time results in extensive damage in all pyramidal cell layers (Werner et al., 2007; Scartabelli et al., 2008). Because it has been shown that tolerated doses of glutamate receptor agonists may be neuroprotective, we seeked to develop pharmacological models of preconditoning by first establishing which was the toxic threshold in our system for for NMDA, that has been used as a preconditioning stimulus in various *in vitro* culture models (Grabb & Choi, 1999; Raval et al., 2003; Soriano et al., 2006). NMDA induced significant increases in CA1 PI fluorescence 24 h later starting at 10 μM when added to the incubation medium for 60 min, at 30 μM when incubated for 30 min, and at 30-100 μM when incubated for 15 min (Fig. 20).

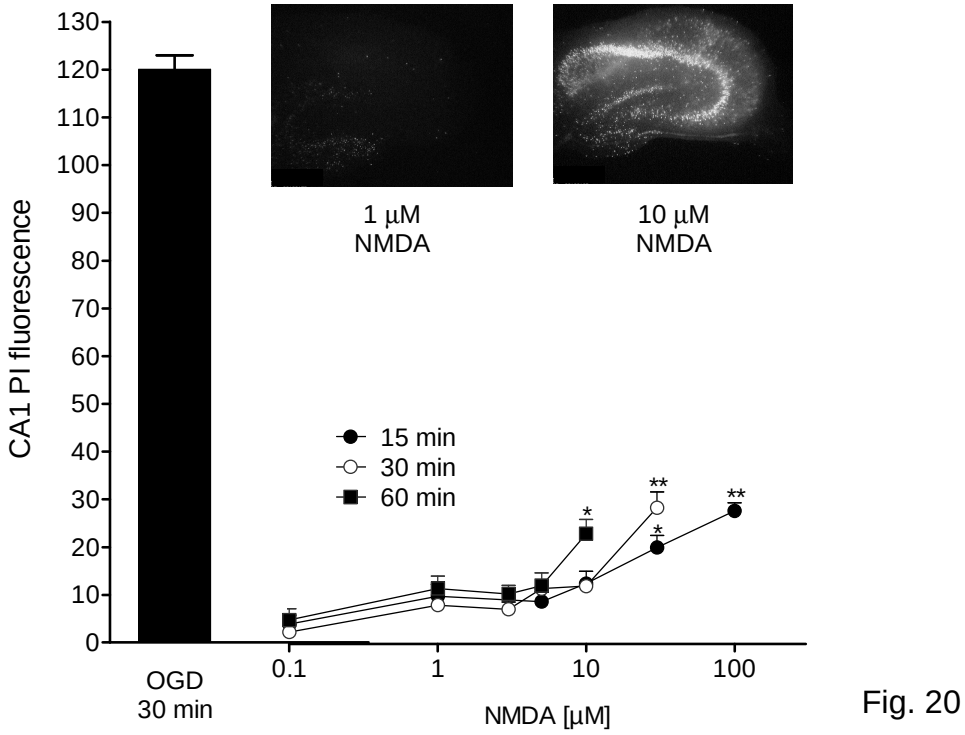


Fig. 20 NMDA toxicity in organotypic hippocampal slices

When the highest concentration of DHPG that was tolerated without producing a toxic response (i.e., 10 μM) was added to the incubation medium for 30 min, the CA1 injury induced 24 h later by a 30 min exposure to OGD was reduced by approximately 50%. Previous exposure to a slightly toxic concentration of DHPG (100 μM for 30 min)

did not induce tolerance but rather exacerbated the subsequent neuronal death induced by OGD (Fig. 21 A and 21 B).

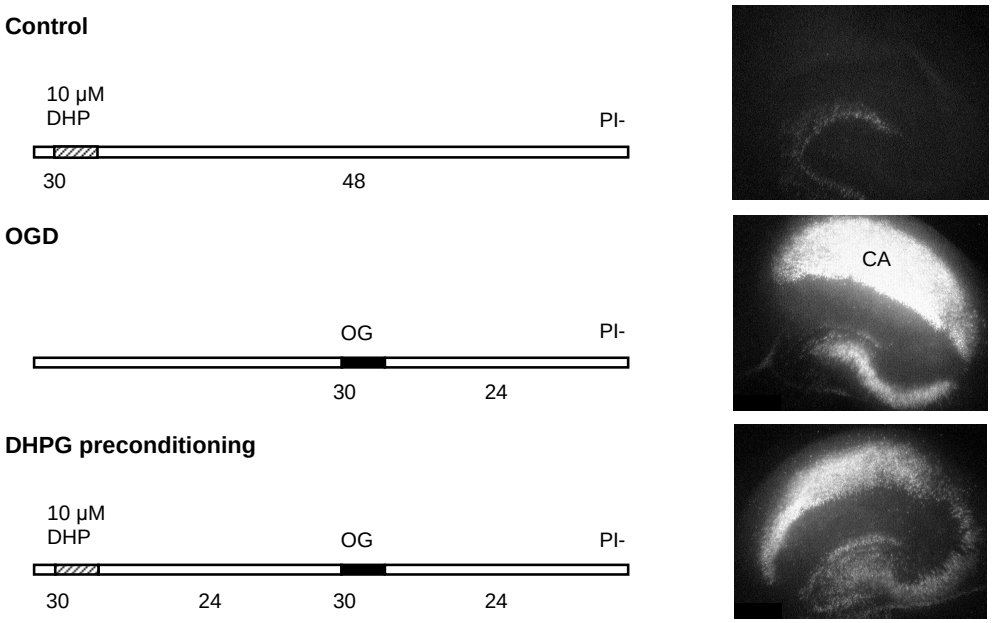


Fig. 21 A

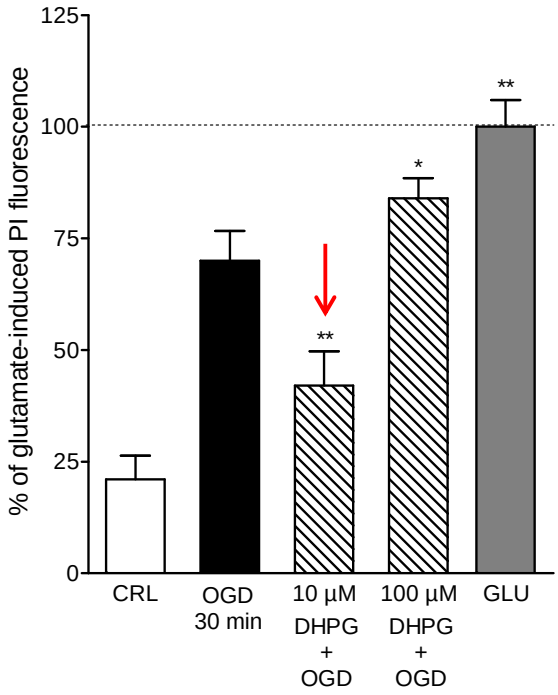


Fig. 21 B

Fig. 21A & 21B. Slices were exposed to 10 µM DHPG for 30 min and then, 24 h later, to 30 min OGD. Quantitative data are expressed as percentage of Glutamate-induced CA1 totoxicity and show that DHPG preconditioning reduced OGD injury. Bars represent the mean ± SEM of at least 4 experiments. **P<0.01 and *P<0.05 vs. AMPA

Similarly, we tested the neuroprotective potential of various concentrations of NMDA that were below (1 and 3 μM for 30 and 60 min, 10 μM for 30 min) or slightly above (10 μM for 60 min) the toxic threshold, and observed that pre-exposure of hippocampal slices to 1 and 3 μM, but not to 10 μM NMDA, was able to reduce the subsequent CA1 injury induced by 30 min OGD (Fig. 22).

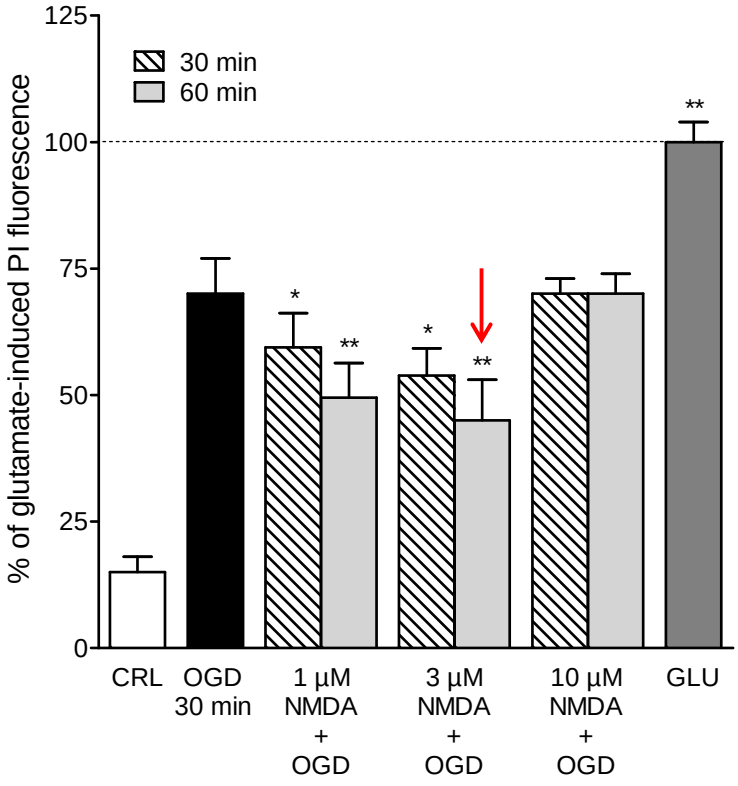


Fig. 22

Fig. 22. Slices were exposed to 1, 3 or 10 μM NMDA for 30 or 60 min and then, 24 h later, to 30 min OGD. Quantitative data are expressed as percentage of glutamate-induced CA1 toxicity and show that preconditioning at 1-3 μM NMDA**,** but not at higher concentrations, significantly reduced OGD injury. Red arrow depicts the paradigm that was selected for all subsequent experiments. Bars represent the mean ± SEM of at least 4 experiments. **P<0.01 and *P<0.05 vs. glutamate.

Pre-exposure to 3 μM NMDA for 60 min produced the highest degree of tolerance (by approximately 45%), and hence we selected this paradigm for our subsequent experiments. Interestingly, both of these pharmacological preconditioning protocols (10 μM DHPG for 30 min and 3 μM NMDA for 60 min) were also able to reduce 24 h later

the neuronal damage induced in organotypic hippocampal slices by a toxic exposure to 10 µM NMDA for 24 h (not shown).

In order to evaluate the mechanisms leading to tolerance towards the OGD insult, we investigated the effects of pharmacological preconditioning on the subsequent neurotoxicity induced by ionotropic glutamate receptor agonists AMPA. Exposure of slices to AMPA (10 µM) for 24 h produced a pattern of neurotoxicity, as observed 24 h later, that was slightly more intense and less selective for CA1 than that induced by 30 min OGD (Fig. 23A).

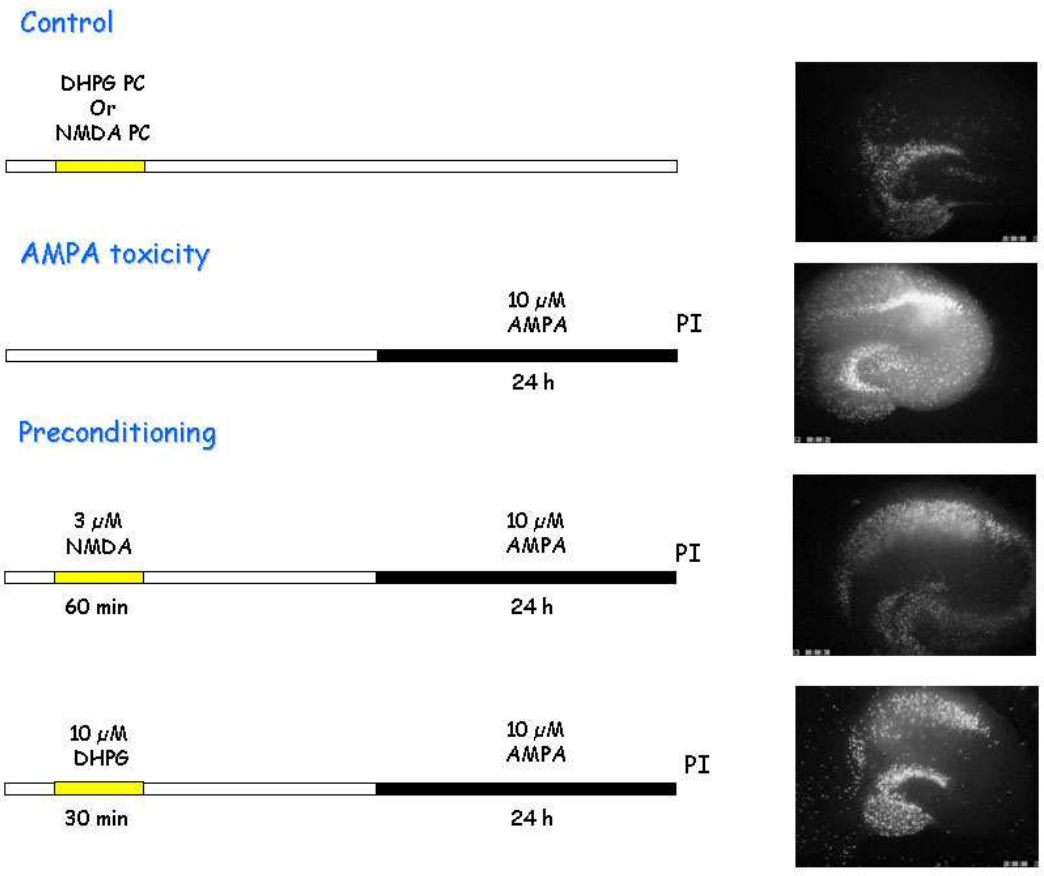


Fig. 23 A

When we tested whether a 24-h preexposure to preconditioning stimuli could affect AMPA toxicity, we observed that the pharmacological preconditioning paradigms reduced the neurotoxicity caused by 10 µM AMPA (Fig. 23B).

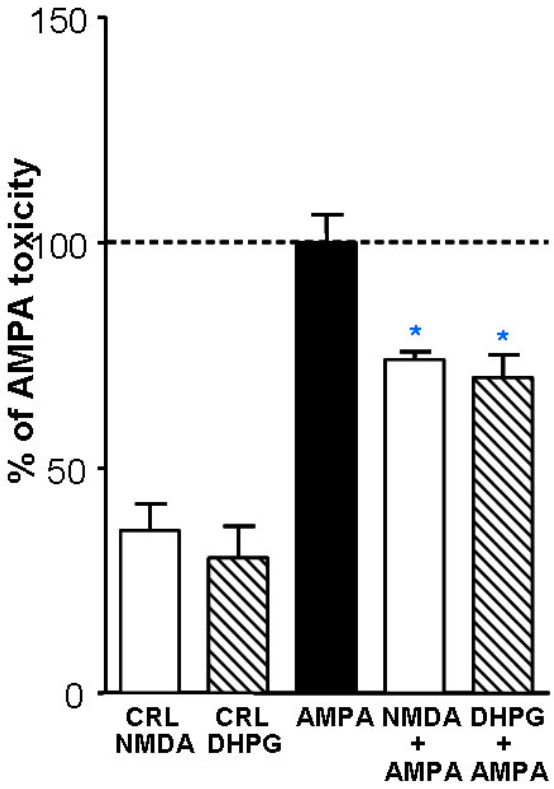


Fig. 23B

Fig. 23A & 23B. Slices were exposed to 3 µM NMDA for 60 min or to 10 µM DHPG for 30 min and then, 24 h later, to 10 µM AMPA for the subsequent 24 h. Quantitative data are expressed as percentage of AMPA-induced CA1 excitotoxicity and show that NMDA and DHPG preconditioning reduced AMPA injury. Bars represent the mean ± SEM of at least 4 experiments. **P<0.01 and *P<0.05 vs. AMPA.

8.2 AMPA currents following NMDA pharmacological preconditioning in organotypic hippocampal slices

To investigate the possible mechanisms by wich pyramidal neurons are able to develop tolerance to NMDA or AMPA excitotoxic insults, whole-cell voltage-clamp recordings were performed on control or preconditioned CA1 pyramidal neurons in rat organotypic hippocampal slices. To this aim, the slices are exposed to preconditionant doses of NMDA or DHPG and, 24h later, AMPA-stimulated inward currents recorded in CA1 pyramidal cells from organotypic hippocampal slices were measured using the patch clamp technich in whole cell configuration (Fig. 24A). Our results show that, when the slices are exposed to both our preconditioning stimuli 24h before the recordings, the AMPA induced-currents are significantly reduced (Fig. 24A).

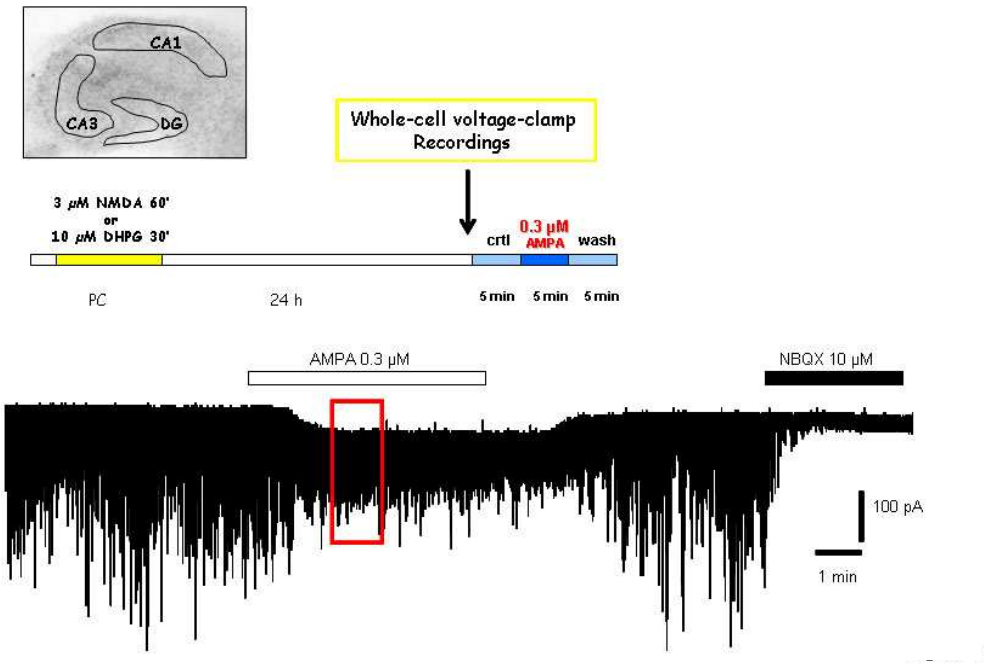


Fig. 24A

Fig. 24A. AMPA-stimulated inward currents recorded in CA1 pyramidal cells from organotypic hippocampal slices. Experimental protocol: NMDA and DHPG preconditioning was performed as described in panel 2. Whole-cell voltage-clamp recordings were performed after 24 h from preconditioning. Example of a typical experiment showing AMPA-induced (open bar) inward currents under control conditions. The figure also shows the inhibitory action of NBQX (10 μM; filled bar) on sEPSCs.

Fig. 24B describe how in control slices (solid circles), AMPA induced a clear increase of the holding current (Ihold) that was significantly reduced in slices preconditioned with DHPG (open circles) or with NMDA (open square). Bar graph showing the AMPA-induced inward current peak value in control and in preconditioned slices.

