

# Neuronal oxidative injury in the development of the epileptic disease: a potential target for novel therapeutic approaches

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## Abstract

Epileptic diseases affect about 50 million people in the world and approximately 30% of patients diagnosed with epilepsy are unresponsive to current medications. For these reasons, primary prevention of epilepsy represents one of the priorities in epilepsy research.

Intracellular oxido-reductive (redox) state is well known to play a crucial role, contributing to the maintenance of the proper function of biomolecules. Therefore, oxidative stress results in functional cellular disruption and cellular damage and may cause subsequent cell death via oxidation of proteins, lipids, and nucleotides.

Recently, the role of oxidative stress in the early stage and in the progression of epileptic disorders has begun to be recognized. The early molecular response to oxidative stress represents a short-term reversible phenomenon that precedes higher and irreversible forms of oxidation.

This article reviews the current understanding of the epileptogenic phenomena related to seizure-induced oxidative injury as potential “critical period” therapeutic targets for the prevention of chronic epileptic disorder.

## Keywords

Epilepsy, Oxidative stress, NMDA receptors.

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## Introduction

Epilepsy is a disorder characterized by chronically recurring seizures and it represents the most frequent neurodegenerative disease after stroke. Epileptic disorders, in general, afflict more than 50 million people worldwide, affecting patients' cognitive function, increasing risk of psychiatric disorders and mortality. Given its invalidating nature with a strong social impact, epileptic diseases economically contribute 0.5% of the global burden of disease.

At present, the conventional antiepileptic drugs (AEDs) show partial or no efficacy in the treatment of some forms of refractory epilepsy such as Temporal Lobe Epilepsy (TLE). Furthermore, generally, AEDs can have important and irreversible side effects that can severely affect the patient's quality of life, even more so in children, in which exposure to powerful anticonvulsant medications will have irreversible consequences on cognitive development.<sup>1</sup> Given the invalidating nature of refractory forms of epilepsy, the need for novel effective treatments is undeniable.

Epilepsy prevention approaches rest upon identification. Understanding of the underlying mechanisms of epilepsy development, and intervention targeted at these mechanisms in an appropriate time period after acute seizure could represent an important tool for novel therapeutic approaches aimed at preventing chronic irreversible epileptic disorder.

Brain injury resulting from seizures is a dynamic process that comprises multiple factors contributing to a hyper-excitable state and neuronal reorganization underlying the chronic epileptic phenotype. In some forms of refractory epileptic disorders, such as TLE, the focus of seizure activity typically involves the hippocampal formation, which may develop pathological lesions, including neuronal loss and aberrant mossy fiber sprouting.<sup>2</sup>

Reactive Oxygen Species are generated during normal cellular metabolism<sup>3</sup> and continuously scavenged by enzymatic and non-enzymatic antioxidant defense systems. However, excessive ROS levels due to increased ROS production and/or decreased antioxidant defense ability, leads to oxidative stress.

Oxidative stress results in functional cellular disruption and cellular damage and may cause subsequent cell death via oxidation of biomolecules such as proteins, lipids, and nucleotides.

The brain is particularly prone to oxidative stress because it utilizes a high amount of oxygen. Furthermore, the high concentrations of polyunsaturated fatty acids, that are prone to peroxidation, and metals, such as iron, which can catalyze hydroxyl radical formation, increase the brain susceptibility to oxidative stress. Therefore, oxidative injury in brain is one of the main causes of a number of neurologic conditions and neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and epilepsy,<sup>4</sup> in which glutamatergic paroxysm-induced mitochondrial dysfunction and oxidative stress are among the crucial epileptogenic factors.

Under physiological conditions, NMDARs are involved in long-term responses and neural plasticity in the brain. Via  $\text{Ca}^{2+}$  influx, NMDARs also modulate intracellular oxidant systems,<sup>5,6</sup> including NADPH oxidase (NOX), which is a major source of superoxide production downstream the NMDAR activation. Changes in redox status participate in normal long-term neuronal responses<sup>7</sup> and also regulate NMDAR channel function.<sup>8</sup> However, oxidative stress can also promote altered function of neuronal cells when NMDAR activation is sustained.<sup>9</sup>

Recent studies reported that the NADPH oxidase complex plays a crucial role in seizure-mediated superoxide production, in neuronal death in the pilocarpine model of epilepsy and in NMDAR subunits expression.<sup>10</sup>

In particular, NR2B subunit is predominantly contained in pre-synaptic autoreceptors that tonically facilitate the glutamate release at excitatory synapses.<sup>11,12</sup> Moreover, recent studies have shown that NR2B is coupled with extracellular signal-regulated kinase 1/2 (ERK1/2) signaling, whereas NR2A subunit is related to BDNF expression,<sup>13</sup> a crucial factor in limbic epileptogenesis and in chronic TLE.<sup>14</sup>

In this article, we focus on the emerging experimental evidences on oxidative stress as contributing factor in epileptogenesis by means the NMDARs' subunits altered expression.

