

## Extra View

# Excitotoxic insults lead to peroxiredoxin hyperoxidation

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Post-mitotic neurons must have strong antioxidant defenses to survive the lifespan of the organism. We recently showed that neuronal antioxidant defenses are boosted by synaptic activity. Elevated synaptic activity, acting via the N-methyl-D-aspartate (NMDA) receptor, enhances thioredoxin activity, facilitates the reduction of hyperoxidized peroxiredoxins, and promotes resistance to oxidative stress. In contrast, blockade of spontaneous synaptic NMDA receptor activity renders neurons highly sensitive to hyperoxidation of peroxiredoxins by oxidative insults. These NMDA receptor-dependent effects are mediated in part by a coordinated program of gene expression changes centered on the thioredoxin-peroxiredoxin system, a thiol-based enzymatic system which is an important reducer of oxidative stressors such as hydroperoxides. We show here that while too little glutamatergic activity can render neurons vulnerable to peroxiredoxin hyperoxidation, so can too much. Exposure of neurons to toxic concentrations of glutamate, activating both synaptic and extrasynaptic NMDA receptors, acutely induces peroxiredoxin hyperoxidation. Thus, the effect of NMDA receptor activity on the activity of neuronal peroxiredoxins follows the classical U-shaped dose response curve.

## Introduction

Correct redox regulation is essential in all cells, especially in post-mitotic cells such as neurons where harmful oxidative damage can accumulate. Oxidative damage and stress occurs when there is an imbalance between production of reactive oxygen species (ROS) and the cell's capacity to neutralize them through its intrinsic antioxidant defenses. Neurons are particularly susceptible to oxidative damage due to high levels of ROS production (through respiration and metabolism) and relatively low levels of certain antioxidant enzymes, particularly catalase.<sup>1,2</sup> Oxidative damage is implicated in the pathogenesis of several neurodegenerative diseases as well as acute cerebrovascular disorders.<sup>1,2</sup> Appropriate

redox balance depends on the activity of antioxidant systems. Key among these are the thiol reducing systems based around thioredoxin and glutathione respectively, which are important reducers of many oxidative stressors such as peroxides.<sup>2,3</sup> The principle source of peroxide is from spontaneous and superoxide dismutase (SOD)-catalysed dismutation of superoxide generated in active mitochondria. However, other sources of peroxides exist, including products of metabolic pathways involving oxidases/oxygenases (e.g., monoamine oxidase).

We recently showed that the vulnerability of neurons to oxidative death triggered by exposure to hydrogen peroxide was regulated by synaptic activity acting via N-methyl-D-aspartate (NMDA) receptor (NMDAR) signaling.<sup>4</sup> Neurons that were experiencing (or had recently experienced) higher levels of synaptic NMDAR activity were far more likely to withstand the oxidative insult than electrically quiet neurons. Accumulation of reactive oxygen species following an oxidative insult was significantly lower in active neurons than inactive ones. Neurons experiencing complete NMDAR blockade were highly vulnerable to peroxide-induced apoptosis *in vitro*, and NMDAR blockade *in vivo* promoted neuronal apoptosis associated with oxidative damage. It is not completely clear why synaptic activity, acting via Ca<sup>2+</sup> signaling, should act to boost antioxidant defences. One possibility is to protect against increased ROS generation associated with high energy demand. Synaptic activity and activity-dependent plasticity place high energy demands on a neuron which must be largely met through oxidative phosphorylation, a process that generates ROS. Thus, active neurons would ordinarily need to have stronger antioxidant defenses than inactive ones in order to maintain the correct redox balance. However, synaptic activity is clearly strengthening antioxidant defenses above and beyond that required to cope with increased metabolic activity, since active neurons become resistant to additional exogenous insults.<sup>4</sup>

Investigations into the mechanism behind this revealed that synaptic activity exerted a number of changes to the thioredoxin-peroxiredoxin system which contributed to the activity-dependent protection.<sup>4</sup> Synaptic activity enhanced thioredoxin activity and facilitated the reduction of hyperoxidized peroxiredoxins, an important class of antioxidant enzymes. These changes were mediated by a coordinated program of gene expression changes. Synaptic NMDAR activity triggered the transcriptional suppression of the thioredoxin inhibitor Txnip, a protein known to