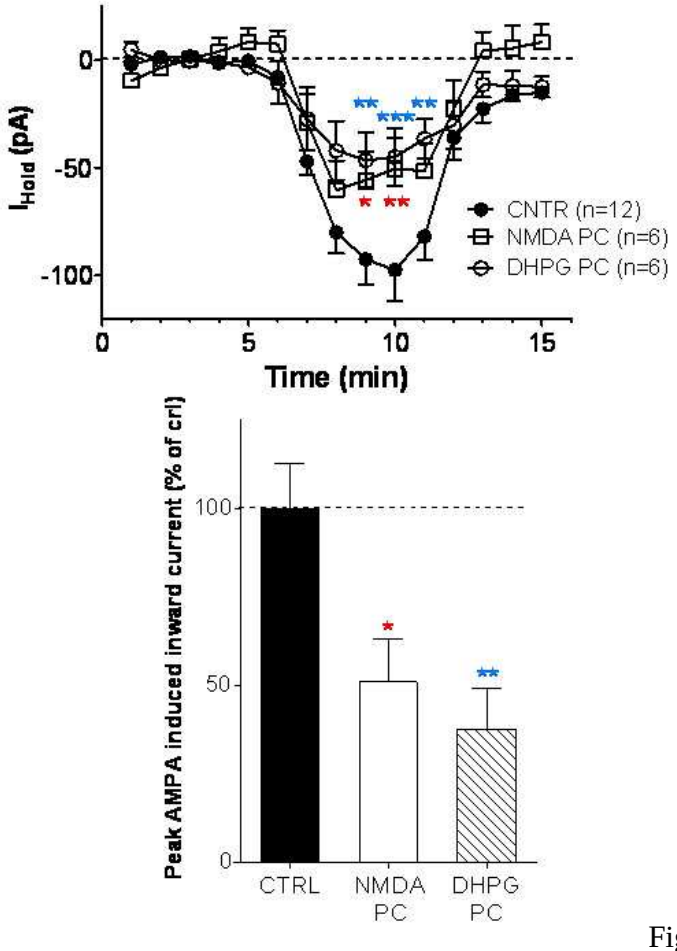


Fig. 24B

Fig. 24B. AMPA-stimulated inward currents recorded in CA1 pyramidal cells from organotypic hippocampal slices. In control slices (solid circles), AMPA induced a clear increase of the holding current (Ihold) that was significantly reduced in slices preconditioned with DHPG (open circles) or with NMDA (open square). Bar graph showing the AMPA-induced inward current peak value in control and in preconditioned slices. The number of cell tested is in parenthesis; data are shown as mean ± SEM, *P 0.05, **P 0.01 using 2 way ANOVA followed by Bonferroni post-tests.

Fig. 24C shows an example of a typical experiment showing AMPA-induced sEPSCs in control or 24 h after NMDA/DHPG preconditioning. Curve represent the cumulative probability plots demonstrating the effect of NMDA/DHPG preconditioning on sEPSC inter-event interval and amplitude. (Kolmogorov- Smirnov (K ± S) statistic P50.01).

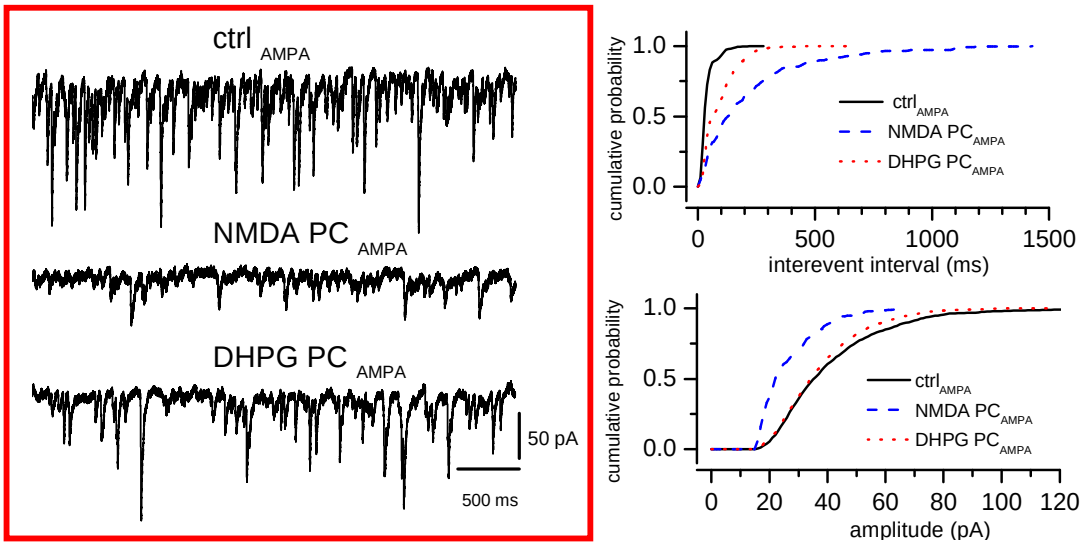


Fig. 24C

Fig. 24C. The traces show an example of a typical experiment showing AMPA-induced sEPSCs in control or 24 h after NMDA/DHPG preconditioning. Curve represent the cumulative probability plots demonstrating the effect of NMDA/DHPG preconditioning on sEPSC inter-event interval and amplitude. (Kolmogorov- Smirnov (K ± S) statistic P50.01).

In Fig. 24D, bar graphs show the value of the AMPA-induced effect on sEPSC frequency and amplitude in control and in NMDA or DHPG preconditioned slices. Our results show that 24 h following NMDA/DHPG preconditioning, the increase on AMPA-induced frequency was significantly reduced. On the other hand, the AMPA-induced amplitude was significantly reduced only after NMDA but not DHPG preconditioning.

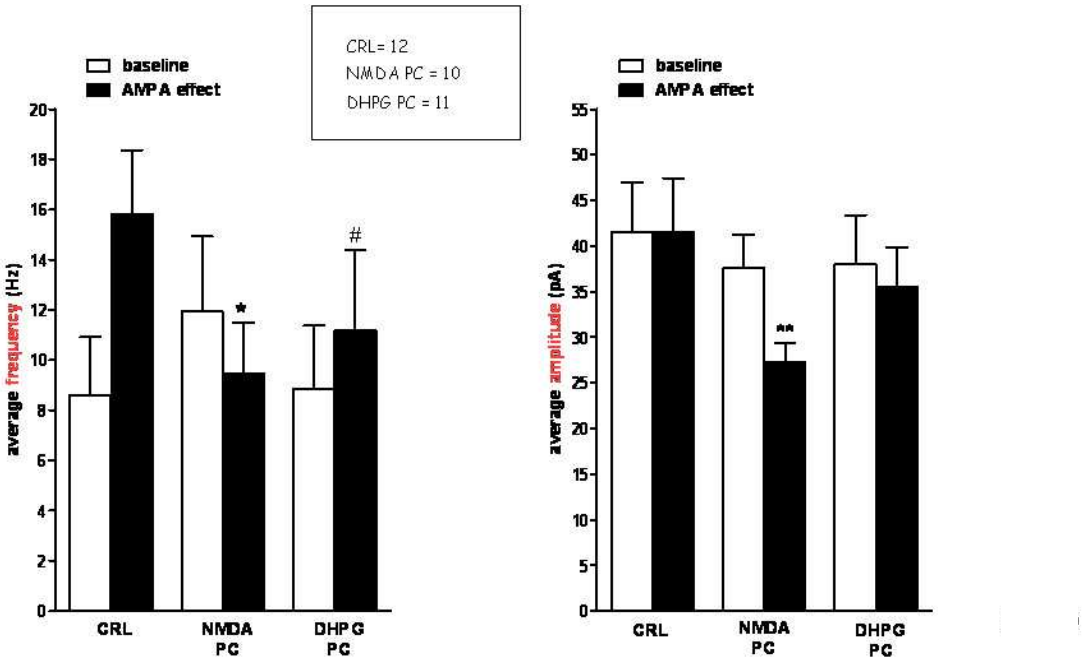


Fig. 24D

Fig. 24D. Bar graphs show the value of the AMPA-induced effect on sEPSC frequency and amplitude in control and in NMDA or DHPG preconditioned slices. Our results show that 24 h following NMDA/DHPG preconditioning, the increase on AMPA-induced frequency was significantly reduced. On the other hand, the AMPA-induced amplitude was significantly reduced only after NMDA but not DHPG preconditioning, Bars represent the mean of at least 4 experiments. *P<0.05 vs. CTRL.

Fig 24E, To investigate a possible involvement of presynaptic terminal and a different mechanisms of NMDA or DHPG induced-ischemic tolerance, AMPA-stimulated inward currents were recorded in CA1 pyramidal cells from organotypic hippocampal slices in the presence of tetrodotoxin (TTX). Application of 1 µM TTX does not modify AMPA-induced inward currents in control (solid circles), in NMDA (open square) and in DHPG (open circle) preconditioned slices, suggesting a possible presynaptic mechanism in the induction of tolerance in both of our preconditioning paradigms. Bar graph showing the AMPA-induced inward current peak value in control and in preconditioned slices.

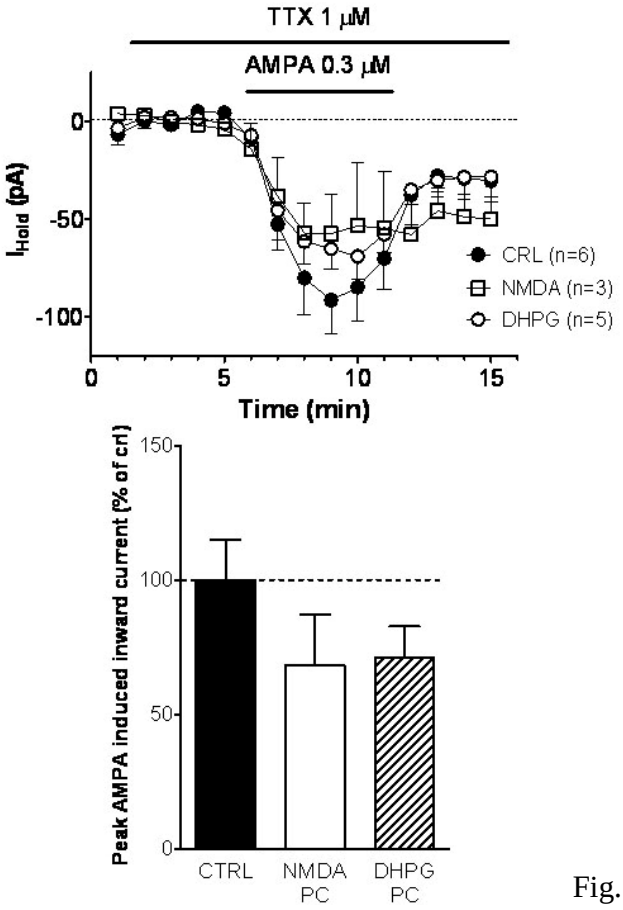


Fig. 24E

Fig. 24E. AMPA-stimulated inward currents recorded in CA1 pyramidal cells from organotypic hippocampal slices in the presence of tetrodotoxin (TTX). Application of 1 µM TTX does not modify AMPA-induced inward currents in control (solid circles), in NMDA (open square) and in DHPG (open circle) preconditioned slices, suggesting a possible presynaptic mechanism in the induction of tolerance in both of our preconditioning paradigms. The numbers of cell tested are in parenthesis. Bar graph showing the AMPA-induced inward current peak value in control and in preconditioned slices.

8.3 Modulation of glutamate receptor subunits following pharmacological preconditioning in organotypic hippocampal slices

To better comprehend the mechanisms leading to tolerance towards the AMPA insult, we investigated the effects of pharmacological preconditioning on the post synaptic density. In particular, we choose the phosphorylation of: NMDA subunits NR2A and NR2B, AMPA subunit GluR1, PSD95 or CAMKII. To this aim, hippocampal slices were treated with NMDA or DHPG preconditioning protocols and homogenate or TIF extract was prepared immediately or after 24 h the preconditioning treatment and processed by western blot analysis. Our results show that following 24 h after NMDA preconditioning, the expression of GluR1 was significantly reduced, while NR2A, NR2B, αCaMKII and PSD-95 immunostaining in the TIF were not affected (Fig. 25A).

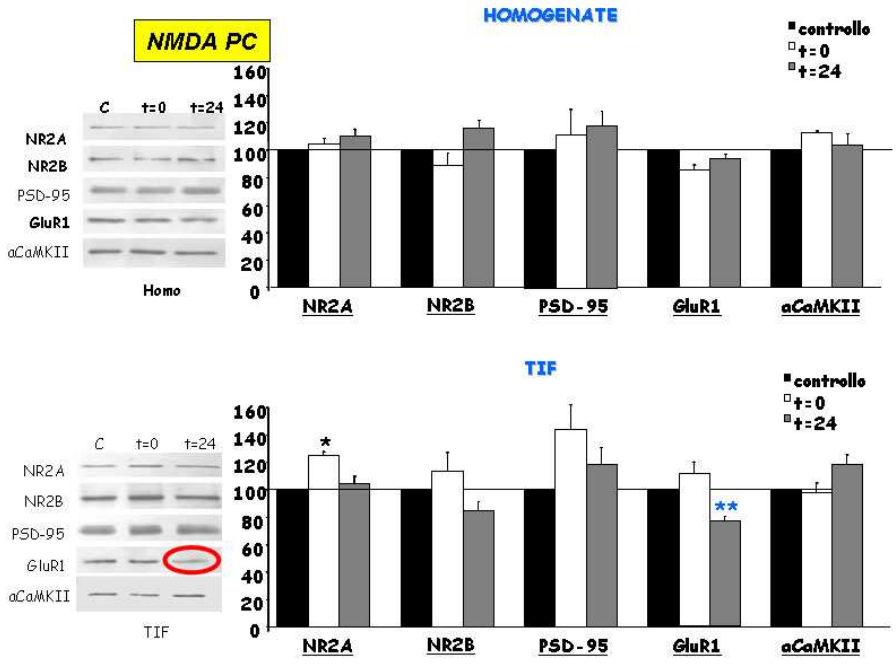


Fig. 25A

Fig. 25A. Expression of GluR1, NR2A and NR2B subunits, αCaMKII and PSD-95, in organotypic slices lysed immediately or 24 h after the preconditioning stimuli. Western blot analysis were performed in the homogenate (Homo) and postsynaptic TIF (triton insoluble fraction). 24 h after NMDA preconditioning, the expression of GluR1 was significantly reduced, while NR2A, NR2B, αCaMKII and PSD-95 immunostaining in the TIF were not affected. Data are expressed as percentage of control. Bars represent the mean ± SEM of at least 3 experiments. **P<0.01 and *P<0.05 vs. CRL

On the contrary, following DHPG preconditioning, we have observed no changes in the expression of GluR1, NR2A, NR2B, αCaMKII and PSD-95 immunostaining in the TIF or homogenate extract (Fig. 25B).

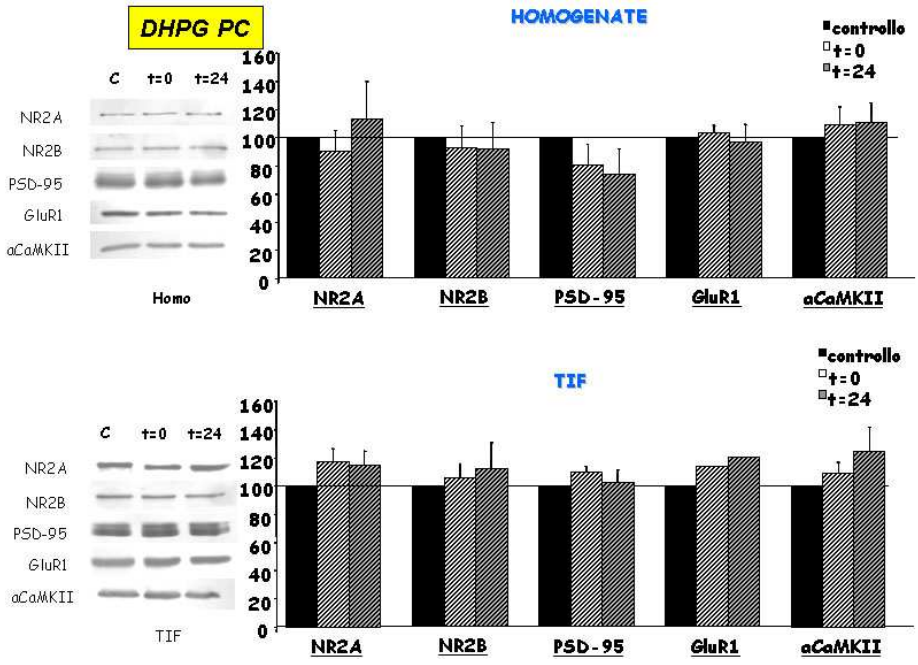


Fig. 25B

Fig. 25B. Expression of GluR1, NR2A and NR2B subunits, αCaMKII and PSD-95, organotypic slices lysed immediately or 24 h after the preconditioning stimuli. Western blot analysis were performed in the homogenate (Homo) and postsynaptic TIF (triton insoluble fraction). Our results show that following DHPG preconditioning, the expression of GluR1, NR2A, NR2B, αCaMKII and PSD-95 immunostaining in the TIF were not affected. Data are expressed as percentage of control. Bars represent the mean ± SEM of at least 3 experiments.

8.4 NMDA but not DHPG pharmacological preconditioning induces GluR1 internalization in organotypic hippocampal slices

To verify a possible AMPA interalization induced by our preconditioning paradigms, we used the cross-linker Bis(Sulfosuccinimidyl) suberate (BS3) in organotypic slices preconditioned 24h before the treatment and then we evaluated the expression level of GluR1. Western blotting analysis reveals that NMDA but not DHPG treatment induces GluR1 internalization, suggesting a different mechanism of induced neuroprotection. (Fig. 26).

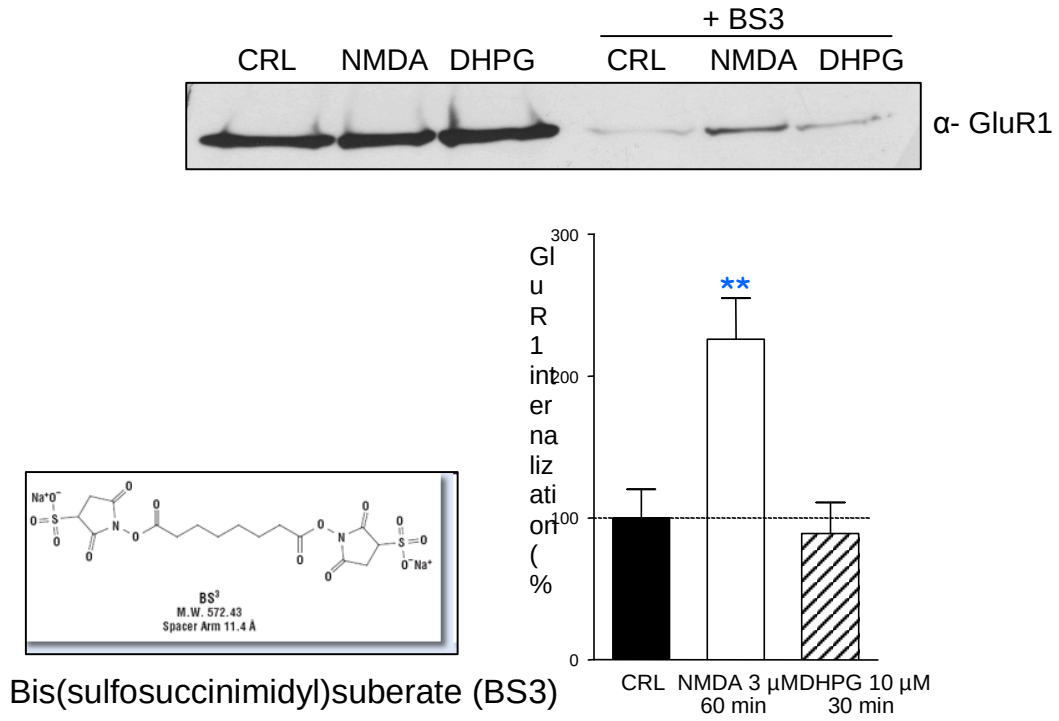


Fig. 26

Fig. 26. Organotypic slices were preconditioned with both NMDA and DHPG and treated, 24 h later, with the cross-linker bis(sulfosuccinimidyl) suberate (*BS3*). The expression levels of GluR1 were then evaluated by western blotting analysis, revealing that NMDA, but not DHPG preconditioning, increased the internalization of GluR1. Data are expressed as percentage of BS3-treated control. Bars represent the mean ± SEM of at least 4 experiments. **P<0.01 and *P<0.05 vs. BS3-treated control

Working Hypothesis: Fig. 27

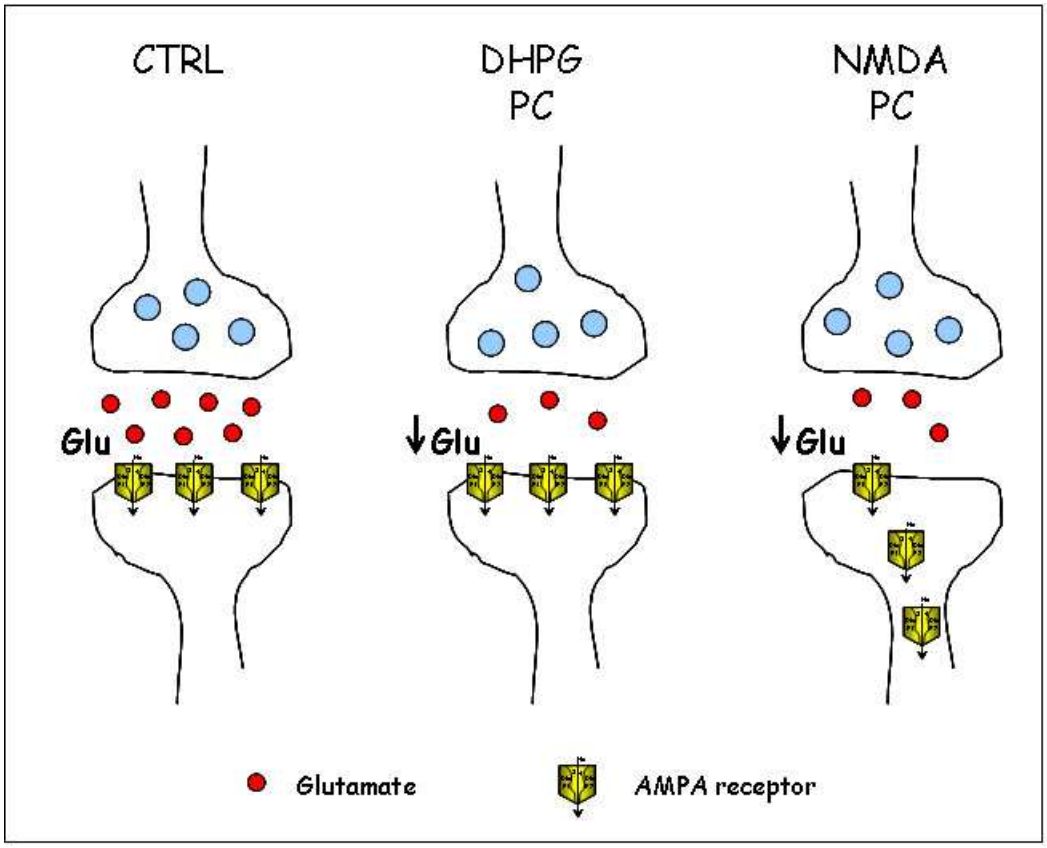


Fig. 27

Fig. 27 Under physiological conditions (CTRL), the presynaptic terminals release glutamate that act on AMPA postsynaptic receptors. We hypothesize that our preconditioning paradigms (DHPG PC and NMDA PC) modulate the level of glutamate release and/or the number of postsynaptic AMPA receptors on to the membrane.

