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Short communication

Mitochondrial ROS cause motor deficits induced by synaptic inactivity: Implications for synapse pruning

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ABSTRACT

Developmental synapse pruning refines burgeoning connectomes. The basic mechanisms of mitochondrial reactive oxygen species (ROS) production suggest they select inactive synapses for pruning: whether they do so is unknown. To begin to unravel whether mitochondrial ROS regulate pruning, we made the local consequences of neuromuscular junction (NMJ) pruning detectable as motor deficits by using disparate exogenous and endogenous models to induce synaptic inactivity *en masse* in developing *Xenopus laevis* tadpoles. We resolved whether: (1) synaptic inactivity increases mitochondrial ROS; and (2) chemically heterogeneous antioxidants rescue synaptic inactivity induced motor deficits. Regardless of whether it was achieved with muscle (α-bungarotoxin), nerve (α-latrotoxin) targeted neurotoxins or an endogenous pruning cue (SPARC), synaptic inactivity increased mitochondrial ROS *in vivo*. The manganese porphyrins MnTE-2-PyP^{5+} and/or MnTnBuOE-2-PyP^{5+} blocked mitochondrial ROS to significantly reduce neurotoxin and endogenous pruning cue induced motor deficits. Selectively inducing mitochondrial ROS—using mitochondria-targeted Paraquat (MitoPQ)—recapitulated synaptic inactivity induced motor deficits; which were significantly reduced by blocking mitochondrial ROS with MnTnBuOE-2-PyP^{5+}. We unveil mitochondrial ROS as synaptic activity sentinels that regulate the phenotypical consequences of forced synaptic inactivity at the NMJ. Our novel results are relevant to pruning because synaptic inactivity is one of its defining features.

1. Introduction

Mitochondrial reactive oxygen species (ROS), namely superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), are of considerable biological interest because they can signal but can also cause damage [1–4]. The factors that control the production and removal of mitochondrial ROS are well understood (reviewed in [5–7]). ATP demand, [NADH] and proton motive force (Δp) control mitochondrial ROS production in forward mode whereas antioxidant enzyme activity controls their removal [5–10]. Key mitochondrial antioxidant enzymes include manganese superoxide dismutase, peroxiredoxin 3 and glutathione peroxidase 1 [11–13]. Using existing knowledge of mitochondrial ROS production and removal (the known), it is possible to make experimentally tractable predictions about situations wherein a role for mitochondrial ROS is suspected but unverified (the unknown).

Developmental synapse pruning refines burgeoning connectomes [14,15]. Neuronal activity regulates pruning: activity protects synapses from and inactivity selects synapses for pruning, respectively [16–18]. Mitochondrial ROS are proposed to regulate pruning because neuronal activity should divergently regulate their production [19,20]. Neuronal activity *should* fulfil essential criteria for comparatively low mitochondrial ROS production (i.e. high respiration, high ATP demand and low Δp [5]). Conversely, neuronal inactivity *should* fulfil essential criteria for significant mitochondrial ROS production in forward mode (i.e. low respiration, low ATP synthesis and [NADH] build-up [5]). In support, skeletal muscle activity and inactivity decreases and increases mitochondrial ROS, respectively [21–24]. Further, surgically abolishing skeletal muscle activity increases mitochondrial ROS *ex vivo* [25–28]. We propose that: mitochondrial ROS are endogenous synaptic activity sentinels.

Whether mitochondrial ROS regulate pruning is unknown because it is a novel idea associated with two formidable technical challenges. First, studying pruning is challenging in the brain owing it its intricate connectome. The neuromuscular junction (NMJ)—a peripheral synapse