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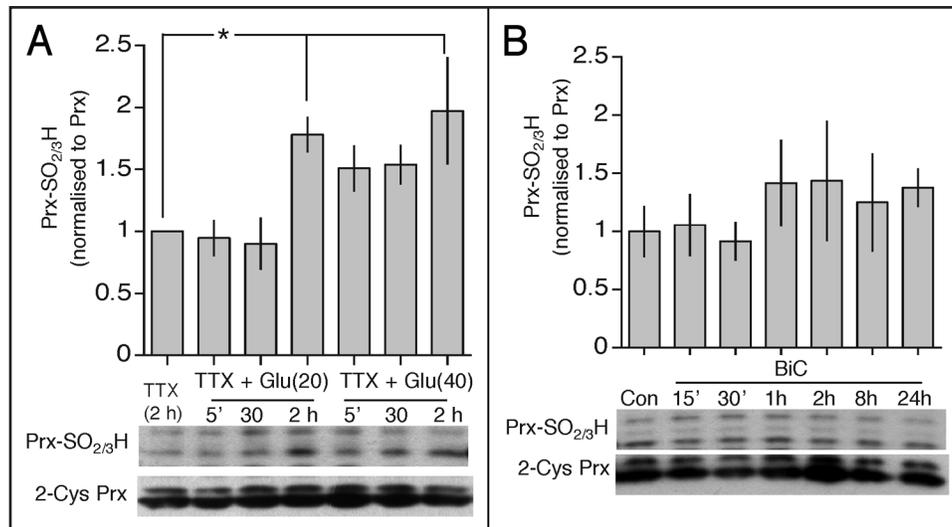


Figure 1. Excitotoxic glutamatergic activity promotes hyperoxidation of peroxiredoxins. (A and B) Lower: Example western analysis of peroxiredoxin (Prx) hyperoxidation in neurons treated as indicated, using an anti-PrxSO<sub>2/3</sub>H specific antibody. Upper: densitometric analysis of PrxSO<sub>2/3</sub>H band intensity of Prx II, normalized to the appropriate Prx II band. \**p* < 0.05 compared to control (1-way Anova followed by Fisher's LSD test, *n* = 5).

enhance oxidative stress. Furthermore, enhanced reduction of hyperoxidized peroxiredoxins was associated with transcriptional induction of two genes, sulfiredoxin and sestrin 2, whose products are reported to mediate this reaction. We did not address which of sestrin 2 or sulfiredoxin was responsible for reducing peroxiredoxin hyperoxidation. However, we recently showed that specific induction of sulfiredoxin is sufficient to prevent peroxiredoxin hyperoxidation in neurons.<sup>5</sup> In any case, induction of one (or both) of these genes cooperated with the suppression of Txnip in boosting antioxidant defenses.

The effect of synaptic NMDAR activity in promoting the reduction of hyperoxidized peroxiredoxins may at first glance appear to be at odds with other studies that report the induction of oxidative stress and free radical production by NMDAR activity. For example, NMDAR activity has been shown to induce superoxide production using electron spin resonance detection.<sup>6</sup> Indeed, agonists of all ionotropic glutamate receptors, as well as glutamate itself can induce rapid ROS generation.<sup>7,8</sup> However, the ROS-generating capacity of agonists of ionotropic glutamate receptors are typically studied in the context of excitotoxic levels of agonists. Increased free radical production is likely a result of consequences of excessive Ca<sup>2+</sup> influx, such as intracellular Ca<sup>2+</sup> deregulation or mitochondrial dysfunction as well as other processes such as strong activation of nitric oxide production.<sup>9,10</sup> In contrast, our stimulation paradigms used to promote synaptic activity are not toxic, quite the reverse—they are neuroprotective.<sup>11–13</sup> Moreover, they do not induce mitochondrial dysfunction.<sup>11</sup> The response of neurons to NMDAR activity follows an inverted U-shaped, or bell shaped curve, i.e., too much or too little can be potentially harmful.<sup>14,15</sup> The studies which report ROS generation are generally concerned with the effect of high toxic levels of NMDAR activity, while we are looking at the central part of the curve where modest levels of NMDAR activity are protective.

## Results

To contrast the effects of synaptic activity with those of toxic doses of glutamate, we have studied peroxiredoxin hyperoxidation in response to excitotoxic concentrations of glutamate (20 and 40 μM). Glutamate was added in the presence of tetrodotoxin to prevent preferential activation of synaptic NMDA receptors (which can happen in its absence<sup>16</sup>). We observed a significant elevation of levels of peroxiredoxin hyperoxidation at two hours post-treatment (Fig. 1A), indicative of oxidative stress. In contrast, stimulating synaptic activity using an established model (bicuculline plus 4-aminopyridine (BiC/4-AP)<sup>13,17</sup>) triggers no peroxiredoxin hyperoxidation at any time point observed (Fig. 1B). This emphasizes the difference between the two stimulation paradigms.