PART II

8.5 Effects of PARP inhibitors on the induction of ischemic or pharmacological preconditioning

To determine the role of PARP-1 in the induction of pharmacological preconditioning, we used TIQ-A, a PARP-1 inhibitor characterized in our laboratory (Chiarugi et al., 2003). Fig. 28 shows that TIQ-A completely prevented the induction of tolerance to OGD induced by both DHPG and NMDA in a dose-dependent manner when present in the incubation medium during exposure of slices to the preconditioning agents (15 min preincubation plus 30 min for DHPG or 60 min for NMDA) and the subsequent 24 h recovery period. Another PARP-1 inhibitor, namely PJ34 (Abdelkarim et al., 2001), also prevented the neuroprotective effects of NMDA preconditioning under the same experimental conditions (data not shown). Our data suggest a possible involvement of PARP in the induction of tolerance.

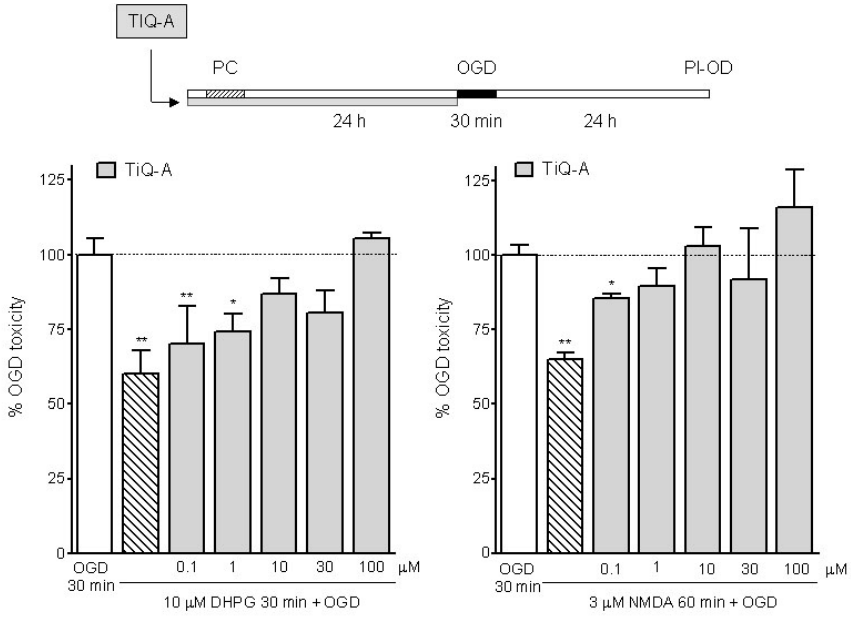


Fig. 28

Fig. 28. The tolerance induced by NMDA and DHPG was significantly reverted by TIQ-A (0.1 -100 µM) in a dose-dependent manner. Data were expressed as percentage of 30 min OGD-induced CA1 toxicity. Bars represent the mean ± SEM of at least 4 experiments. **P<0.01 and *P<0.05 vs. 30 min OGD.

8.6 Involvement of PARP on the induction of ischemic tolerance

To directly evaluate the activation of PARP-1 under our experimental conditions, we examined the formation of its product, the PAR polymer, in organotypic hippocampal slices immediately after their exposure to preconditioning concentrations of NMDA and DHPG (Fig. 29).

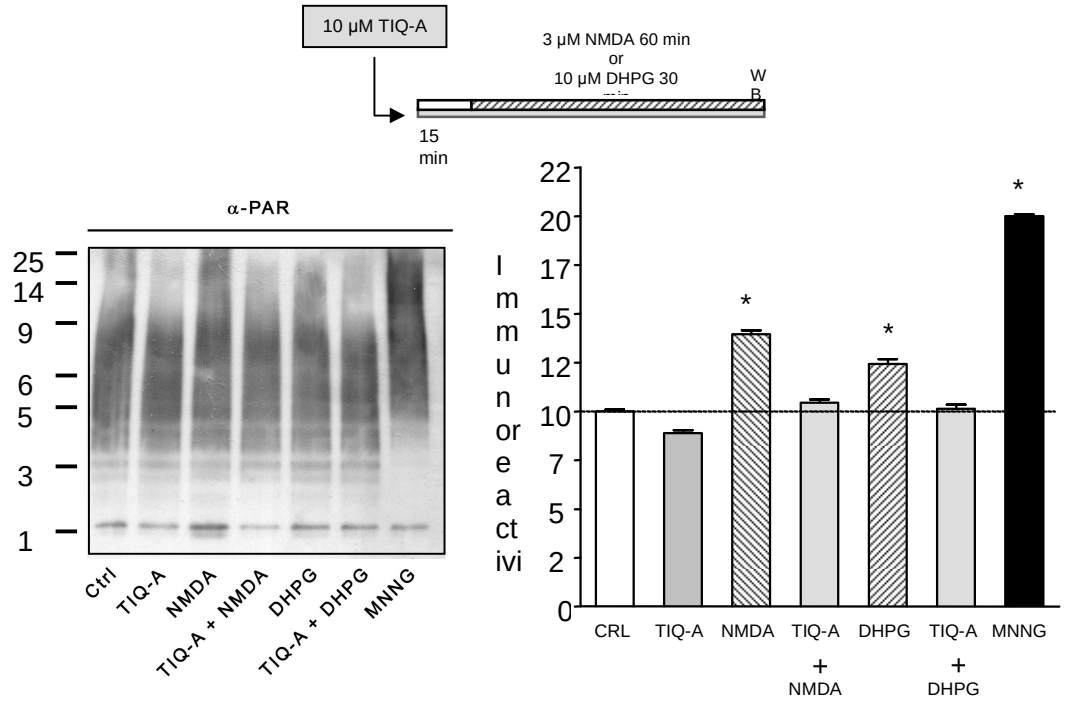


Fig. 29

Fig. 29. Hippocampal slices were exposed to 10 µM TIQ-A, 3 µM NMDA or NMDA +TIQ-A for 60 min, 10 µM DHPG or DHPG + TIQ-A for 30 min, or 100 µM MNNG for 5 min and then lysed and processed for Western blot with an anti-PAR antibody (Alexis). PARP activity, as detected by the formation of PAR, was increased after exposure to preconditioning concentrations of NMDA and DHPG. This increase was prevented when TIQ-A was added to the medium. Quantitative analysis of Western blots are expressed as percentage of control PARP activity. Bars represent the mean ± SEM of at least 4 experiments. **P<0.01 and *P<0.05 vs. control

Western blot analysis with an anti-PAR antibody revealed that both preconditioning protocols induced an increase in PAR formation that was significant but relatively modest as compared to that evoked by the alkylating agent MNNG, and was completely prevented by the presence of 10 µM TIQ-A in the incubation medium.

Interestingly, the important activation of PARP-1 induced by MNNG was associated with a dramatic decrease of cellular NAD^+ and ATP levels that was evident up to 4 h after exposure to the alkylating agent, whereas NMDA and DHPG produced only slight, transient and non-significant reductions (Fig. 9B). At any rate, the reductions in NAD^+ and ATP observed immediately after exposure to NMDA, DHPG and MNNG appeared to be PARP-1-dependent in that they were all prevented by the use of TIQ-A (Table 2).

Table 1. NAD^+/ATP consumption in preconditioned hippocampal slices is dependent on PARP-1 activity.

	NAD^+		**ATP**	
	Absorbance (nm)	(%)	Counts per second	(%)
Basal	0.32 ± 0.08	(100)	106,434 ± 25,551	(100)
10 µM TiQ-A 60 min	0.36 ± 0.12	(112)	95,903 ± 35,256	(90)
3 µM NMDA 60 min	0.28 ± 0.07	(86)	80,611 ± 21,062	(75)
NMDA + TiQ-A	0.30 ± 0.07	(93)	116,007 ± 31,790	(108)
10 µM DHPG 30 min	0.24 ± 0.05	(74)	68,191 ± 15,791	(64)
DHPG + TiQ-A 5 min	0.37 ± 0.21	(115)	104,486 ± 30,906	(98)
100 µM MNNG	0.19 ± 0.04*	(58)	41,808 ± 13,017*	(39)
MNNG + TiQ-A	0.26 ± 0.11	(80)	104,572 ± 50,000	(98)

Tab. 2 Slices were exposed to drugs as indicated and then assayed to determine NAD^+ or ATP cellular levels immediately after the treatment. TIQ-A was added to the incubation medium 15 min prior to and during the period of incubation with the other agents. Results are expressed as nm of absorbance for NAD^+ and as counts per minute for ATP, as well as percentage of basal NAD^+ or ATP levels in untreated slices. The modest nonsignficant decrease in NAD^+ and ATP levels induced by preconditioning with NMDA and DHPG, as well as the robust decrease induced by MNNG, were all prevented by TIQ-A. Values represent the mean ± SEM of at least 3 experiments run in triplicate. *P<0.05 vs. basal (ANOVA + Tukey's *w* test).

8.7 Involvement of caspase-3 in the induction of ischemic tolerance

Because caspase-3 activation has been shown to be essential for neuroprotection in preconditioning (McLaughlin et al., 2003; Garnier et al., 2003), we examined its role in our models by using the caspase inhibitor Z-VAD-FMK. Fig. 30A shows that neuroprotection induced by both DHPG and NMDA preconditioning was prevented when 100 μM Z-VAD-FMK was added to the incubation medium during exposure of slices to the preconditioning agents and the subsequent 24 h recovery period. We then examined the activity of caspase 3/7 in preconditioned slices and following incubation with 10 μM staurosporine for 24 h, a condition that is known to induce apoptotic cell death in this system (Meli et al., 2004).

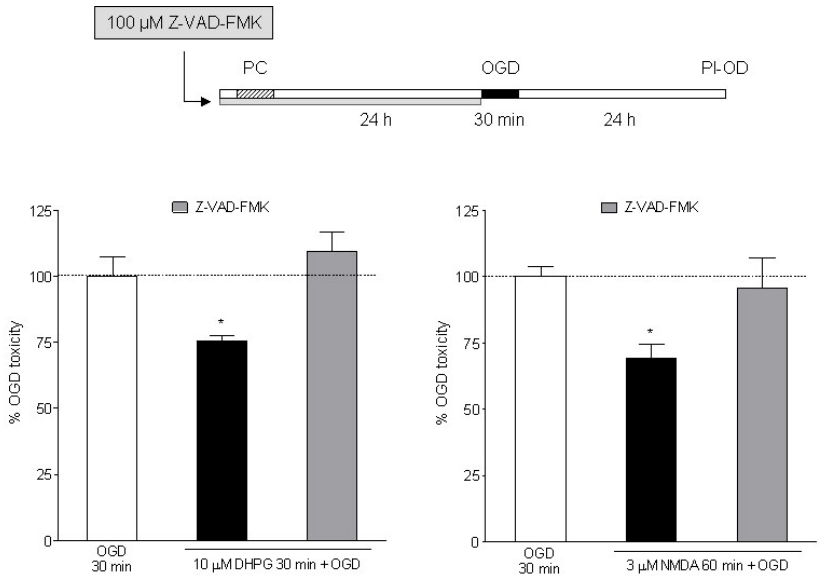


Fig. 30A

Fig. 30 A Caspase activity is required for the induction of DHPG and NMDA preconditioning in rat organotypic hippocampal slices. Schematic diagram showing that slices were incubated with the caspase inhibitor Z-VAD-FMK 15 min before preconditioning (PC), during PC, and during the subsequent 24 h recovery period. CA1 injury was assessed 24 h later by assessing the optical density of PI fluorescence (PI-OD) in this region. Quantitative analysis of CA1 PI fluorescence expressed as percentage of 30 min OGD-induced CA1 toxicity, showing that the tolerance induced by DHPG (B), NMDA were significantly reverted by 100 μM Z-VAD-FMK. Bars represent the mean ± SEM of at least 4 experiments. *P<0.05 vs. 30 min OGD (ANOVA + Tukey's *w* test).

Exposure of hippocampal slices to DHPG and NMDA preconditioning produced an increase in caspase-3/7 activity 1 h later that was significant but modest as compared to that induced by staurosporine and was completely prevented by the presence of 100 μM Z-VAD-FMK in the incubation medium (Fig. 30B).

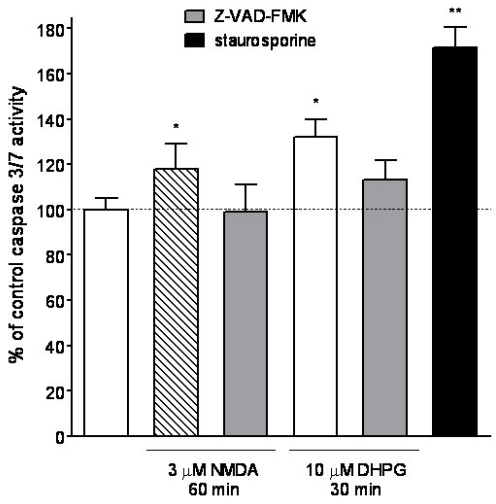


Fig. 30B

Fig. 30 B Caspase 3/7 activation in preconditioned hippocampal slices. Slices were exposed to either the NMDA (3 μM for 60 min) or DHPG (10 μM for 30 min) preconditioning protocol and processed 1 h later. Z-VAD-FMK (100 μM) was added to the incubation medium 15 min before and during the preconditioning exposure. Caspase 3/7 activity, as detected by the Caspase-Glo®3/7 Assay kit, was increased in preconditioned slices, but to a lesser extent as compared to incubation with 10 μM staurosporine for 24 h. Data are expressed as percentage of control caspase 3/7 activity. Bars represent the mean ± SEM of at least 3 experiments. **P<0.01 and *P<0.05 vs. control (ANOVA + Tukey's *w* test).

Sublethal caspase activation has been shown to result in PARP-1 cleavage that leads to ischemic tolerance (Garnier et al., 2003). Hence, we examined whether caspase-3/7 activation could determine PARP-1 cleavage under our experimental conditions by using an antibody that detects endogenous levels of full length PARP-1 (116 kDa), as well as the large fragment (89 kDa) of PARP-1 resulting from caspase cleavage (Fig. 10C). Western blot analysis revealed that PARP-1 was cleaved only when organotypic slices were exposed to 10 μM staurosporine for 24 h and not when they were collected and processed immediately (Fig. 30C) or 24 h (data not shown) after exposure to either of the three preconditioning paradigms used in this study.

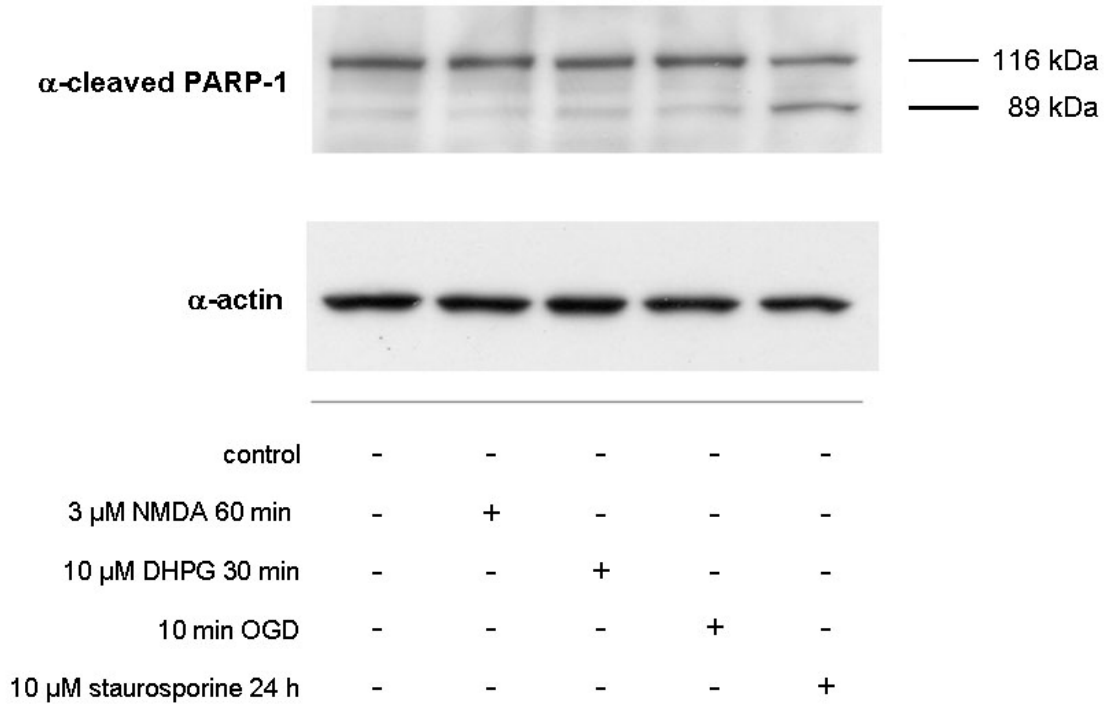


Fig. 30C

Fig. 30 C PARP-1 is not cleaved following pharmacological and ischemic preconditioning in rat organotypic hippocampal slices. Slices were exposed to either 3 µM NMDA for 60 min, 10 µM DHPG for 30 min or 10 min OGD and then lysed and processed for Western blot with an antibody directed against both full length PARP-1 (116 kDa) and the large fragment (85 kDa) resulting from caspase cleavage. PARP-1 was not cleaved in our three preconditioning models as compared to incubation with 10 µM staurosporine for 24 h. The numbers on the right indicate the position of the molecular mass markers in kilodaltons (kDa).