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between a motor neuron and skeletal muscle fibre—is an ideal alternative owing to its relative simplicity, size and accessibility [29]. The NMJ is tractable because its development requires pruning to transition from the poly to mono-innervated state [29–31]. Second, pruning occurs over a protracted time-course (order of weeks) and encompasses *in utero* and *ex utero* developmental stages [30]. It is, however, possible to use neurotoxins to broadly recapitulate pruning by forcing synaptic inactivity in species that develop *ex utero*, such as *Xenopus laevis* (*X. laevis*) [32]. Neurotoxins obviate the need for invasive surgical procedures that can provoke adaptive redox responses to injury [33–35]. A key advantage: neurotoxins make the micro functional outcomes of pruning (partial loss of innervation) detectable as motor deficits at the macro level owing to their mass action [36]. Whether they induce pruning is unclear; but their ability to, at worst, mimic its functional consequences (i.e. motor deficits owing to lost innervation) is clear. To begin to resolve whether mitochondrial ROS regulate pruning, we used multiple forced synaptic inactivity models to determine if: synaptic inactivity increases mitochondrial ROS; and chemically heterogeneous antioxidants rescue synaptic inactivity induced motor deficits.

2. Results

2.1. The manganese porphyrin MnTE-2-PyP^{5+} reduces α-BTX—a post-synaptic inactivity model—induced motor deficits

The neurotoxin α-bungarotoxin (α-BTX) induces forced synaptic inactivity by ligating the nicotinic acetylcholine receptor to competitively antagonise cholinergic neurotransmission ([36,37] Fig. 1A). We used α-BTX to induce forced synaptic inactivity in *X. leavis* tadpoles. To verify α-BTX induced motor deficits, we assessed evoked swimming frequency: α-BTX decreased evoked swimming frequency by 15.5 ± 1.4% compared to control (Fig. 1B). To determine if α-BTX increases mitochondrial ROS *in vivo*, we used two intensity based mitochondria-targeted (i.e. triphenylphosphonium cation conjugated) probes: mito-dihydroethidium (Mito-SOX) and mitochondria peroxy yellow 1 (MitoPY1) [38–40]. Free radicals (e.g. $O_2^{\cdot-}$) oxidise Mito-SOX to fluorescent ethidium or 2-hydroxyethidium products whereas H_2O_2 and/or peroxynitrite oxidise MitoPY1 to a fluorescent phenol by a boronate de-protection mechanism [41–47]. α-BTX increased Mito-SOX and MitoPY1 oxidation by 40.5 ± 4.9% and 30.5 ± 3.5%, respectively, compared to control (Fig. 1C-D).

To determine if mitochondrial ROS play a functional role, we used chemically heterogeneous antioxidants (Supplementary Table 1). Specifically, Mn(III) *meso*-tetrakis(2-pyridyl)porphyrin (MnTE-2-PyP^{5+}), Mn(III) *meso*-tetrakis(*N*-(*n*-butoxyethyl)pyridinium-2-yl (MnTnBuOE-2-PyP^{5+}), Mn(III) *meso*-tetrakis(4-carboxylatophenyl)porphyrin (MnTBAP^{3-}) and 2,2,6,6-tetramethyl-4-[5-(triphenylphosphonio)pentoxy] piperidin-1-oxy (MitoTempol) [48–52]. MnTBAP^{3-} and MitoTempol were inefficacious: failing to rescue α-BTX induced motor deficits or reduce MitoSOX and MitoPY1 oxidation. MnTnBuOE-2-PyP^{5+} successfully reduced MitoPY1 oxidation by 20.3 ± 4.2% compared to α-BTX, but failed to significantly reduce α-BTX induced MitoSOX oxidation or motor deficits. MnTE-2-PyP^{5+} successfully blocked mitochondrial ROS reducing Mito-SOX and MitoPY1 oxidation by 38.6 ± 4.7% and 31.2 ± 5.0%, respectively, compared to α-BTX (Fig. 1C-D). MnTE-2-PyP^{5+} significantly reduced α-BTX induced motor