Prevention of epilepsy in human subjects has been largely unsuccessful to this point. Understanding of the underlying oxidative stress-related mechanisms of epilepsy development, and intervention targeted at these mechanisms in an appropriate time period, could represent a successful strategy for the prevention of chronic epileptic disorders.

### **Oxidative stress and NMDARs' subunits expression**

NMDARs containing NR2A or NR2B subunits differ in kinetic properties, sensitivity to various ligands, permeability to divalent ions, and interactions with intracellular proteins.<sup>15</sup> In the hippocampal area, AMPA and NMDA receptor subunit mRNA levels change dynamically during development, and in models of epilepsy, as animals progress from the latent to the chronic limbic seizure phase. These early stoichiometric changes in subunit composition may underlie plastic structural changes, such as aberrant mossy fiber sprouting, and cause an excitation/inhibition imbalance in neural networks that results in epileptogenesis.<sup>16,17</sup> Therefore, understanding the molecular mechanisms accounting for these changes in NMDAR subunit composition could be of critical importance to developing therapeutic interventions.

In *vitro* and in *vivo* studies applying the pilocarpine (PILO) model of TLE, provided direct evidence that short-term exposure to PILO induces early over-expression of functional NR2B subunits in hippocampal neuronal cultures and in rat hippocampus by means of cooperative interaction between (i) NMDAR-enhanced NADPH oxidase activity with a resultant superoxide production and (ii) ERK1/2 phosphorylation which is activated independently by m1R-mediated IP3 synthesis accompanied by increased expression of NR2B subunits.<sup>10</sup>

NADPH oxidase is a cytoplasmic enzyme identified in many cell types (including neurons) that transfers an electron from NADPH to molecular oxygen to generate superoxide. Recent studies have suggested a mechanism by which  $\text{Ca}^{2+}$  influx through NMDAR channels leads to production of superoxide, and have described NADPH oxidase as the primary source of NMDA-induced superoxide production in neurons and a potential target to influence NMDAR-mediated excitotoxicity in different models of epilepsy in rat.

With respect to oxidative stress, thiol residues constitute some of the most important redox-sensitive functional groups of proteins and play a critical

regulatory role in numerous functions, including protein import, modulation of enzymatic signal transduction cascades, regulation of transcription factors and in the mitochondrial electron transport system.

Evaluating the role of disulfide formation as critical molecular switch during the early response to PILO in relation to altered expression of NMDARs subunits, our findings suggest that the activation of NADPH oxidase constitutes the major biochemical link between the NMDA–Ca<sup>2+</sup> transduction pathway and redox-sensitive regulatory transcription factors leading to the NR2B subunit over-expression after PILO exposure.

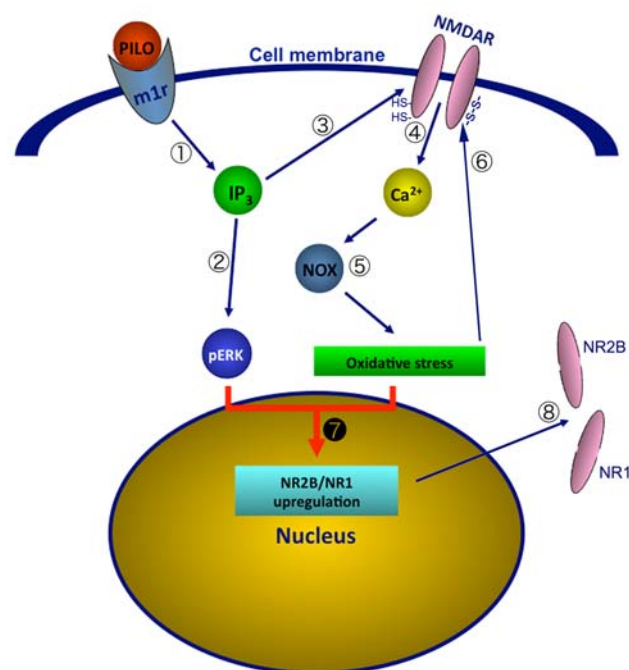
Furthermore, the direct NMDAR activation in hippocampal neurons mediated by the exposure to free-Mg<sup>2+</sup> medium, elicits a NMDAR-Ca<sup>2+</sup>-dependent increase in thiol oxidation but no changes in NMDAR subunits expression were detected, suggesting that, in the PILO model, the NMDA–Ca<sup>2+</sup>-NADPH oxidase pathway represents only one arm of a more complex pathway, which leads to early induction of NR2B over expression.