It is worth noting that the difference between these two stimulation paradigms is not simply due to degree of NMDA receptor activity. If this was true then exposure of neurons to lower doses of glutamate (e.g., 5 and 10 μM) would trigger the same effects as BiC/4-AP treatment. However, these stimuli do not trigger the same anti-oxidative effects: reduction of peroxiredoxin hyperoxidation or sulfiredoxin/sestrin 2 upregulation is not promoted, nor is protection against oxidative stress.<sup>4</sup> The key difference is that the strong antioxidant signaling described in<sup>4</sup> relies on the trans-synaptic activation of synaptic NMDA receptors. Chronic activation of all (synaptic and extrasynaptic NMDA receptors) is unable to recapitulate trans-synaptically evoked signaling, regardless of dose. It is currently unclear how synaptic and extrasynaptic NMDARs would be coupled to different signaling pathways in the context of the effects described here. It could be that they are coupled differently (either physically or functionally) as a result of their differing location. Another contributing factor could be the way in which these distinct pools are activated: brief saturating activation in the case of trans-synaptic activation of synaptic NMDARs vs. chronic

low level activation of extrasynaptic NMDARs by bath/ambient glutamate. Differences in the properties of intracellular  $\text{Ca}^{2+}$  transients evoked by these different stimuli could differentially affect signaling, even if the overall  $\text{Ca}^{2+}$  load were similar (reviewed in ref. 18).

The observation that exogenous glutamate exposure induced hyperoxidation of peroxiredoxins is interesting in view of the fact that this event also occurs *in vivo* following an excitotoxic insult—namely ischemia followed by reperfusion.<sup>4</sup> Thus, it is possible that ROS generation triggered by excessive glutamate build up following an ischemic episode contributes to neuronal damage through inactivation of peroxiredoxins, in concert with the many other damaging events that take place, such as stress-activated protein kinase activation, calpain activation, nitric oxide production as well as inflammatory events and ionic imbalance.<sup>19–24</sup>

## Materials and Methods

**Neuronal cultures and stimulation.** Cortical rat neurons were cultured as described<sup>25</sup> from E21 rats except that growth medium was supplemented with B27 (Invitrogen). Stimulations were done in both cases after a culturing period of 8–10 days during which cortical neurons develop a network of processes, express functional NMDA-type and AMPA/kainate-type glutamate receptors, and form synaptic contacts. Stimulations were performed after transferring neurons into defined medium lacking trophic support “TMo”:<sup>13</sup> 10% MEM (Invitrogen), 90% Salt-Glucose-Glycine (SGG) medium;<sup>26</sup> (SGG: 114 mM NaCl, 0.219%  $\text{NaHCO}_3$ , 5.292 mM KCl, 1 mM  $\text{MgCl}_2$ , 2 mM  $\text{CaCl}_2$ , 10 mM HEPES, 1 mM Glycine, 30 mM Glucose, 0.5 mM sodium pyruvate, 0.1% Phenol Red; osmolarity 325 mosm/l, hereafter TMo). Bursts of action potential firing were induced by treatment of neurons with 50  $\mu\text{M}$  bicuculline, and burst frequency was enhanced by addition of 250  $\mu\text{M}$  4-amino pyridine.<sup>27</sup> Glutamate was applied at the indicated concentration in the presence of TTX (2  $\mu\text{M}$ , Tocris).

**Western blotting and antibodies.** Total cell lysates were boiled at 100°C for 5 min in 1.5x sample buffer (1.5 M Tris pH 6.8; Glycerol 15%; SDS 3%;  $\beta$ -mercaptoethanol 7.5%; bromophenol blue 0.0375%). Gel electrophoresis and western blotting were performed using Xcell Surelock system (Invitrogen) using precast gradient gels (4–20%) according to the manufacturer’s instructions. The gels were blotted onto PVDF membranes, which were then blocked for one hour at room temperature with 5% (w/v) non-fat dried milk in TBS with 0.1% Tween 20. The membranes were then incubated at 4°C overnight with the primary antibodies diluted in blocking solution: 2-Cys Prx (1:500, Abcam), Prx- $\text{SO}_{2/3}\text{H}$  (1:1,000, Abcam). For visualisation of western blots, HRP-based secondary antibodies were used followed by chemiluminescent detection on Kodak X-Omat film. Western blots were analysed by digitally scanning the blots, followed by densitometric analysis (ImageJ). All analysis of Prx- $\text{SO}_{2/3}\text{H}$  levels involved normalizing to the loading control: total 2-Cys Prx as determined using an anti-2-Cys Prx antibody. Blots were first probed with the anti-Prx- $\text{SO}_{2/3}\text{H}$  antibody, then stripped and re probed with anti-2-Cys Prx to determine loading.

## Concluding Remarks and Future Perspectives

The detailed activity-dependent changes in gene expression which contribute to enhanced antioxidant defenses have hitherto focused on the thioredoxin system.<sup>4</sup> However, these changes do not account for the full effects of synaptic activity, suggesting that other enzymic systems may also be targeted. It will be of interest to know how, or whether, synaptic activity boosts the capacity of the other key antioxidant system in neurons—that based on glutathione. Another outstanding issue is the degree to which protective changes in gene expression occur *in vivo* following an episode of NMDA receptor activity. Given that an episode of ischemia/reperfusion triggers hyperoxidation of peroxiredoxins,<sup>4</sup> one might expect that a protective preconditioning episode reduces this. If this is indeed the case, this would beg the question as to whether the changes in gene expression, such as upregulation of the peroxiredoxin regenerating enzyme, sulfiredoxin, occur in response to preconditioning.

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