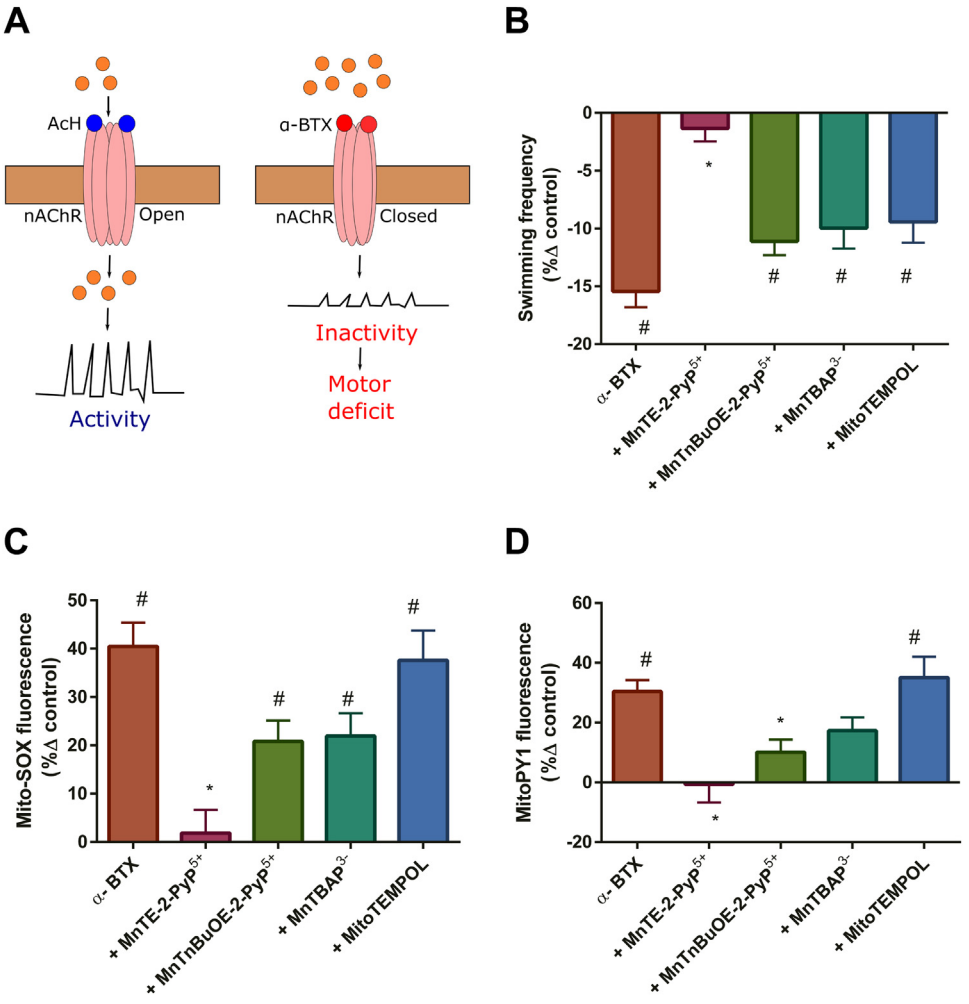

Fig. 1. α-BTX induced motor deficits are redox regulated. (A) α-BTX mechanism of action scheme. Left: skeletal muscle activity stems from acetylcholine (ACh) binding to post-synaptic nicotinic acetylcholine receptor (nAChR) to permit Na^+ entry. Right: α-BTX ligates the nAChR to render it impermeable to Na^+ leading to forced post-synaptic inactivity induced motor deficits. (B) Evoked swimming frequency (expressed as %δ control) by condition (α-BTX, α-BTX plus: MnTE-2-PyP^{5+}, MnTnBuOE-2-PyP^{5+}, MnTBAP^{3-} or MitoTempol; $n = 10$ in each condition). (C) Mito-SOX oxidation (expressed as %δ control) by condition ($n = 10$ per condition). (D) MitoPY1 oxidation (expressed as %δ control) by condition ($n = 10$ per condition). Error bars are SEM. Concentrations and incubations: α-BTX (8 μM for 30 min), MnTE-2-PyP^{5+}, MnTnBuOE-2-PyP^{5+}, MnTBAP^{3-} conditions = 1 μM for 30 min. MitoTempol = 20 μM for 30 min. # denotes significant difference vs control. * denotes significant difference vs α-BTX.