Substantial progress has been made in our understanding of how PILO causes long-term physiological changes in neurons. Previous work showed that m1R signaling leads to increased IP<sub>3</sub> production in hippocampal neurons without a concurrent intracellular Ca<sup>2+</sup> release.<sup>18</sup> Our studies show that m1R-mediated IP<sub>3</sub> synthesis, constitutes the biochemical signal leading to NMDAR activation, thiol oxidation and NR2B subunit over-expression.

Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are essential components of pathways through which signals received at membrane receptors are transduced to specific changes in protein function and gene expression. Extracellular stimuli such as neurotransmitters, neurotrophins and growth factors in the brain regulate critical cellular events, including synaptic transmission, neuronal plasticity, morphological differentiation and survival. ERK1/2 are abundantly expressed in the central nervous system and are activated in response to various physiological stimuli associated with synaptic activity and plasticity, but are also activated during pathological events such as brain ischemia and epilepsy. The present findings show that IP<sub>3</sub>-mediated ERK1/2 phosphorylation is a redox-independent pathway necessary, but not sufficient, for NR2B subunit over-expression. Therefore, the authors suggest that the early m1R-enhanced IP<sub>3</sub> synthesis plays a crucial role by activating two different biochemical pathways: Ca<sup>2+</sup>-dependent oxidation (via NMDARs) and ERK1/2 activation. Both pathways are required for NR2A up-regulation.

### Oxidative stress and NMDARs function

The cysteine residues on NMDARs are targets for S-nitrosylation and disulfide bond formation, implicating a critical role for reactive oxygen and nitrogen species in modulating NMDAR function.<sup>8</sup> Our results suggest that oxidation of thiols contained in NMDARs plays a potentially protective – but ultimately ineffective – role in preventing NMDAR-mediated Ca<sup>2+</sup> dysregulation after PILO treatment. Interestingly, NMDAR activation is a requisite proximal step that leads to oxidative stress, which, in turn, modulates NMDAR function. Taken together, these findings provide evidence that NMDAR-mediated oxidative stress contributes in complex ways to epileptogenesis by playing an essential role in the upregulation of NR2B, and by directly modulating the site-specific entry of Ca<sup>2+</sup> through NMDA channels, an important signal for initiating the cascade of molecular events that may contribute to delayed neuronal cell death in the context of epilepsy.



**Figure 1.** Schematic diagram of PILO-induced changes in NMDARs.

(1) PILO activation of m1R causes IP<sub>3</sub> production, which in turn, independently activates ERK1/2 (2) and increases activation of NMDARs (3). Enhanced influx of Ca<sup>2+</sup> through NMDARs (4) leads to activation of NOX (5) and generation of superoxide. This results in oxidative modification of cell surface NMDARs with impairment of receptor function (6). Additionally, the NOX-dependent oxidative stress together with ERK1/2 activation leads to upregulation of NR2B and NR1 subunits.

## Conclusions

In summary, this study provides evidence in the PILO model of TLE that muscarinic mR1-mediated stimulation of IP<sub>3</sub> synthesis is a pivotal biochemical event for activating two independent pathways, which together, are critical for aberrant NMDAR subunit expression: Ca<sup>2+</sup>-dependent NADPH oxidase activation and ERK1/2 phosphorylation (Fig 1). Importantly, these are *early* biochemical phenomena that are required to induce NR2B over-expression. Early up-regulation of NR2B subunit may be involved in the induction and maintenance of epileptogenesis, since NR1/NR2B heterodimers are primarily expressed as facilitatory presynaptic autoreceptors on hippocampal neurons. In conclusion, these findings provide mechanistic insights into the biochemical phenomena underlying the oxidative response to an epileptogenic stimulus, and may inform development of new therapeutic approaches for chronic epileptic disorders prevention.

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