Working Hypothesis: Fig. 31

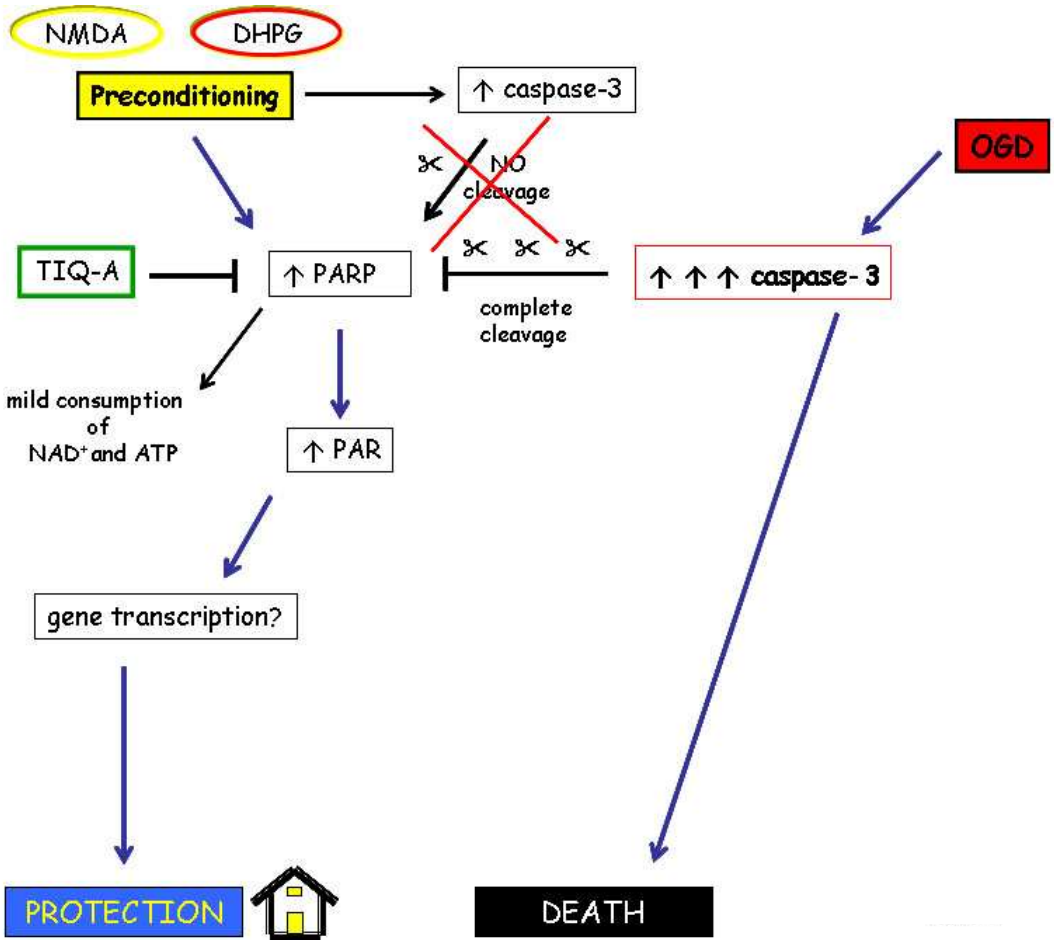


Fig. 31

PART III

8.8 Effects of HDACs inhibitors on OGD and on the induction of ischemic tolerance in organotypic hippocampal slices

As previously reported (Faraco et al., 2006), pharmacological inhibition of histone deacetylases by SAHA reduces MCAO induced-ischemic injury in the mouse brain. We tested the effect of HDAC inhibitors on OGD in our model of organotypic hippocampal slices. Fig. 32 shows that exposing hippocampal slices to SAHA (10 µM) or sodium butirrate (100 µM) during 30 min OGD and during the subsequent 24 h recovery period, we observed a significant neuroprotection approximately 25% in CA1 region. On the contrary, culture slices esposed to 10 µM SAHA for 1 or 3 h, 24 h before 30 min OGD were not able to be preconditioned (10 µM SAHA is not able to induce tolerance).

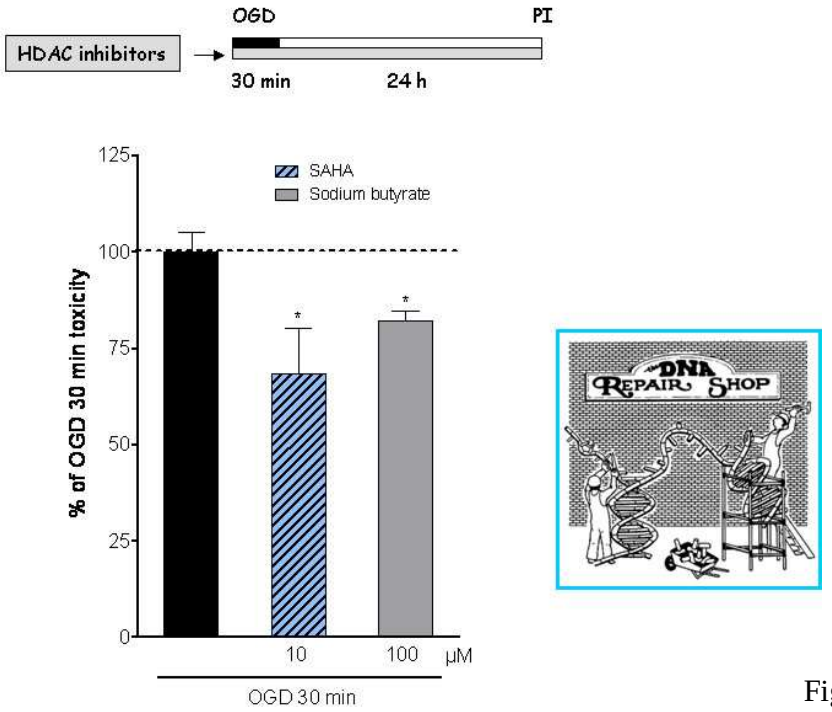


Fig. 32

Fig. 32 The HDAC inhibitors SAHA and sodium butirrate was added to the incubation medium 15 min prior, during 30 min OGD and during the subsequent 24 h recovery period. Data are expressed as percentage of 30 min OGD-induced CA1 toxicity. Bars represent the mean ± SEM of at least 4 experiments. **P<0.01 and *P<0.05 vs. 30 min OGD.

8.9 Involvement of HDACs on the induction of ischemic tolerance

To determine the role of HDACs in the induction of pharmacological preconditioning, we used SAHA (1 and10 µM) and sodium butirrate (10 and 100 µM), two HDACs inhibitors. Fig. 33 shows that both inhibitors completely prevented the induction of tolerance to OGD induced by NMDA in a dose-dependent manner when present in the incubation medium during exposure of slices to the preconditioning agents (15 min preincubation plus 60 min for NMDA) and the subsequent 24 h recovery period.

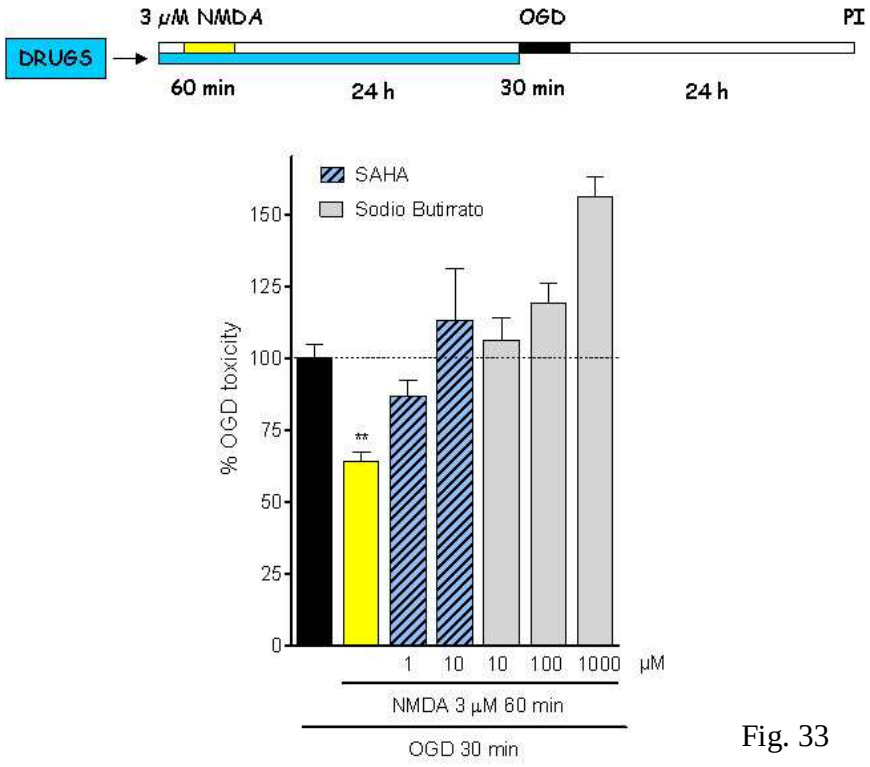


Fig. 33

Fig. 33 The HDAC inhibitors SAHA and sodium butirrate was added to the incubation medium 15 min prior, during preconditioning and during the subsequent 24 h recovery period. Data are expressed as percentage of 30 min OGD-induced CA1 toxicity. The tolerance induced by NMDA was significantly reverted by SAHA and sodium butirrate when incubated in a dose dependent manner. Bars represent the mean ± SEM of at least 4 experiments. **P<0.01 and *P<0.05 vs. 30 min OGD.

In order to establish which was the critical period for the beneficial involvement of HDACs in the development of tolerance to OGD, we added SAHA to the incubation medium only during the 60 min exposure to a preconditioning dose of NMDA (Fig.

34B, “Pre”) or during the subsequent 3, 6 or 24 h recovery period (Fig. 14B). Our results show that the development of tolerance to OGD could be abolished only when HDACs were inhibited during the exposure to the preconditioning stimulus and until 6h after treatment, not at early time points (Fig. 34 A and 34 B).

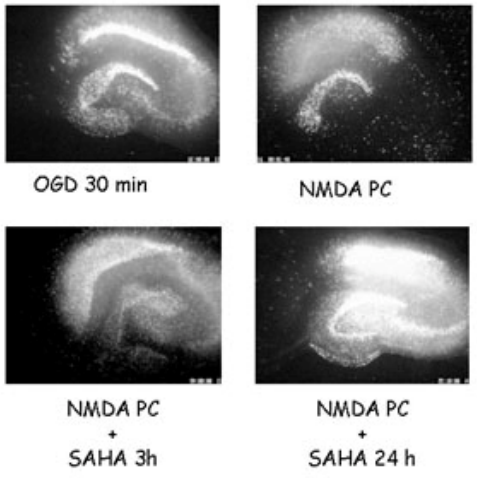


Fig. 34A

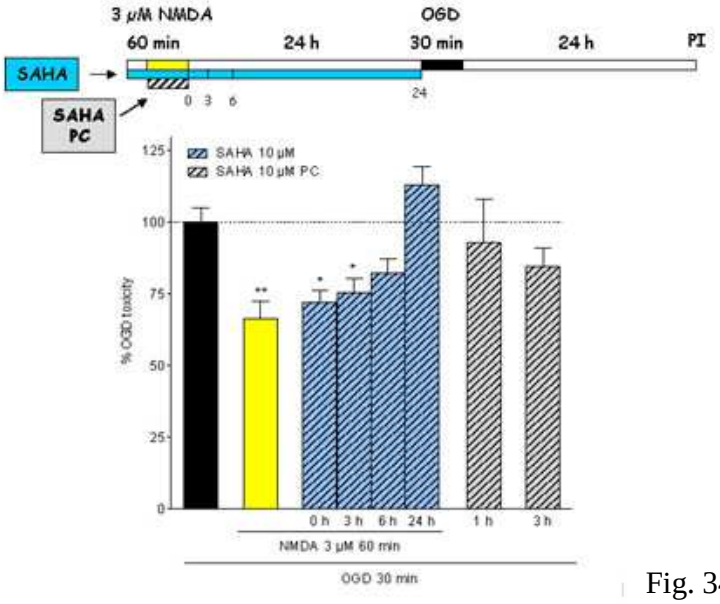


Fig. 34B

Fig. 34 a & 34B. Slices were exposed to 3 µM NMDA for 60 min then, 24 h later, to 30 min OGD. The HDAC inhibitor SAHA was added to the incubation medium 15 min prior, during 30 min OGD and during the subsequent 24 h recovery period. Data are expressed as percentage of 30 min OGD-induced CA1 toxicity. The tolerance induced by NMDA was significantly reverted by SAHA in a time-dependent manner. SAHA was not able by itself to induce tolerance when added to the incubation medium for 1 or 3h, 24 h before 30 min OGD challenge. Bars represent the mean ± SEM of at least 4 experiments. **P<0.01 and *P<0.05 vs. 30 min OGD.

8.10 Interplay between PARP and HDACs activities on the induction of ischemic tolerance

We have previously demonstrated the involvement of PARP in the induction of ischemic tolerance in the same preconditioning paradigm (PART II). To evaluate the activity of HDACs in our preconditioning model and the possible interplay with PARP, we carried out western blotting experiments using antibodies against the poly(ADP-ribose) (PAR) polymer product and against the histone H3 acetylated in lysine (K) 18 (Fig. 35).

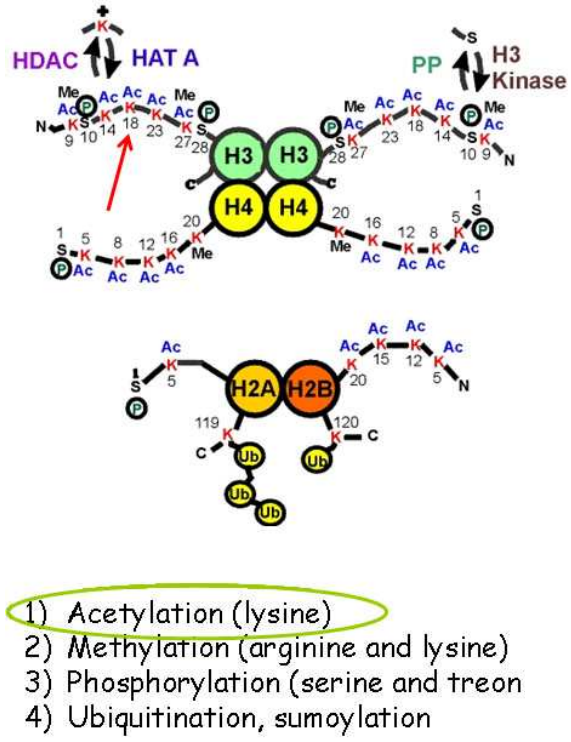


Fig. 35

3h after the preconditioning stimulus we observed a sligh increase in H3 (K18) acetylation as compared with the dramatic increase induced by the HDAC inhibitor SAHA (Fig. 36A).

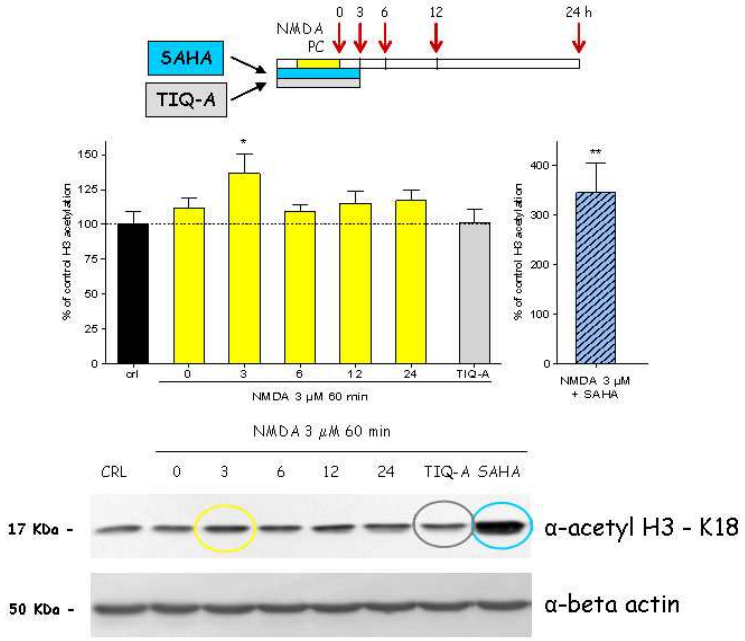


Fig. 36A

Fig. 36A Hippocampal slices were exposed to 3 µM NMDA for 60 min and then left to recover for 3, 6, 12 or 24 h. Slices were then lysed and processed for Western blot with an anti-acetyl H3 (K18) antibody (Cell Signaling). We observed a significant NMDA-induced increase in H3 acetylation 3 h after the preconditioning stimulus, that was prevented when TIQ-A was added to the incubation medium during 60 min of NMDA treatment and during the subsequent 3 h, as compared with the dramatic increase induced by the HDAC inhibitor SAHA. Quantitative analysis of Western blots are expressed as percentage of control H3 acetylation. Bars represent the mean ± SEM of at least 4 experiments. **P<0.01 and *P<0.05 vs. control.

Futhermore, our results show that the formation of PAR was increased by preconditioning doses of NMDA in a TIQ-A-dependent manner (Fig. 36 B)., Finally, we observed that the increase in H3 (K18) acetylation induced by NMDA preconditioning, was significantly prevented by TIQ-A, whereas the increase in PAR formation was similarly prevented by SAHA (Fig. 16 B), thus suggesting a possible interaction between PARP and HDAC activities in the development of ischemic tolerance. We can exclude a direct action on PARP pure enzyme, because the HDAC inhibitor SAHA is not able to block it as compared with the endogenus inhibitor nicotinamide or a potent PARP inhibitor PJ34 (Fig. 36 C).

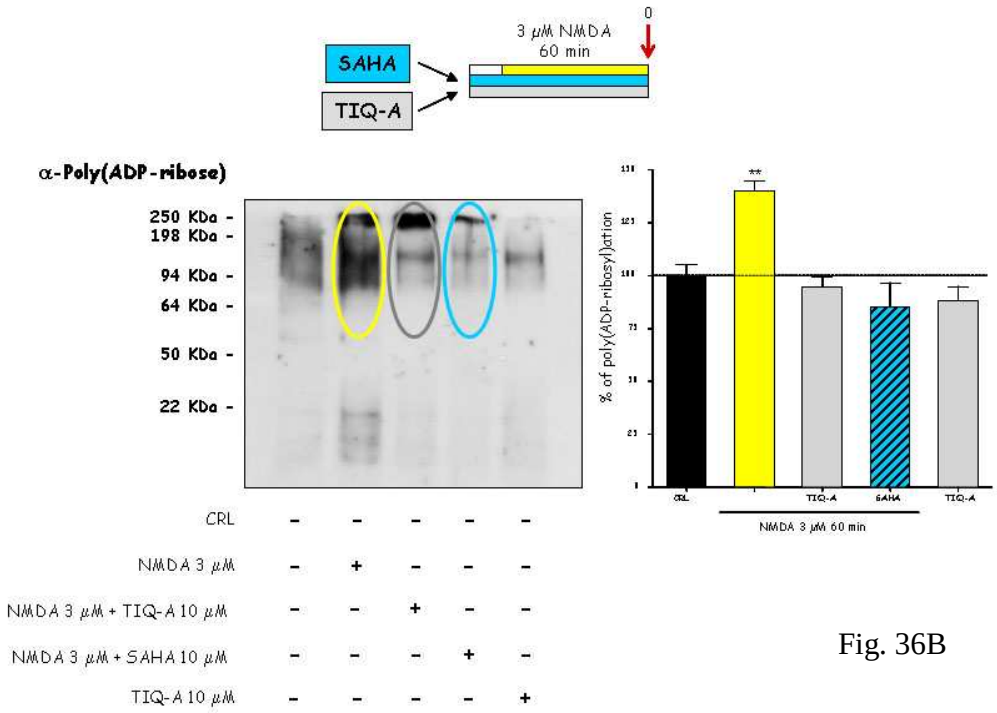


Fig. 36B

Fig. 36B Hippocampal slices were exposed to 3 µM NMDA, NMDA + TIQ-A or NMDA + SAHA for 60 min and then lysed and processed for Western blot with an anti-PAR antibody (Alexis). PARP activity, as detected by the formation of PAR, was increased after exposure to a preconditioning concentration of NMDA. This increase was prevented when TIQ-A or SAHA were added to the medium. Quantitative analysis of Western blots are expressed as percentage of control PARP activity. Bars represent the mean ± SEM of at least 4 experiments. **P<0.01 and *P<0.05 vs. control.

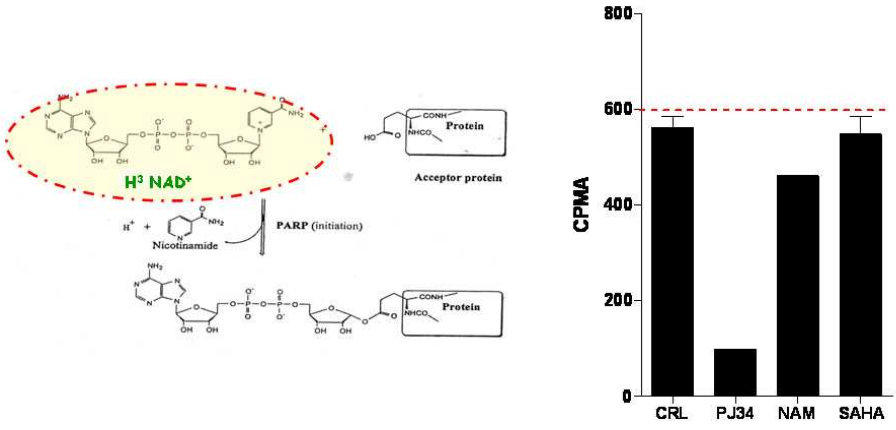


Fig. 36C

Fig. 36C The activity of purified PARP was measured using a radiolabeled assay. The enzyme uses [3H]NAD to form PAR polymers and the extent of PAR formation was assessed by quantification of radiation. Our results show that the HDAC inhibitor SAHA (10 µM) does not directly inhibit purified PARP, as compared with 100 µM of the endogenous inhibitor nicotinamide (NAM) or the inhibitor PJ34 (10 µM). Data are expressed as counts per minute (CPM). Bars represent the mean ± SEM of at least 3 experiments. **P<0.01 and *P<0.05 vs. control.

Working Hypothesis: Fig. 37

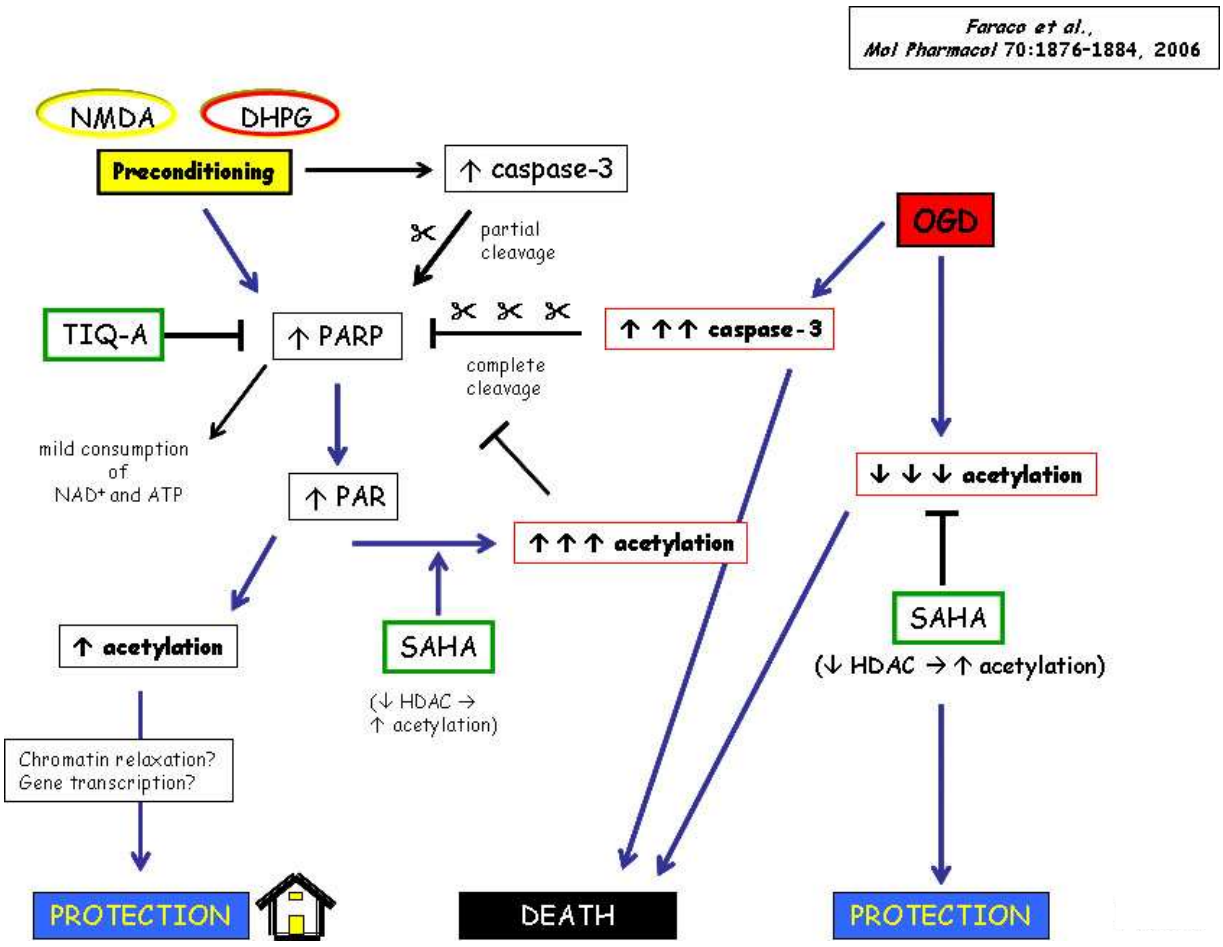


Fig. 37

9. DISCUSSION

Ischemic tolerance is an evolutionary conserved cellular defense program in which exposure to a subtoxic preconditioning stimulus results in resistance to a subsequent lethal ischemic insult (Gidday, 2006; Steiger & Hanggi, 2007; Stenzel-Poore et al., 2007; O'Duffy et al., 2007; Obrenovitch, 2008; Dirnagl et al., 2009). The key element in this process is the preconditioning stimulus and its ability to regulate neuronal activity, involving a number of transductional and traslational pathways that generate an array of mechanisms in which specific transducers lead to neuroprotection by modifying the gene expression of vulnerable cells.

A number of possible molecular mediators of ischemic tolerance, that determine either inhibition of programmed cell death or augmentation of programmed cell survival processes, have been proposed. Among these, the induction pathways triggered by activation of ionotropic (iGlu) and metabotropic glutamate (mGlu) receptors have received particular attention. Subtoxic concentrations of *N*-methyl-*D*-aspartate (NMDA) have been used as preconditioning stimuli (Grabb & Choi, 1999; Raval et al., 2003; Soriano et al., 2006) and shown to produce neuroprotection through various mechanisms, including moderate increases in the concentrations of intracellular calcium, the rapid release of brain-derived neurotrophic factor (BDNF), the formation of NO, the activation of the Ras/ extracellular signal-regulated kinase (Erk) and phosphatidylinositol 3 (PI3)-kinase/Akt pathways, and the increased activity of the transcription factor NFκB (Gidday et al., 1999; Shamloo et al., 1999; Gonzalez-Zulueta et al., 2000; Bickler and Fahlman, 2004). Similarly, activation of group I mGlu receptors of the mGlu1 and mGlu5 subtypes with sublethal concentrations of (*S*)-3,5-dihydroxypghenylglycine (DHPG) has recently been demonstrated to represent an effective preconditioning stimulus that is able of attenuating the toxic effects of NMDA and oxygen-glucose deprivation (OGD) (Blaabjerg et al., 2003; Werner et al., 2007).

In our experiments, we observed that DHPG (10 µM), a group I mGlu receptor agonist, or NMDA (3 µM) applied to the slices for 30 min 24 h before the lethal OGD or AMPA toxic challenge, significantly attenuated CA1 injury. These observations suggest that activation of group I mGlu receptors or NMDA receptors are sufficient to trigger mechanisms culminating in an increased resistance to ischemic insults (ischemic tolerance).

The endogenous molecular mechanisms of increased neuronal resistance induced by preconditioning represent attractive targets for the development of therapeutic strategies, but these processes are still not clearly understood.

We have examined two different aspects in the induction of ischemic tolerance: the modulation of glutamate receptors in the Post Synaptic Density and the changes in gene expression.

For the first point, we have investigated whether MAGUK proteins and glutamate receptor responses could be modified in rat organotypic hippocampal slice models of pharmacological preconditioning and how AMPA-stimulated inward currents could be modified after preconditioning stimulus.

A previous report of Blaabjerg and colleagues have supported the role of mGluR1 in neuroprotection against NMDA-induced cell death. In particular, their results show that selective activation of group-I-mGluRs with 10 µM DHPG (2h before recording and application of NMDA) protect neurons against a subsequent excitotoxic stimulation of NMDA receptors in a concentration-dependent manner in hippocampal CA1 and CA3 subfields in a model of organotypic hippocampal slices (Blaabjerg et al., 2003). These data show that DHPG-mediated neuroprotection against NMDA toxicity occurs via the mGluR1 receptor (infact, neuroprotection could be reversed by inhibition of the mGluR1 receptor with LY367385, but not by the mGluR5 receptor antagonist MPEP) and that neuroprotective DHPG treatment can greatly reduce NMDA-stimulated currents. A similar observation has been made in cerebellar granule cells, in which treatment with *trans*-ACPD or (*R,S*)-DHPG significantly reduces Ca^{2+} influx induced by application of NMDA or glutamate (Pizzi et al., 1996b) and in mouse cortical cultures where DHPG reduced NMDA whole-cell currents (Yu et al., 1997).

Our results also show that AMPA-stimulated inward currents in voltage-clamped CA1 pyramidal cells were significantly decreased, suggesting that activation of group-I-mGluRs or NMDARs could lead to inhibition of AMPA-receptor function. Our data suggest that a possible mechanism may involve modifications of AMPA receptors and its downregulation were observed. Infact, 24 h following preconditioning stimulation with NMDA preconditioning we observed a reduction in GluR1 AMPA subunit expression level in TIF preparation and an increase of the same protein after treatment

with a crosslinker BS3, indicating an internalization of AMPA receptor NMDA-preconditioning induced. No changes were observed in NMDA receptor subunits (NR2a and NR2B) or MAGUK proteins (PSD95 and CAMKII) after 24 h of preconditioning stimulation in the same experimental conditions. The internalization of AMPA receptors could explain NMDA but not DHPG neuroprotection. A possible mechanism by which DHPG induces neuroprotection is due probabily to a modulation of presynaptic terminals that could reduce the glutamate release (Blaabjerg et al., 2003). Our data support this thesis because the value of the AMPA-induced effect on sEPSC frequency but not amplitude in DHPG preconditioned slices as compared with control slices was significantly reduced 24 h following NMDA/DHPG preconditioning. On the other hand, the AMPA-induced amplitude was significantly reduced only after NMDA but not DHPG preconditioning indicating a presynaptic involvement for DHPG neuroprotection and a postsynaptic involvement for NMDA neuroprotection.

For the second point, we have choose to investigate the contribution of two classes of enzymes involved in gene modulation and early studied in our laboratory: PARP and HDACs. We know that both NMDA (Eliasson et al., 1997; Lo et al., 1998; Mandir et al., 2000) and DHPG (Meli et al. 2005) can stimulate the activity of PARP-1, and that the dynamic chromatin remodeling events in hippocampal nerurons are associated with NMDA receptor-mediated activation and histone modulation (Tian et al., 2009).

Our study shows that PARP-1 exerts a neuroprotective role in the development of ischemic tolerance following our preconditioning paradigms and this activation was relatively mild, and was not associated with a significant consumption of cellular NAD^+ and ATP. Caspase-3/7 activity also appeared to be necessary for the development of ischemic tolerance in these models, but the activation of this enzymatic pathway was modest and did not lead to significant cleavage of PARP-1.

In an astrocyte-neuron co-culture model, "chemical" ischemic preconditioning leads to mild caspase-3 activation and PARP-1 cleavage, suggesting a cause-effect relationship between inhibition of PARP-1-mediated cell death pathways and increased resistance to a subsequent lethal OGD challenge (Garnier et al., 2003). Caspase-3 activation and PARP-1 cleavage were also observed in a rat model of ischemic

preconditioning *in vivo*, and were shown to be triggered by sublethal zinc accumulation and the consequent activation of $p75^{NTR}$ in neurons (Lee et al., 2008). In a very recent report, caspase-3 and PARP-1 appear not to be required for the development of ischemic tolerance in a mouse model of focal ischemia (Faraco et al., 2010). Depending on the degree and nature of the insult, and especially in conditions that more closely resemble the mild or sublethal progressive stress of endogenous preconditioning and perhaps chronic neurodegeneration, PARP-1 activation may play a neuroprotective role.

The modest activation of caspase-3 induced by ischemic preconditioning is still able to cleave and inactivate PARP-1 (Garnier et al., 2003; Lee et al., 2008), and this mechanism has been proposed to confer resistance to neurons against subsequent insults, including ischemia, that would otherwise induce PARP-1-mediated cell death. Conversly, our DHPG and NMDA preconditioning protocols induced a similar modest increase in the activity of caspase-3 and 7, as compared to what observed with staurosporine, but were unable to produce significant cleavage of PARP-1. Hence, it appears that in our system PARP-1 cleavage occurs only following the substantial activation of caspase-3/7 induced by conditions that produce apoptotic cell death, i.e., incubation with 10 staurosporine for 24 h (Meli et al., 2004), whereas following DHPG and NMDA preconditioning caspase 3/7 is only modestly activated and PARP-1 is not cleaved, but rather appears to be necessary for the development of tolerance to subsequent insults in neurons.

On the other hand, despite the established role of PARP-1 in post-ichemic cell death, several reports have demonstrated that PARP-1 activation in physology or in conditions of mild cellular stress may be beneficial via multiple mechanisms, including DNA excision repair (Nagayama et al., 2000), histone acetylation (Cohen-Armon et al., 2007) and the activation of the transcription factor NF-κB (Chiarugi & Moskowitz, 2003), that may support the survival of neurons by inducing the expression of antiapoptotic genes (Bhakar et al., 2002). NF-kB is a key transcription factor in the regulation of a set of proteins, and PARP has been shown to act as a coactivator in the NF-kB-mediated transcription (Oliver et al., 1999). It is nowaday established that PARP-1 also regulates the expression of various proteins at the transcriptional level.

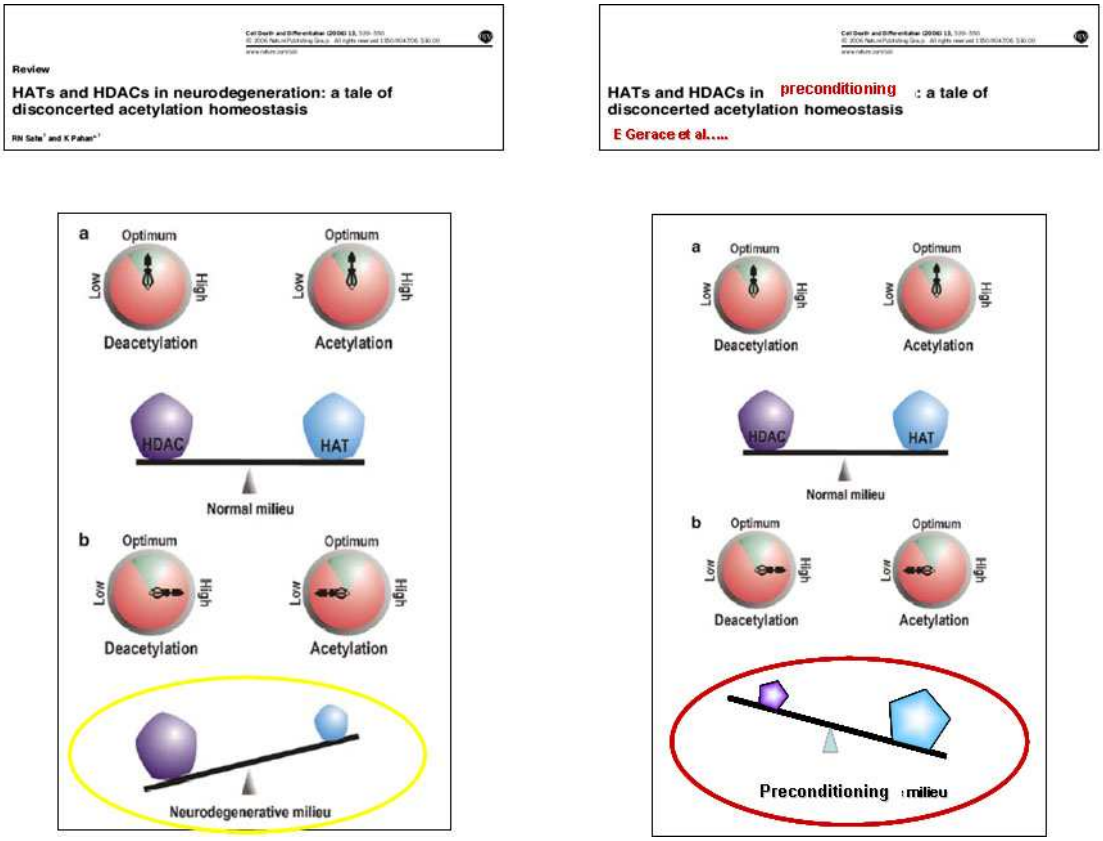
Poly(ADP-ribosylation) of histones may also contribute to the transcription-promoting effect of PARP-1, because poly(ADP-ribose) confers negative charge to histones, leading to electrostatic repulsion between histones and DNA. Thus, poly(ADP-ribosylation) can loosen the chromatin structure and can thereby make genes more accessible for the transcriptional machinery (Miyamoto et al., 1999).

The induction of ischemic tolerance is associated with a substantial change in gene expression, suggesting that preconditioning stimulates a fundamental genomic reprogramming of cells that confers cytoprotection and survival (Stenzel-Poore et al., 2007). The genomic response after ischemic preconditioning is a signature of the complex interplay of multiple signalling pathways. To this aim we investigated the role of HDAC in our preconditioning paradigms and a possible interplay between PARP and HDAC products. We know that histone acetylation enhances gene transcription while histone deacetylation decrease gene transcription and that protein acetylation is dynamic and maintained by two classes of functionally antagonistic enzymes: histone acetylases (HATs) and HDACs, which add or remove acetyl groups from protein substrates, respectively (Sun et al.; 2003).

A recent paper by Ting have demonstrated a crucial role for HDAC inhibition in protecting the heart against I/R injury in both early and delayed pharmacologic preconditioning and an essential role of p38 in mediating protection elicited by HDAC inhibition. HDAC inhibition causes a dramatic increase in p38 activity in pharmacologically preconditioned hearts, further supporting that HDAC inhibition with p38 activation is critical to achieve cardioprotection (Ting et al.; 2007).

Our results show that HDAC inhibitors are not able to induce tolerance by itself in a model of organotypic slices while are able to revert the neuroprotection induced by NMDA preconditioning stimulus. A possible mechanism of NMDA neuroprotection could be do to an increase in histones acetylation that confer in a gene molulation and finally in cell resistance and neuroprotection. According to this point, 3h after the NMDA preconditioning stimulus we observed a sligh increase in H3 (K18) acetylation as compared with the dramatic increase induced by the HDAC inhibitor SAHA (Fig. 36A). Faraco and colleagues have demonstrated that in the ischemic brain tissue subjected to 6 h of middle cerebral artery occlusion, histone H3 acetylation levels

drastically decreased, without evidence for a concomitant change of histone acetyl-transferase or deacetylase activities. Treatment with SAHA (50 mg/kg i.p.) increased histone H3 acetylation within the normal brain (of approximately 8-fold after 6 h) and prevented histone deacetylation in the ischemic brain (Faraco et al.; 2006). The acetylation level of histones seems to be very important for the destiny of cell survival. During neurodegeneration, critical loss of HAT protein level ensue a rebated HAT dose and activity. This unbalances the acetylation towards excessive deacetylation of target moieties (see the figure 38). We can add that in preconditionant conditions, an excessive level of histone acetylation induce to cell death probabily by upregulating gene expression and limiting the normal preconditionant mechanisms that induce to neuronal resistance.



Saha RN, Pahan K. (2006) Cell Death Differ. 13(4):539-50.

Fig. 38

Futhermore, we observed that the increase in H3 (K18) acetylation was significantly prevented by the PARP inhibitor TIQ-A, whereas the increase in PAR formation induced by the same NMDA preconditioning stimulus was similarly prevented by HDAC inhibitor SAHA (Fig. 36 B). This suggest a possible interaction between PARP and HDAC activities in the development of ischemic tolerance. We can exclude a direct action on PARP pure enzyme, because we have observed that SAHA is not able to block PARP activity as compared with the endogenus inhibitor nicotinamide or a potent PARP inhibitor PJ34 in an *in vitro* dosage (Fig. 36 C).

A recent paper pubblished by Krishnakumar suggests a possible interplay between PARP-1 and histone 1 at promoter specifies transcriptional outcomes caused by a similar nucleosome-binding properties and effects on chromatine structure *in vitro* (Krishnakumar et al., 2008). A review of Schreiber talks about poly(ADP-ribosylation) of histones H1 and H2B and relaxation of DNA structure and repair mediated by this covalent interaction (Schreiber et al.; 2006). Cohen-Armon and colleagues had demonstrated that poly (ADP-ribosylation) mediate erk-induced acetylation of core histones. In particular, phosphorylated ERK shuttles between the cytoplasm and nucleus and interact with PARP-1, enhancing PARP-1 activation and auto-polyADP-ribosylation. PolyADP-ribosylated PARP-1 acts as an anchoring protein for phosphorylated ERK2 in the nucleus and acts as a scaffold protein, enhancing ERK-catalyzed phosphorylation of transcription factor Elk1(see the figure 39).

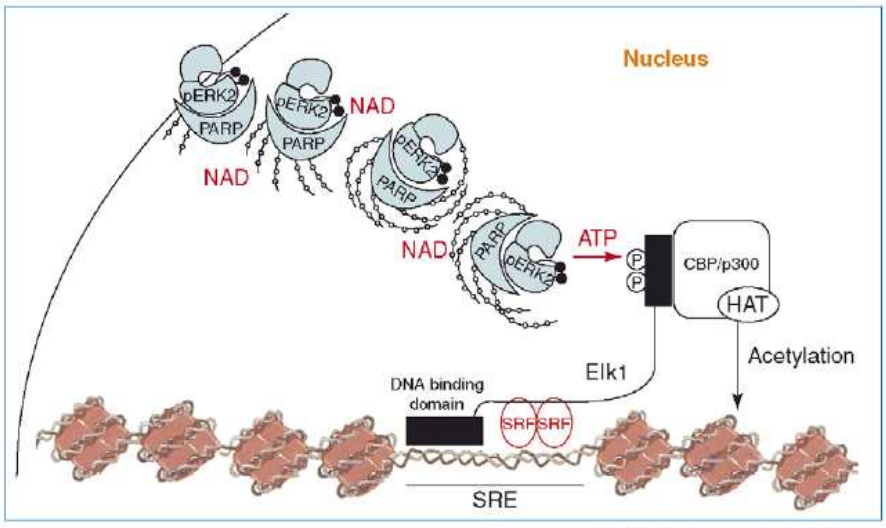


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Fig. 39

We hypothesized that Erk phosphorylated could be a link that can explain the interplay between poly(ADP-ribosyl)ation and H3 acetylation induced by NMDA preconditioning protocol in our organotypic hippocampal slices. Our results showed that the formation of PAR was increased by preconditioning doses of NMDA in a TIQ-A or SAHA - dependent manner. We observed that the increase in H3 (K18) acetylation induced by NMDA preconditioning, was significantly prevented by TIQ-A. Erk could be responsible for the interplay between poly(ADP-ribosyl)ation and histone acetylation in our model of preconditioning. Futhermore, our results show that NMDA preconditioning induce an increase in Erk½ phosphorylation suggesting a new via diverse signal transduction mechanisms (date not shown).

In conclusion, our results suggest that under conditions of sublethal cellular stress, such as those evoked by DHPG and NMDA preconditioning in our *in vitro* models of ischemic tolerance, mild activation of caspase 3/7, PARP-1 and HAT may produce a neuroprotective response. Because PARP inhibitors and HDAC inhibitors have entered the stage of clinical testing for the treatment of cancer (Horvath and Szabo, 2007; Liang and Tan, 2010) and are currently under investigation as potential therapeutic agents in stroke and neurotrauma (Komjati et al., 2005; Moroni and Chiarugi, 2009), our results indicate that caution should be exercised when considering the use of PARP-1or HDACs inhibitors in the presence of chronic neurodegenerative diseases.

10. CONCLUSIONS

In conclusion, our results show that:

1. Organotypic hippocampal slices exposed to sublethal doses of DHPG or NMDA develop tolerance to a toxic challenge of 30 min OGD applied 24 h later.

2. Organotypic hippocampal slices exposed to 3 µM NMDA for 60 min or to 10 µM DHPG for 30 min develop tolerance to a subsequent toxic challenge of 10 µM AMPA for 24 h.

3. The reduced AMPA response and toxicity following **DHPG** preconditioning are NOT mediated by GluR1 internalization but only by a pre-synaptic mechanism.

4. The reduced AMPA response and toxicity following **NMDA** preconditioning appears to be mediated by a post-synaptic GluR1 internalization and by a simultaneous pre-synaptic mechanism.

5. PARP and HDACs are involved in the development of ischemic tolerance following DHPG and NMDA preconditioning, possibly by playing a neuroprotective role.

6. The activation of PARP following DHPG or NMDA preconditioning leads to mild consumption of cellular NAD+ and ATP. Under our experimental conditions, caspase-3 is activated but leads only to a minimal cleavage of PARP.

7. The increase of acetylation in K18 of H3 following NMDA preconditioning is prevented by the PARP inhibitor TIQ-A. The activation of PARP following NMDA preconditioning is prevented by the HDAC inhibitor SAHA.

8. We hypothesize an interaction between PARP and histone acetylation in the development of ischemic tolerance.

11. BIBLIOGRAPHY

Aarts M, Liu Y, Liu L, Besshoh S, Arundine M, Gurd JW, Wang YT, Salter MW, Tymianski M. (2002) Treatment of ischemic brain damage by perturbing NMDA receptor- PSD-95 protein interactions. Science.298(5594):846-50.

Abdelkarim GE, Gertz K, Harms C, Katchanov J, Dirnagl U, Szabó C, Endres M. (2001) Protective effects of PJ34, a novel, potent inhibitor of poly(ADP-ribose) polymerase (PARP) in in vitro and in vivo models of stroke. Int J Mol Med. 7(3):255-60.

Althaus FR, Höfferer L, Kleczkowska HE, Malanga M, Naegeli H, Panzeter P, Realini C. (1993) Histone shuttle driven by the automodification cycle of poly(ADP-ribose)polymerase. Environ Mol Mutagen. 22(4):278-82. Review.

Anderson CM, & Swanson RA, (2000). Astrocyte glutamate transport: review of properties, regulation, and physiological functions. *Glia* **32**: 1–14.

Atochin DN, Clark J, Demchenko IT, Moskowitz MA, Huang PL (2003). Rapid cerebral ischemic preconditioning in mice deficient in endothelial and neuronal nitric oxide synthases. *Stroke* **34**; 1299–1303.

Attwell D, (2000). Brain uptake of glutamate: food for thought. *Journal of Nutrition* **130**: 1023S–1025S.

Bakondi E, Bai P, Szabó E E, Hunyadi J, Gergely P, Szabó C, Virág L. (2002) Detection of poly(ADP-ribose) polymerase activation in oxidatively stressed cells and tissues using biotinylated NAD substrate. J Histochem Cytochem. 50(1):91-8.

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

Bauer PI, Farkas G, Buday L, Mikala G, Meszaros G, Kun E, Farago A. (1992) Inhibition of DNA binding by the phosphorylation of poly ADP-ribose polymerase protein catalysed by protein kinase C. Biochem Biophys Res Commun.187(2):730-6.

Benveniste M, Mayer ML. (1993) Multiple effects of spermine on N-methyl-D-aspartic acid receptor responses of rat cultured hippocampal neurones. J Physiol.464:131-63.

Berger NA, Sims JL, Catino DM, Berger SJ. (1983) Poly(ADP-ribose) polymerase mediates the suicide response to massive DNA damage: studies in normal and DNA-repair defective cells. Princess Takamatsu Symp.13:219-26.

Bergeron MJ, Leclerc S, Laniel MA, Poirier GG, Guérin SL. (1997) Transcriptional regulation of the rat poly(ADP-ribose) polymerase gene by Sp1. Eur J Biochem. 250(2):342-53.

Bernaudin M et al. (2002). Normobaric hypoxia induces tolerance to focal permanent cerebral ischemia in association with an increased expression of hypoxiainducible factor-1 and its target genes, erythropoietin and VEGF, in the adult mouse brain. *J. Cereb. Blood Flow Metab*. **22**; 393–403.

Bertos, N. R., Wang, A. H. & Yang, X. J. (2001). Class II histone deacetylases: structure, function, and regulation. *Biochem. Cell Biol.* **79**, 243–252

Bhakar AL, Tannis LL, Zeindler C, Russo MP, Jobin C, Park DS, MacPherson S, Barker PA. (2002) Constitutive nuclear factor-kappa B activity is required for central neuron survival. J Neurosci. 22(19):8466-75.

Bickler PE & Fahlman CS (2004). Moderate increases in intracellular calcium activate neuroprotective signals in hippocampal neurons. *Neuroscience,* **127**; 673–683.

Blaabjerg M, Fang LW, Zimmer J, Baskys A (2003). Neuroprotection against NMDA excitotoxicity by group I metabotropic glutamate receptors is associated with reduction of NMDA stimulated currents. *Exp. Neurol.,* **183**; 573–580.

Blondeau N, Widmann C, Lazdunski M, Heurteaux C (2001). Activation of the nuclear factor-kB is a key event in brain tolerance. *J. Neurosci*. **21**; 4668–4677.

Bolden, J. E., Peart, M. J. & Johnstone, R. W. (2006) Anticancer activities of histone deacetylase inhibitors. *Nature Rev. Drug Discov.* **5**, 769–784.

Bordet R, Deplanque D, Maboudou P, Puisieux F, Pu Q, Robin E, Martin A, Bastide M, Leys D, Lhermitte M, Dupuis B. (2000) Increase in endogenous brain superoxide dismutase as a potential mechanism of lipopolysaccharide-induced brain ischemic tolerance. J Cereb Blood Flow Metab.20(8):1190-6.

Boulikas T. (1990) Poly(ADP-ribosylated) histones in chromatin replication. J Biol Chem. 265(24):14638-47.

Bradley SR, Levey AI, Hersch SM, Conn PJ. 1996 Immunocytochemical localization of group III metabotropic glutamate receptors in the hippocampus with subtype-specific antibodies. J Neurosci. 16(6):2044-56.

Bruno V, Battaglia G, Copani A, Giffard RG, Raciti G, Raffaele R, Shinozaki H, Nicoletti F. (1995) Activation of class II or III metabotropic glutamate receptors protects cultured cortical neurons against excitotoxic degeneration. Eur J Neurosci 7(9):1906-13.

Buller AL, Larson HC, Schneider BE, Beaton JA, Morrisett RA, Monaghan DT. (1994) The molecular basis of NMDA receptor subtypes: native receptor diversity is predicted by subunit composition. J Neurosci. 14(9):5471-84.

Bürkle A. (2001) PARP-1: a regulator of genomic stability linked with mammalian longevity. Chembiochem.2(10):725-8. Review.

Butler, R. & Bates, G. P. (2006) Histone deacetylase inhibitors as therapeutics for polyglutamine disorders. *Nature Rev. Neurosci.* **7**, 784–796

Burnashev N, Zhou Z, Neher E, Sakmann B. (1995) Fractional calcium currents through recombinant GluR channels of the NMDA, AMPA and kainate receptor subtypes. J Physiol. 485 (Pt 2):403-18.

Calabresi P, Saulle E, Marfia GA, Centonze D, Mulloy R, Picconi B, Hipskind RA, Conquet F, and Bernardi G (2001). Activation of metabotropic glutamate receptorsubtype 1/protein kinase C/mitogen-activated protein kinase pathway is required for postischemic long-term potentiation in the striatum. *Mol Pharmacol* **60**:808–815.

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

Carey N, La Thangue NB: (2006) Histone deacetylase inhibitors: gathering pace. Curr Opin Pharmacol, 6:369-375.

Castillo PE, Malenka RC, Nicoll RA. (1997) Kainate receptors mediate a slow postsynaptic current in hippocampal CA3 neurons. Nature. 388(6638):182-6.

Chatterjee S, Berger SJ, Berger NA. (1999b) Poly(ADP-ribose) polymerase: a guardian of the genome that facilitates DNA repair by protecting against DNA recombination. Mol Cell Biochem. 193(1-2):23-30.

Chatterton JE, Awobuluyi M, Premkumar LS, Takahashi H, Talantova M, Shin Y, Cui J, Tu S, Sevarino KA, Nakanishi N, Tong G, Lipton SA, Zhang D. (2002) Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits. Nature. 15(6873):793-8.

Chavis P, Shinozaki H, Bockaert J, Fagni L. (1994) The metabotropic glutamate receptor types 2/3 inhibit L-type calcium channels via a pertussis toxin-sensitive G-protein in cultured cerebellar granule cells. J Neurosci. 14(11 Pt 2):7067-76.

Chiarugi A. (2002) Poly(ADP-ribose) polymerase: killer or conspirator? The 'suicide hypothesis' revisited. Trends Pharmacol Sci. 23(3):122-9.

Chiarugi A, Meli E, Calvani M, Picca R, Baronti R, Camaioni E, Costantino G, Marinozzi M, Pellegrini-Giampietro DE, Pellicciari R, Moroni F. (2003) Novel isoquinolinone-derived inhibitors of poly(ADP-ribose) polymerase-1: pharmacological characterization and neuroprotective effects in an in vitro model of cerebral ischemia. J Pharmacol Exp Ther. 305(3):943-9.

Chiarugi A, Moskowitz MA. (2003) Poly(ADP-ribose) polymerase-1 activity promotes NF-kappaB-driven transcription and microglial activation: implication for neurodegenerative disorders. J Neurochem. 85(2):306-17.

Chittajallu R, Vignes M, Dev KK, Barnes JM, Collingridge GL, Henley JM. (1996) Regulation of glutamate release by presynaptic kainate receptors in the hippocampus. Nature. 379(6560):78-81.

Christopherson KS, Hillier BJ, Lim WA, Bredt DS. (1999) PSD-95 assembles a ternary complex with the N-methyl-D-aspartic acid receptor and a bivalent neuronal NO synthase PDZ domain. J Biol Chem. 274(39):27467-73.

Cohen-Armon M, Visochek L, Rozensal D, Kalal A, Geistrikh I, Klein R, Bendetz-Nezer S, Yao Z, Seger R. (2007) DNA-independent PARP-1 activation by phosphorylated ERK2 increases Elk1 activity: a link to histone acetylation. Mol Cell. 25(2):297-308.

Cohen-Armon M. (2007) PARP-1 activation in the ERK signaling pathway. Trends Pharmacol Sci. 28(11):556-60

Cosi C, Suzuki H, Milani D, Facci L, Menegazzi M, Vantini G, et al. (1994) Poly(ADP-ribose) polymerase: early involvement in glutamateinduced neurotoxicity in cultured cerebellar granule cells. J Neurosci Res;39:38–46.

d'Adda di Fagagna F, Hande MP, Tong WM, Lansdorp PM, Wang ZQ, Jackson SP. (1999) Functions of poly(ADP-ribose) polymerase in controlling telomere length and chromosomal stability. Nat Genet. 23(1):76-80.

Daikhin Y, & Yudkoff M, (2000). Compartmentation of brain glutamate metabolism in neurons and glia. *Journal of Nutrition* **130**: 1026S–1031S.

Dahl NA & Balfour WM (1964). Prolonged anoxic survival due to anoxia pre-exposure: brain ATP, lactate, and pyruvate. *Am. J. Physiol*. **207**; 452–456.

Dantzer F, Nasheuer HP, Vonesch JL, de Murcia G, Ménissier-de Murcia J. (1998) Functional association of poly(ADP-ribose) polymerase with DNA polymerase alpha-primase complex: a link between DNA strand break detection and DNA replication. Nucleic Acids Res. 26(8):1891-8.

Dave KR, Lange-Asschenfeldt C, Raval AP, Prado R, Busto R, Saul I, Pérez-Pinzón MA. (2005) Ischemic preconditioning ameliorates excitotoxicity by shifting glutamate/gamma-aminobutyric acid release and biosynthesis. J Neurosci Res. 1;82(5):665-73.

Davidovic L, Vodenicharov M, Affar EB, Poirier GG. (2001) Importance of poly(ADP-ribose) glycohydrolase in the control of poly(ADP-ribose) metabolism. Exp Cell Res. 268(1):7-13. Review.

del Río E, McLaughlin M, Downes CP, and Nicholls DG (1999). Differential coupling of G-protein-linked receptors to Ca2_ mobilization through inositol(1,4,5)trisphosphate or ryanodine receptors in cerebellar granule cells in primary culture. *Eur J Neurosci* **11**:3015–3022.

de Murcia G, Ménissier de Murcia J. (1994) Poly(ADP-ribose) polymerase: a molecular nick-sensor. Trends Biochem Sci.19(4):172-6. Review. Erratum in: Trends Biochem Sci 1994 Jun;19(6):250.

de Murcia G, Schreiber V, Molinete M, Saulier B, Poch O, Masson M, Niedergang C, Ménissier de Murcia J. (1994) Structure and function of poly(ADP-ribose) polymerase. Mol Cell Biochem. 138(1-2):15-24. Review.

de Murcia JM, Niedergang C, Trucco C, Ricoul M, Dutrillaux B, Mark M, Oliver FJ, Masson M, Dierich A, LeMeur M, Walztinger C, Chambon P, de Murcia G. (1997) Requirement of poly(ADP-ribose) polymerase in recovery from DNA damage in mice and in cells. Proc Natl Acad Sci U S A. 94(14):7303-7.

Ding YH, Young CN, Luan X, Li J, Rafols JA, Clark JC, McAllister JP 2nd, Ding Y. (2005). Exercise preconditioning ameliorates inflammatory injury in ischemic rats during reperfusion. *Acta Neuropathol.* **109**; 237-46

Dingledine R, Borges K, Bowie D, Traynelis SF. (1999). The glutamate receptor ion channels. *Pharmacol Rev.* **51**: 7-61.

Dirnagl U, Simon RP & Hallenbeck JM (2003). Ischemic tolerance and endogenous neuroprotection. *Trends Neurosci* **26**; 248–254.

Dirnagl U, Meisel A. (2008) Endogenous neuroprotection: mitochondria as gateways to cerebral preconditioning? Neuropharmacology.55(3):334-44.

Dirnagl U, Becker K, Meisel A. (2009) Preconditioning and tolerance against cerebral ischaemia: from experimental strategies to clinical use.
Lancet Neurol.8(4):398-412. Review.

Doucet-Chabeaud G, Godon C, Brutesco C, de Murcia G, Kazmaier M. (2001) Ionising radiation induces the expression of PARP-1 and PARP-2 genes in Arabidopsis. Mol Genet Genomics. 265(6):954-63.

Du Y, Li C, Hu WW, Song YJ, Zhang GY. (2009) Neuroprotection of preconditioning against ischemic brain injury in rat hippocampus through inhibition of the assembly of GluR6-PSD95-mixed lineage kinase 3 signaling module via nuclear and non-nuclear pathways. Neuroscience. 161(2):370-80.

El-Husseini AE, Schnell E, Chetkovich DM, Nicoll RA, Bredt DS. (2000) PSD-95 involvement in maturation of excitatory synapses. Science.290(5495):1364-8.

Eliasson MJ, Sampei K, Mandir AS, Hurn PD, Traystman RJ, Bao J, et al. (1997) Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. Nat Med;3:1089–95.

Endres M, Wang ZQ, Namura S, Waeber C, Moskowitz MA. (1997) Ischemic brain injury is mediated by the activation of poly(ADPribose) polymerase. J Cereb Blood Flow Metab;17:1143–51.

Endres M, Scott G, Namura S, Salzman AL, Huang PL, Moskowitz MA, et al. (1998) Role of peroxynitrite and neuronal nitric oxide synthase in the activation of poly(ADP-ribose) synthetase in a murine model of cerebral ischemia-reperfusion. Neurosci Lett 248:41–4.

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

Endres M, Gertz K, Lindauer U, Katchanov J, Schultze J, Schröck H, Nickenig G, Kuschinsky W, Dirnagl U, Laufs U. (2003). Mechanisms of stroke protection by physical activity. *Ann Neurol.* **54**; 582-90.

Fagni L, Chavis P, Ango F, and Bockaert J (2000). Complex interactions between mGluRs, intracellular Ca^{2+} stores and ion channels in neurons. *Trends Neurosci* **23**:80–88.

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

Faraco G, Blasi F, Min W, Wang ZQ, Moroni F, Chiarugi A. (2010) Brain ischemic preconditioning does not require PARP-1. Stroke. 41(1):181-3. Epub 2009 Nov 5.

Fischle, W. *et al.* (2001). Human HDAC7 histone deacetylase activity is associated with HDAC3 *in vivo*. *J. Biol. Chem.* **276**, 35826–35835

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

Frerking M, Schmitz D, Zhou Q, Johansen J, Nicoll RA. (2001) Kainate receptors depress excitatory synaptic transmission at CA3-->CA1 synapses in the hippocampus via a direct presynaptic action. J Neurosci. 21(9):2958-66.

Fukata Y, Adesnik H, Iwanaga T, Bredt DS, Nicoll RA, Fukata M. (2006) Epilepsy-related ligand/receptor complex LGI1 and ADAM22 regulate synaptic transmission.
Science. 313(5794):1792-5.

Gan, L. & Mucke, L. (2008) Paths of convergence: sirtuins inaging and neurodegeneration. *Neuron* **58**, 10–14

Gao, L., Cueto, M. A., Asselbergs, F. & Atadja, P. (2002) Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family. *J. Biol. Chem.* **277**, 25748–25755

Gardoni F, Polli F, Cattabeni F, Di Luca M. (2006) Calcium-calmodulin-dependent protein kinase II phosphorylation modulates PSD-95 binding to NMDA receptors. Eur J Neurosci.24(10):2694-704.

Garnier, P., Ying, W. & Swanson, R. A. (2003). Ischemic preconditioning by caspase cleavage of poly(ADPribose) polymerase-1. *J. Neurosci*. **23**; 7967–7973.

Geiger JR, Melcher T, Koh DS, Sakmann B, Seeburg PH, Jonas P, Monyer H. (1995) Relative abundance of subunit mRNAs determines gating and Ca2+ permeability of AMPA receptors in principal neurons and interneurons in rat CNS. Neuron. 15(1):193-204.

Gidday JM, Fitzgibbons JC, Shah AR & Park TS (1994). Neuroprotection from ischemic brain injury by hypoxic preconditioning in the neonatal rat. *Neurosci.Lett* **168**; 221–224.

Gidday JM, Shah AR, Maceren RG, Wang Q, Pelligrino DA, Holtzman DM, Park TS. (1999). Nitric oxide mediates cerebral ischemic tolerance in a neonatal rat model of hypoxic preconditioning. *J Cereb Blood Flow Metab*. **19**; 331-40.

Gidday JM (2006). Cerebral preconditioning and ischaemic tolerance. *Nat Rev Neurosci* **7**; 437–448.

Giovannelli L, Cozzi A, Guarnieri I, Dolara P, Moroni F. (2002) Comet assay as a novel approach for studying DNA damage in focal cerebral ischemia: differential effects

of NMDA receptor antagonists and poly(ADP-ribose) polymerase inhibitors. J Cereb Blood Flow Metab 22:697–704

Gonzalez-Zulueta M, Feldman AB, Klesse LJ, Kalb RG, Dillman JF, Parada LF, Dawson TM, Dawson VL. (2000). Requirement for nitric oxide activation of p21(ras)/extracellular regulated kinase in neuronal ischemic preconditioning. *Proc Natl Acad Sci U S A.* **97**; 436-41.

Grabb MC & Choi DW (1999). Ischemic tolerance in murine cortical cell culture: critical role for NMDA receptors. *J. Neurosci.,* **19**; 1657–1662.

Grabb MC, Lobner D, Turetsky DM, Choi DW. (2002) Preconditioned resistance to oxygen-glucose deprivation-induced cortical neuronal death: alterations in vesicular GABA and glutamate release. Neuroscience. 115(1):173-83.

Gregoretti, I. V., Lee, Y. M. & Goodson, H. V. (2004) Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. *J. Mol. Biol.* 338, 17–31.

Hallows, W. C., Lee, S. & Denu, J. M. (2006). Sirtuins deacetylate and activate mammalian acetyl-CoA synthetases. *Proc. Natl Acad. Sci. USA* **103**, 10230–10235

Hauschildt S, Scheipers P, Bessler WG, Mülsch A. (1992) Induction of nitric oxide synthase in L929 cells by tumour-necrosis factor alpha is prevented by inhibitors of poly(ADP-ribose) polymerase. Biochem J. 288 (Pt 1):255-60.

Hayashi T, Saito A, Okuno S, Ferrand-Drake, M, Chan PH (2003). Induction of GRP78 by ischemic preconditioning reduces endoplasmic reticulum stress and prevents delayed neuronal cell death. *J. Cereb. Blood Flow Metab.* **23**; 949–961.

Heurteaux C, Lauritzen I, Widmann C, Lazdunski, M. (1995). Essential role of adenosine, adenosine A1 receptors, and ATP-sensitive K+ channels in cerebral ischemic preconditioning. *Proc. Natl Acad. Sci. USA* **92**; 4666–4670.

Heuss C, Scanziani M, Gähwiler BH, Gerber U. (1999) G-protein-independent signaling mediated by metabotropic glutamate receptors. Nat Neurosci. 2(12):1070-7.

Hollmann M, O'Shea-Greenfield A, Rogers SW, Heinemann S. (1989). Cloning by functional expression of a member of the glutamate receptor family. *Nature* **342**:643-8.

Hollmann M, Hartley M, Heinemann S. (1991) Ca2+ permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. Science. May 10;252(5007):851-3.

Homburg S, Visochek L, Moran N, Dantzer F, Priel E, Asculai E, et al. (2000) A fast signal induced activation of poly(ADPribose) polymerase: a downstream target of phospholipase C. J Cell Biol;150:293–307

Horvath EM, Szabó C. (2007) Poly(ADP-ribose) polymerase as a drug target for cardiovascular disease and cancer: an update. Drug News Perspect. 20(3):171-81. Review.

Hubbert, C. *et al.* (2002). HDAC6 is a microtubule-associated deacetylase. *Nature* 417, 455–458

Johnson JW, & Ascher P. (1987). Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature* 11:529-31.

Jones NM & Bergeron M (2004). Hypoxia-induced ischemic tolerance in neonatal rat brain involves enhanced ERK1/2 signaling. *J. Neurochem*. 89; 157–167.

Jones, P. *et al.* (2008). Probing the elusive catalytic activity of vertebrate class IIa histone deacetylases. *Bioorg. Med. Chem. Lett.* **18**, 1814–1819

Kanai Y, Tanuma S, Sugimura T. (1981) Immunofluorescent staining of poly(ADP-ribose) in situ in HeLa cell chromosomes in the M phase. Proc Natl Acad Sci U S A.78(5):2801-4.

Karikó K, Weissman D, Welsh FA. (2004) Inhibition of toll-like receptor and cytokine signaling--a unifying theme in ischemic tolerance. J Cereb Blood Flow Metab. Review.; **24**(11):1288-304.

Kazantsev AG, Thompson LM. (2008) Therapeutic application of histone deacetylase inhibitors for central nervous system disorders. Nat Rev Drug Discov. 7(10):854-68. Review.

Kawaichi M, Ueda K, Hayaishi O. (1981) Multiple autopoly(ADP-ribosyl)ation of rat liver poly(ADP-ribose) synthetase. Mode of modification and properties of automodified synthetase. J Biol Chem. 256(18):9483-9.

Kennedy MB. (1998) Signal transduction molecules at the glutamatergic postsynaptic membrane. Brain Res Brain Res Rev. 26(2-3):243-57. Review.

Kim, E., Sheng, M., (2004). PDZ domain proteins of synapses. Nat. Rev. eurosci. 5, 771–781.

Kirino T, Tsujita Y & Tamura A (1991). Induced tolerance to ischemia in gerbil hippocampal neurons. *J Cereb Blood Flow Metab* 11; 299–307.

Kitagawa K, Matsumoto M, Tagaya M et al. (1990). 'Ischemic tolerance' phenomenon found in the brain. *Brain Res* 528; 21–24.

Kitagawa K, Matsumoto M, Kuwabara K et al. (1991). 'Ischemic tolerance' phenomenon detected in various brain regions. *Brain Res* 561; 203–211.

Kleczkowska HE, Althaus FR. (1996) The role of poly(ADP-ribosyl)ation in the adaptive response. Mutat Res. 358(2):215-21. Review.

Komjáti K, Besson VC, Szabó C. (2005) Poly (adp-ribose) polymerase inhibitors as potential therapeutic agents in stroke and neurotrauma. Curr Drug Targets CNS Neurol Disord. 4(2):179-94. Review.

Kouzarides, T. (2007) Chromatin modifications and their function. *Cell* **128**, 693–705.

Krishnakumar R, Gamble MJ, Frizzell KM, Berrocal JG, Kininis M, Kraus WL. (2008) Reciprocal binding of PARP-1 and histone H1 at promoters specifies transcriptional outcomes. Science. 319(5864):819-21.

Lahm, A. *et al.* (2007). Unraveling the hidden catalytic activity of vertebrate class IIa histone deacetylases. *Proc. Natl Acad. Sci. USA* 104, 17335–17340

Langley B, Gensert JM, Beal MF, Ratan RR. (2005) Remodeling chromatin and stress resistance in the central nervous system: histone deacetylase inhibitors as novel and broadly eff ective neuroprotective agents. Curr Drug Targets CNS Neurol Disord 4: 41–50

Lau A, Tymianski M. (2010) Glutamate receptors, neurotoxicity and neurodegeneration. Pflugers Arch. 460(2):525-42.

Leduc Y, Lawrence JJ, De Murcia G, Poirier GG. (1988) Cell cycle regulation of poly(ADP-ribose) synthetase in FR3T3 cells. Biochim Biophys Acta 968(3):275-82.

Lee JY, Kim YJ, Kim TY, Koh JY, Kim YH. (2008) Essential role for zinc-triggered p75NTR activation in preconditioning neuroprotection. J Neurosci. 28(43):10919-27.

Le Page C, Sanceau J, Drapier JC, Wietzerbin J. (1998) Inhibitors of ADP-ribosylation impair inducible nitric oxide synthase gene transcription through inhibition of NF kappa B activation. Biochem Biophys Res Commun. 243(2):451-7.

Liang H, Tan AR. (2010) Iniparib, a PARP1 inhibitor for the potential treatment of cancer, including triple-negative breast cancer. IDrugs. 13(9):646-56. Review.

Lindahl T, Satoh MS, Dianov G. (1995) Enzymes acting at strand interruptions in DNA. Philos Trans R Soc Lond B Biol Sci.347(1319):57-62. Review.

Lipton SA, Rosenberg PA. (1994) Excitatory amino acids as a final common pathway for neurological disorders. N Engl J Med 330:613–22.

Liu J, Narasimhan P, Yu F, Chan PH. (2005) Neuroprotection by hypoxic preconditioning involves oxidative stress-mediated expression of hypoxia-inducible factor and erythropoietin. Stroke. 36(6):1264-9.

Liu, H., Hu, Q., Kaufman, A., D'Ercole, A. J. & Ye, P. (2008). Developmental expression of histone deacetylase 11 in the murine brain. *J. Neurosci. Res.* 86, 537–543

Lo EH, Bosque-Hamilton P, Meng W. (1998) Inhibition of poly(ADP-ribose) polymerase: reduction of ischemic injury and attenuation of N-methyl-D-aspartate-induced neurotransmitter dysregulation. Stroke. 29(4):830-6.

Love S, Barber R, Wilcock GK. (1999) Neuronal accumulation of poly(ADP-ribose) after brain ischaemia. Neuropathol Appl Neurobiol;25:98–103

Love S, Barber R, Wilcock GK. (2000) Neuronal death in brain infarcts in man. Neuropathol Appl Neurobiol;26:55–66.

Majdzadeh, N., Morrison, B. E. & D'Mello, S. R. (2008) Class IIA HDACs in the regulation of neurodegeneration. *Front. Biosci.* 13, 1072–1082

Mandir AS, Poitras MF, Berliner AR, Herring WJ, Guastella DB, Feldman A, Poirier GG, Wang ZQ, Dawson TM, Dawson VL. (2000) NMDA but not non-NMDA excitotoxicity is mediated by Poly(ADP-ribose) polymerase. J Neurosci. 20(21):8005-11.

Martin, M., Kettmann, R. & Dequiedt, F. Class IIa (2007) histone deacetylases: regulating the regulators. *oncogene* 26, 5450–5467

Maruta H, Matsumura N, Tanuma S. (1997) Role of (ADP-ribose)n catabolism in DNA repair. Biochem Biophys Res Commun. 236(2):265-9.

Masson J, Sagne C, Hamon M, El Mestikawy S, (1999). Neurotransmitter transporters in the central nervous system. *Pharmacological Reviews* 51: 439–464.

Matsuyama, A. *et al.* (2002). *In vivo* destabilization of dynamic microtubules by HDAC6-mediated deacetylation. *EMBo J.* 21, 6820–6831

Mayer ML, Westbrook GL, Guthrie PB. (1984) Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. Nature. 17-23;309(5965):261-3.

Mauceri D, Cattabeni F, Di Luca M, Gardoni F. (2004) Calcium/calmodulin-dependent protein kinase II phosphorylation drives synapse-associated protein 97 into spines. J Biol Chem. 279(22):23813-21.

Mauceri D, Gardoni F, Marcello E, Di Luca M. (2007) Dual role of CaMKII-dependent SAP97 phosphorylation in mediating trafficking and insertion of NMDA receptor subunit NR2A. J Neurochem. 100(4):1032-46.

Mayer ML, Vyklicky L Jr, Clements J. (1989) Regulation of NMDA receptor desensitization in mouse hippocampal neurons by glycine. Nature. 30;338(6214):425-7.

Mazen A, Menissier-de Murcia J, Molinete M, Simonin F, Gradwohl G, Poirier G, de Murcia G. (1989) Poly(ADP-ribose)polymerase: a novel finger protein. Nucleic Acids Res.17(12):4689-98.

McLaughlin B, Hartnett KA, Erhardt JA, Legos JJ, White RF, Barone FC, Aizenman E (2003). Caspase 3 activation is essential for neuroprotection in preconditioning. *Proceedings of the National Academy of Sciences of the USA* 100; 715–720.

Meldrum DR, Cleveland JC Jr, Rowland RT, Banerjee A, Harken AH & Meng X (1997). Early and delayed preconditioning: differential mechanisms and additive protection. *Am J Physiol* 273; H725–H733.

Meldrum BS, (2000). Glutamate as a neurotransmitter in the brain: review of physiology and pathology. *Journal of Nutrition* 130: 1007S–1015S.

Meli E, Picca R, Attucci S, Cozzi A, Peruginelli F, Moroni F, Pellegrini-Giampietro DE (2002). Activation of mGlu1 but not mGlu5 metabotropic glutamate receptors contributes to post-ischemic neuronal injury in vitro and in vivo. *Pharmacology Biochemistry and Behavior* 73; 439–446.

Meli E, Pangallo M, Baronti R, Chiarugi A, Cozzi A, Pellegrini-Giampietro DE, Moroni F. (2003) Poly(ADP-ribose) polymerase as a key player in excitotoxicity and post-ischemic brain damage. Toxicol Lett. 139(2-3):153-62.

Meli E, Pangallo M, Picca R, Baronti R, Moroni F, Pellegrini-Giampietro DE. (2004) Differential role of poly(ADP-ribose) polymerase-1in apoptotic and necrotic neuronal death induced by mild or intense NMDA exposure in vitro. Mol Cell Neurosci. 25(1):172-80.

Meli E, Baronti R, Pangallo M, Picca R, Moroni F, Pellegrini-Giampietro DE. (2005) Group I metabotropic glutamate receptors stimulate the activity of poly(ADP-ribose) polymerase in mammalian mGlu1-transfected cells and in cortical cell cultures. Neuropharmacology. 49 Suppl 1:80-8.

Meller R, Minami M, Cameron JA et al. (2005). CREB-mediated Bcl-2 protein expression after ischemic preconditioning. *J Cereb Blood Flow Metab* 25; 234–246.

Michan, S. & Sinclair, D. (2007) Sirtuins in mammals: insights into their biological function. *Biochem. J.* 404, 1–13

Michishita, E., Park, J. Y., Burneskis, J. M., Barrett, J. C. & Horikawa, I. (2005). Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. *Mol. Biol. Cell* 16, 4623–4635

Michishita, E. *et al.* (2008) .SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. *Nature* 452, 492–496

Minucci S, Pelicci PG: (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat Rev Cancer, 6:38-51.

Miyamoto T, Kakizawa T, Hashizume K. (1999) Inhibition of nuclear receptor signalling by poly(ADP-ribose) polymerase. Mol Cell Biol. 19(4):2644-9.

Moroni F. (2008) Poly(ADP-ribose)polymerase 1 (PARP-1) and postischemic brain damage. Curr Opin Pharmacol.8(1):96-103. Epub 2007 Nov 26. Review.

Moroni F, Chiarugi A. (2009) Post-ischemic brain damage: targeting PARP-1 within the ischemic neurovascular units as a realistic avenue to stroke treatment. FEBS J. 276(1):36-45.

Murry CE, Jennings RB & Reimer KA (1986). Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 74; 1124–1136.

Nagayama T, Simon RP, Chen D, Henshall DC, Pei W, Stetler RA, et al. (2000) Activation of poly(ADP-ribose) polymerase in the rat hippocampus may contribute to cellular recovery following sublethal transient global ischemia. J Neurochem;74:1636–45.

Nakanishi S, Nakajima Y, Masu M, Ueda Y, Nakahara K, Watanabe D, Yamaguchi S, Kawabata S, Okada M (1998). Glutamate receptors: brain function and signal transduction. *Brain Res Brain Res Rev* 26: 230–235.

Niethammer M, Kim E, Sheng M. (1996) Interaction between the C terminus of NMDA receptor subunits and multiple members of the PSD-95 family of membrane-associated guanylate kinases. J Neurosci. 6(7):2157-63.

North, B. J., Schwer, B., Ahuja, N., Marshall, B. & Verdin, E. (2005) Preparation of enzymatically active recombinant class III protein deacetylases. *Methods* **36**, 338–345.

Obrenovitch TP. (2008) Molecular physiology of preconditioning-induced brain tolerance to ischemia. Physiol Rev. 88(1):211-47. Review.

O'Duffy AE, Bordelon YM, McLaughlin B. (2007) Killer proteases and little strokes--how the things that do not kill you make you stronger. J Cereb Blood Flow Metab. 27(4):655-68. Review.

Oei SL, Ziegler M. (2000) ATP for the DNA ligation step in base excision repair is generated from poly(ADP-ribose). J Biol Chem. 275(30):23234-9.

Ohishi H, Ogawa-Meguro R, Shigemoto R, Kaneko T, Nakanishi S, Mizuno N. (1994) Immunohistochemical localization of metabotropic glutamate receptors, mGluR2 and mGluR3, in rat cerebellar cortex. Neuron. 13(1):55-66.

Oliver FJ, Ménissier-de Murcia J, Nacci C, Decker P, Andriantsitohaina R, Muller S, de la Rubia G, Stoclet JC, de Murcia G. (1999) Resistance to endotoxic shock as a consequence of defective NF-kappaB activation in poly (ADP-ribose) polymerase-1 deficient mice. EMBO J. 18(16):4446-54.

Otsuka A, Hanafusa T, Miyagawa J, Kono N, Tarui S. (1991) Nicotinamide and 3-aminobenzamide reduce interferon-gamma-induced class II MHC (HLA-DR and -DP) molecule expression on cultured human endothelial cells and fibroblasts. Immunopharmacol Immunotoxicol. 13(3):263-80.

Pellegrini-Giampietro DE, Cherici G, Alesiani M, Carl`a V, Moroni F. (1990) Excitatory amino acid release and free radical formation may cooperate in the genesis of ischemia-induced neuronal damage. J Neurosci;10:1035–41.

Pellegrini-Giampietro DE, Cozzi A, Peruginelli F, Leonardi P, Meli E, Pellicciari R, Moroni F (1999a). 1-Aminoindan-1,5-dicarboxylic acid and (S)-(þ)-2-(30-carboxybicyclo[1.1.1]pentyl)-glycine, two mGlu1 receptor-preferring antagonists, reduce neuronal death in in vitro and in vivo models of cerebral ischemia. *European Journal of Neuroscience* 11; 3637–3647.

Pellegrini-Giampietro DE, Peruginelli F, Meli E, Cozzi A, Albani Torregrossa S, Pellicciari R, Moroni F (1999b). Protection with metabotropic glutamate 1 receptor antagonists in models of ischemic neuronal death: time-course and mechanisms. *Neuropharmacology* 38; 1607–1619.

Pellegrini-Giampietro DE (2003). The distinct role of mGlu1 receptors in postischemic neuronal death. *Trends Pharmacol Sci* 24:461–470.

Pérez-Pinzón MA. (2004) Neuroprotective effects of ischemic preconditioning in brain mitochondria following cerebral ischemia. J Bioenerg Biomembr.36(4):323-7. Review.

Petrie, K. *et al.* (2003). The histone deacetylase 9 gene encodes multiple protein isoforms. *J. Biol. Chem.* 278, 16059–16072

Petroff OA, (2002). GABA and glutamate in the human brain. *Neuroscientist* 8, 562–573.

Pignataro G, Meller R, Inoue K et al. (2008). In vivo and in vitro characterization of a novel neuroprotective strategy for stroke: ischemic postconditioning. *J Cereb Blood Flow Metab* 28; 232–241.

Pizzi M, Galli P, Consolandi O, Arrighi V, Memo M, Spano PF. (1996) Metabotropic and ionotropic transducers of glutamate signal inversely control cytoplasmic Ca2+ concentration and excitotoxicity in cultured cerebellar granule cells: pivotal role of protein kinase C. Mol Pharmacol. 49(4):586-94.

Pleschke JM, Kleczkowska HE, Strohm M, Althaus FR. (2000) Poly(ADP-ribose) binds to specific domains in DNA damage checkpoint proteins. J Biol Chem. 275(52):40974-80.

Ralph GS et al. (2004). Identification of potential stroke targets by lentiviral vector mediated overexpression of HIF-1̣ and HIF-2̣ in a primary neuronal model of hypoxia. *J. Cereb. Blood Flow Metab*. 24; 245–258.

Ransom RW, Stec NL. (1988) Cooperative modulation of [3H]MK-801 binding to the N-methyl-D-aspartate receptor-ion channel complex by L-glutamate, glycine, and polyamines. J Neurochem. 51(3):830-6.

Ratan RR, Siddiq A, Smirnova N, Karpisheva K, Haskew-Layton R, McConoughey S, Langley B, Estevez A, Huerta PT, Volpe B, Roy S, Sen CK, Gazaryan I, Cho S, Fink M, LaManna J. (2007) Harnessing hypoxic adaptation to prevent, treat, and repair stroke. J Mol Med.;85(12):1331-8.

Raval AP, Dave KR, Mochly-Rosen D, Sick TJ, Pérez-Pinzón MA. (2003) Epsilon PKC is required for the induction of tolerance by ischemic and NMDA-mediated preconditioning in the organotypic hippocampal slice. J Neurosci. 23(2):384-91.

Ravati A, Ahlemeyer B, Becker A, Klumpp S, Krieglstein J. (2001) Preconditioning-induced neuroprotection is mediated by reactive oxygen species and activation of the transcription factor nuclear factor-kappaB. J Neurochem. 78(4):909-19.

Ren P, Kang Z, Gu G, Liu Y, Xu W, Tao H, Zhang JH, Sun X, Ji H. (2008) Hyperbaric oxygen preconditioning promotes angiogenesis in rat liver after partial hepatectomy. Life Sci. 83(7-8):236-41. Epub 2008 Jun 24.

Rock DM, Macdonald RL. (1992) The polyamine spermine has multiple actions on N-methyl-D-aspartate receptor single-channel currents in cultured cortical neurons. Mol Pharmacol. 41(1):83-8.

Roopra A, Huang Y, Dingledine R (2001): Neurological disease: listening to gene silencers. Mol Interv, 1:219-228

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

Rumbaugh G, Sia GM, Garner CC, Huganir RL. (2003) Synapse-associated protein-97 isoform-specific regulation of surface AMPA receptors and synaptic function in cultured neurons. J Neurosci. 23(11):4567-76.

Saha RN, Pahan K. (2006) HATs and HDACs in neurodegeneration: a tale of disconcerted acetylation homeostasis. Cell Death Differ. 13(4):539-50.

Samper E, Goytisolo FA, Ménissier-de Murcia J, González-Suárez E, Cigudosa JC, de Murcia G, Blasco MA. (2001) Normal telomere length and chromosomal end capping in poly(ADP-ribose) polymerase-deficient mice and primary cells despite increased chromosomal instability. J Cell Biol. 154(1):49-60.

Sans N, Petralia RS, Wang YX, Blahos J 2nd, Hell JW, Wenthold RJ. (2000) A developmental change in NMDA receptor associated proteins at hippocampal synapses. J. Neurosci. 20, 1260– 1271

Sattler R, Xiong Z, Lu WY, Hafner M, MacDonald JF, Tymianski M. (1999) Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. Science.284(5421):1845-8.

Saxena A, Wong LH, Kalitsis P, Earle E, Shaffer LG, Choo KH. (2002) Poly(ADP-ribose) polymerase 2 localizes to mammalian active centromeres and interacts with PARP-1, Cenpa, Cenpb and Bub3, but not Cenpc. Hum Mol Genet. 11(19):2319-29.

Sauve, A. A., Wolberger, C., Schramm, V. L. & Boeke, J. D. (2006) The biochemistry of sirtuins. *Annu. Rev. Biochem.* 75, 435–465.

Scartabelli T, Gerace E, Landucci E, Moroni F, Pellegrini-Giampietro DE. (2008) Neuroprotection by group I mGlu receptors in a rat hippocampal slice model of cerebral

ischemia is associated with the PI3K-Akt signaling pathway: a novel postconditioning strategy?
Neuropharmacology. 55(4):509-16.

Schmitz D, Mellor J, Nicoll RA. (2001) Presynaptic kainate receptor mediation of frequency facilitation at hippocampal mossy fiber synapses. Science. 291(5510):1972-6. Epub 2001 Feb 8. Erratum in: Science 2001 Jun 1;292(5522):1656.

Schraufstatter IU, Hyslop PA, Hinshaw DB, Spragg RG, Sklar LA, Cochrane CG. (1986) Hydrogen peroxide-induced injury of cells and its prevention by inhibitors of poly(ADP-ribose) polymerase. Proc Natl Acad Sci U S A. 83(13):4908-12.

Schreiber SS, Baudry M, 1995. Selective neuronal vulnerability in the hippocampus-a role for gene expression? Trends Neurosci 18(10): 446-51.

Schreiber V, Hunting D, Trucco C, Gowans B, Grunwald D, De Murcia G, De Murcia JM. (1995) A dominant-negative mutant of human poly(ADP-ribose) polymerase affects cell recovery, apoptosis, and sister chromatid exchange following DNA damage. Proc Natl Acad Sci U S A. 92(11):4753-7.

Schreiber V, Dantzer F, Ame JC, de Murcia G. (2006) Poly(ADP-ribose): novel functions for an old molecule. Nat Rev Mol Cell Biol. 7(7):517-28. Review.

Schurr A, Reid KH, Tseng MT, West C, Rigor BM. (1986) Adaptation of adult brain tissue to anoxia and hypoxia in vitro. Brain Res. 374(2):244-8.

Schwer, B., North, B. J., Frye, R. A., Ott, M. & Verdin, E. (2002). The human silent information regulator (Sir)2 homologue hSIRT3 is a mitochondrial nicotinamide adenine dinucleotide-dependent deacetylase. *J. Cell Biol.* 158, 647–657

Shall S, de Murcia G. (2000) Poly(ADP-ribose) polymerase-1: what have we learned from the deficient mouse model? Mutat Res. 460(1):1-15.

Shamloo M, Rytter A, Wieloch T. (1999) Activation of the extracellular signal-regulated protein kinase cascade in the hippocampal CA1 region in a rat model of global cerebral ischemic preconditioning. Neuroscience. 93(1):81-8.

Simbulan-Rosenthal CM, Rosenthal DS, Hilz H, Hickey R, Malkas L, Applegren N, Wu Y, Bers G, Smulson ME. (1996) The expression of poly(ADP-ribose) polymerase during differentiation-linked DNA replication reveals that it is a component of the multiprotein DNA replication complex. Biochemistry.35(36):11622-33.

Skaper SD. (2003) Poly(ADP-Ribose) polymerase-1 in acute neuronal death and inflammation: a strategy for neuroprotection. Ann N Y Acad Sci; 993:217–28.

Sims JL, Benjamin RC. (1987) Alteration of poly(adenosine diphosphoribose) metabolism by ethanol: mechanism of action. Arch Biochem Biophys. 253(2):357-66.

Smith QR, (2000). Transport of glutamate and other amino acids at the blood–brain barrier. *Journal of Nutrition* 130: 1016S–1022S.

Smith S, de Lange T. (2000) Tankyrase promotes telomere elongation in human cells. Curr Biol. 10(20):1299-302.

Smith S. (2001) The world according to PARP. Trends Biochem Sci.26(3):174-9. Review.

Soriano FX, Papadia S, Hofmann F, Hardingham NR, Bading H, Hardingham GE. (2006) Preconditioning doses of NMDA promote neuroprotection by enhancing neuronal excitability. J Neurosci. 26(17):4509-18.

Southwood, C. M., Peppi, M., Dryden, S., Tainsky, M. A. & Gow, A. (2007). Microtubule deacetylases, SirT2 and HDAC6, in the nervous system. *Neurochem. Res.* 32, 187–195

Steiger HJ, Hänggi D. (2007) Ischaemic preconditioning of the brain, mechanisms and applications. Acta Neurochir (Wien). 149(1):1-10.. Review.

Stenzel-Poore MP et al. (2003). Effect of ischaemic preconditioning on genomic response to cerebral ischemia: similarity to neuroprotective strategies in hibernation and hypoxia-tolerant states. *Lancet* 362; 1028–1037.

Stenzel-Poore MP, Stevens SL, King JS, Simon RP. (2007) Preconditioning reprograms the response to ischemic injury and primes the emergence of unique endogenous neuroprotective phenotypes: a speculative synthesis. 38(2 Suppl):680-5. Review.

Strosznajder RP, Gadamski R, Czapski GA, Jesko H, Strosznajder JB. (2003) Poly(ADP-ribose) polymerase during reperfusion after transient forebrain ischemia: its role in brain edema and cell death. J Mol Neurosci;20:61–72

Sun, J. M., Spencer, V. A., Chen, H. Y., Li, L. & Davie, J. R. (2003) Measurement of histone acetyltransferase and histone deacetylase activities and kinetics of histone acetylation. *Methods* 31, 12–23.

Szabo C, Cuzzocrea S, Zingarelli B, O'Connor M, Salzman AL. (1997) Endothelial dysfunction in a rat model of endotoxic shock. Importance of the activation of poly(ADP-ribose) synthetase by peroxynitrite. J Clin Invest;100:723–35.

Szabó A, Hake P, Salzman AL, Szabó C. (1998) 3-Aminobenzamide, an inhibitor of poly (ADP-ribose) synthetase, improves hemodynamics and prolongs survival in a porcine model of hemorrhagic shock. Shock. 10(5):347-53.

Szabó E, Virág L, Bakondi E, Gyüre L, Haskó G, Bai P, Hunyadi J, Gergely P, Szabó C. (2001) Peroxynitrite production, DNA breakage, and poly(ADP-ribose) polymerase activation in a mouse model of oxazolone-induced contact hypersensitivity. J Invest Dermatol. 117(1):74-80.

Takagi N, Logan R, Teves L, Wallace MC, Gurd JW. (2000) Altered interaction between PSD-95 and the NMDA receptor following transient global ischemia. J Neurochem 74(1):169-78.

Tanabe T, Mikami A, Niidome T, Numa S, Adams BA, Beam KG. (1993) Structure and function of voltage-dependent calcium channels from muscle. Ann N Y Acad Sci. 707:81-6. Review.

Tanaka Y, Koide SS, Yoshihara K, Kamiya T. (1987) Poly (ADP-ribose) synthetase is phosphorylated by protein kinase C in vitro. Biochem Biophys Res Commun. 148(2):709-17.

Tanaka H et al. (2004). Ischemic preconditioning: neuronal survival in the face of caspase-3 activation. *J. Neurosci*. 24; 2750–2759.

Tang, Y. et al. (2006). Effect of hypoxic preconditioning on brain genomic response before and following ischemia in the adult mouse: identification of potential neuroprotective candidates for stroke. *Neurobiol. Dis*. 21; 18–28.

Tanuma SI, Enomoto T, Yamada MA. (1978) Changes in the level of poly ADP-ribosylation during a cell cycle. Exp Cell Res. 117(2):421-30.

Tapiero H, Mathé G, Couvreur P, Tew KD. (2002) Glutamine and glutamate. Biomed Pharmacother. 56(9):446-57. Review.

Thiagalingam, S. *et al.* (2003) Histone deacetylases: unique players in shaping the epigenetic histone code. *Ann. NY Acad. Sci.* 983, 84–100.

Tian F, Hu XZ, Wu X, Jiang H, Pan H, Marini AM, Lipsky RH. (2009) Dynamic chromatin remodeling events in hippocampal neurons are associated with NMDA receptor-mediated activation of Bdnf gene promoter 1. J Neurochem. 109(5):1375-88

Tokime T, Nozaki K, Sugino T, Kikuchi H, Hashimoto N, Ueda K.(1998) Enhanced poly(ADP-ribosyl)ation after focal ischemia in rat brain. J Cereb Blood Flow Metab 18:991–7.

Tong, J. J., Liu, J., Bertos, N. R. & Yang, X. J. (2002). Identification of HDAC10, a novel class II human histone deacetylase containing a leucine-rich domain. *Nucleic Acids Res.* 30, 1114–1123

Tramontano F, Malanga M, Farina B, Jones R, Quesada P. (2000) Heat stress reduces poly(ADPR)polymerase expression in rat testis. Mol Hum Reprod.6(7):575-81.

Traynelis SF, Cull-Candy SG. (1990) Proton inhibition of N-methyl-D-aspartate receptors in cerebellar neurons. Nature.345(6273):347-50.

Tzschentke TM, (2002). Glutamatergic mechanisms in different disease states: overview and therapeutical implications – an introduction. *Amino Acids* 23: 147–152.

Ullrich O, Grune T. (2001) Proteasomal degradation of oxidatively damaged endogenous histones in K562 human leukemic cells. Free Radic Biol Med. 31(7):887-93.

Uchida M, Hanai S, Uematsu N, Sawamoto K, Okano H, Miwa M, Uchida K. (2001) Genetic and functional analysis of PARP, a DNA strand break-binding enzyme. Mutat Res. 477(1-2):89-96.

Ullrich O, Diestel A, Bechmann I, Homberg M, Grune T, Hass R, Nitsch R. (2001) Turnover of oxidatively damaged nuclear proteins in BV-2 microglial cells is linked to their activation state by poly-ADP-ribose polymerase. FASEB J. 15(8):1460-2.

Vaquero, A. *et al.* (2006). SirT2 is a histone deacetylase with preference for histone H4 Lys16 during mitosis. *Genes Dev.* 20, 1256–1261

Vignes M, Collingridge GL. (1997) The synaptic activation of kainate receptors. Nature. 388(6638):179-82.

Virág L, Salzman AL, Szabó C. (1998) Poly(ADP-ribose) synthetase activation mediates mitochondrial injury during oxidant-induced cell death. J Immunol. 161(7):3753-9.

Virág L, Szabó C. (2001) Purines inhibit poly(ADP-ribose) polymerase activation and modulate oxidant-induced cell death. FASEB J. 15(1):99-107.

Yang, W. M., Tsai, S. C., Wen, Y. D., Fejer, G. & Seto, E. (2002) Functional domains of histone deacetylase-3. *J. Biol. Chem.* 277, 9447–9454.

Yano S, Morioka M, Fukunaga K, Kawano T, Hara T, Kai Y, Hamada J, Miyamoto E, Ushio Y. (2001). Activation of Akt/protein kinase B contributes to induction of ischemic tolerance in the CA1 subfield of gerbil hippocampus. *J Cereb Blood Flow Metab*. 21; 351-60.

Yildirim F, Gertz K, Kronenberg G, Harms C, Fink KB, Meisel A, Endres M. (2008) Inhibition of histone deacetylation protects wildtype but not gelsolin-deficient mice from ischemic brain injury. Exp Neurol. 210(2):531-42.

Yoshida M, Nakakimura K, Cui YJ, Matsumoto M, Sakabe T. (2004) Adenosine A(1) receptor antagonist and mitochondrial ATP-sensitive potassium channel blocker attenuate the tolerance to focal cerebral ischemia in rats. J Cereb Blood Flow Metab. 24(7):771-9.

Yu SP, Sensi SL, Canzoniero LM, Buisson A, Choi DW. (1997) Membrane-delimited modulation of NMDA currents by metabotropic glutamate receptor subtypes 1/5 in cultured mouse cortical neurons. J Physiol. 499 (Pt 3):721-32.

Wang, S., Yan-Neale, Y., Zeremski, M. & Cohen, D. (2004) Transcription regulation by histone deacetylases. *Novartis Found. Symp.* 259, 238–245.

Wang, A. H. & Yang, X. J. (2001). Histone deacetylase 4 possesses intrinsic nuclear import and export signals. *Mol. Cell. Biol.* 21, 5992–6005

Wen, Y. D. *et al.* (2000). The histone deacetylase-3 complex contains nuclear receptor corepressors. *Proc. Natl Acad. Sci. USA* 97, 7202–7207

Wick A, Wick W, Waltenberger J, Weller M, Dichgans J, Schulz JB. (2002). Neuroprotection by hypoxic preconditioning requires sequential activation of vascular endothelial growth factor receptor and Akt. *J Neurosci.* 22; 6401-7.

Wiesener MS et al. (2003). Widespread hypoxia-inducible expression of HIF-2 in distinct cell populations of different organs. *FASEB J.* 17; 271–273.

Williams K. Interactions of polyamines with ion channels. Biochem J. 325 (Pt 2):289-97. Review. Erratum in: Biochem J 1997 Sep 15;326(Pt 3):943.

Whitacre CM, Hashimoto H, Tsai ML, Chatterjee S, Berger SJ, Berger NA. (1995) Involvement of NAD-poly(ADP-ribose) metabolism in p53 regulation and its consequences. Cancer Res. 55(17):3697-701.

Zhang, C. L., McKinsey, T. A. & Olson, E. N. (2002) Association of class II histone deacetylases with heterochromatin protein 1: potential role for histone methylation in control of muscle differentiation. *Mol. Cell. Biol.* 22, 7302–7312

Zhang, Y. *et al.* (2003) HDAC-6 interacts with and deacetylates tubulin and microtubules *in vivo*. *EMBo J*. 22, 1168–1179.

Zhao H (2007). The protective effect of ischemic postconditioning against ischemic injury: from the heart to the brain. *J Neuroimmune Pharmacol* 2; 313–318.

Zingarelli B, Virág L, Szabó A, Cuzzocrea S, Salzman AL, Szabó C. (1998) Oxidation, tyrosine nitration and cytostasis induction in the absence of inducible nitric oxide synthase. Int J Mol Med. 1(5):787-95.