JNK signalling regulates antioxidant responses in neurons

Chris Ugboe, Nathan Garnham, Laura Fort-Aznar, Gareth J.O. Evans, Sangeeta Chawla, Sean T. Sweeney

A B S T R A C T

Reactive oxygen species (ROS) are generated during physiological bouts of synaptic activity and as a consequence of pathological conditions in the central nervous system. How neurons respond to and distinguish between ROS in these different contexts is currently unknown. In Drosophila mutants with enhanced JNK activity, lower levels of ROS are observed and these animals are resistant to both changes in ROS and changes in synapse morphology induced by oxidative stress. In wild type flies, disrupting JNK-AP-1 signalling perturbs redox homeostasis suggesting JNK activity positively regulates neuronal antioxidant defense. We validated this hypothesis in mammalian neurons, finding that JNK activity regulates the expression of the antioxidant gene Srxn-1, in a c-Jun dependent manner. We describe a conserved 'adaptive' role for neuronal JNK in the maintenance of redox homeostasis that is relevant to several neurodegenerative diseases.

1. Introduction

Active neurons generate reactive oxygen species (ROS) predominantly as a by-product of mitochondrial respiration. ROS levels are neutralized by constitutive and adaptive reductive mechanisms operating in neurons and glia, including the glutathione system [1-3]. In this manner, the amplitude and temporal dynamics of the ROS signal are controlled, damage is limited and transient ROS signals can be interpreted in part to support the growth and plasticity of neurons [4,5]. A physiological level of ROS has been demonstrated to regulate a range of nervous system processes, including neuronal development, synaptic plasticity, and neural circuit tuning [5-7].

In many neurodegenerative disorders the reductive capacity of neurons is overwhelmed, contributing to disease progression [4,8]. These damaging levels of ROS, termed oxidative stress, overwhelm neuronal antioxidant defenses. A central component of the neuronal response to ROS is the activation of c-Jun N-terminal kinase, JNK. ROS have been shown to activate JNK-AP-1 signalling [9] which regulates neuronal growth and plasticity [10-13]. In Drosophila models, excessive ROS driving increases in synaptic growth are seen in both activity generated excitotoxicity and lysosomal storage disease [5,12,14]. Similar changes in synaptic structure can be induced directly by application of oxidants such as paraquat [12] or diethylmaleate (DEM) [5], or through genetic activation of JNK via manipulation of the JNKK hemipterus [15] (hep) or the JNKK Wallenda [11] (wnd), upstream activators of JNK.

Whether JNK activation by ROS in neurons is protective or degenerative is unknown. Recent evidence indicates JNK-AP-1 activity can prevent degeneration of injured axons [16] while JNK inhibition prevents neuronal loss after injury by promoting AKT signalling [17].

We recently found that knockdown of enzymes involved in antioxidant defense, superoxide dismutase and catalase, reshapes neuronal morphology at the Drosophila larval neuromuscular junction (NMJ) [5]. Similarly, mis-expressing antioxidant enzymes to manipulate levels of individual ROS species has profound effects on synaptic and dendritic growth and arborisation [5,12]. This suggests that plasticity at the synapse is finely tuned to the level of ROS. Given that JNK is known to regulate synaptic plasticity in response to ROS and that knockdown of antioxidant genes can change synapse structure, we hypothesized that JNK-induced structural changes in neurons are mediated in part by an antioxidant response.

To understand if JNK activity regulates antioxidant responses in neurons, we monitored ROS levels in Drosophila with mutations in highwire (hiw) and puckered (puc) mutants. In hiw mutants, unrestrained
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specificity phosphatase, transcriptionally activated by AP-1 that coun-

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to investigate how JNK activity influences ROS levels under these conditions, we reared (Fig. 1 A). To understand whether DEM influences glutathione levels, is sufficient to induce an overgrowth at the larval NMJ, compared to larvae reared on normal food (Fig. 1 D). Our data indicates that activation of JNK in neurons drives antioxidant responses to shape neuronal morphology.

2. Results

2.1. JNK activity drives antioxidant responses in Drosophila

To quantify levels of ROS in Drosophila, we used the Amplex red assay for detection of hydrogen peroxide [20]. We have previously found that food containing 5 mM and 10 mM DEM which depletes glutathione levels, is sufficient to induce an overgrowth at the larval NMJ [5], with 10 mM concentrations being detrimental to survival (Fig. S1, 6% of flies pupate). To understand whether DEM influences ROS levels in flies, we raised flies on food containing ethanol (vehicle) or DEM (Fig. S1, 6% of flies pupate). To understand whether DEM influences glutathione levels, is sufficient to induce an overgrowth at the larval NMJ, compared to larvae reared on normal food (Fig. 1 D). Our data indicates that activation of JNK in neurons drives antioxidant responses to shape neuronal morphology.

The elaborate synaptic morphology of hiw mutant flies has been extensively described [11] and is a consequence of activation of the JNK-AP-1 pathway. We therefore chose to evaluate the synaptic morphology of puc<sup>hiw</sup>+/+ neurons by analyzing the NMJ at Muscle 6/7, hemi-segment A3 in wild type, puc<sup>hiw</sup>+/+ mutants and wild type flies expressing dominant negative fos (fos<sup>ΔN</sup>), jun (jun<sup>ΔN</sup>) and ask1 (ask1<sup>ΔN</sup>), reared on food containing ethanol or DEM. Scale bar = 30 μm. E Quantification of mean normalized bouton number from (D) genotypes. Data are plotted as mean ± SEM (**p < 0.01; ***p < 0.001; one-way ANOVA; minimum 15 flies per genotype). F Schematic showing the contribution of JNK to neuronal antioxidant stress responses in Drosophila. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

*wallenda* (wnd) activity activates JNK and downstream AP-1 signalling to generate exuberant synaptic overgrowth [11,18]. Puc encodes a dual specificity phosphatase, transcriptionally activated by AP-1 that counteracts JNK activity [19]. In both the hiw and puc<sup>hiw</sup>+/+ mutant backgrounds we identified enhanced antioxidant defenses and low levels of ROS. In hiw, this enhanced antioxidant defense can be reversed in neurons by down-regulating JNK-AP-1 signalling. Conversely, both puc<sup>hiw</sup>+/+ and hiw flies are resistant to chemically induced oxidative stress, while inhibition of JNK-AP-1 signalling increases ROS levels in wild type neurons. We show that in mammalian neurons exposed to oxidative stress, the JNK-AP-1 pathway coordinates the transcription and translation of the antioxidant gene Sulfiredoxin-1, which was localised predominantly in synaptic compartments. Our data indicates that activation of JNK in neurons drives antioxidant responses to shape neuronal morphology.

2.2. DEM induces oxidative stress in primary mammalian neurons

JNK activity in mammalian neurons is often associated with apoptosis and activated JNK is commonly used as a hallmark of cell death in neurodegenerative disease. Given that JNK-AP-1 signalling in the fly generates an antioxidant response and decreasing JNK activity in fly neurons increases ROS levels, we hypothesized that antioxidant responses in mammalian neurons would also be regulated by JNK-AP-1. Having previously validated the effects of DEM in the fly, we established a mammalian model of oxidative stress, using DEM to deplete cellular glutathione. We used this model to assay antioxidant responses after DEM treatment. We first characterized the effect of DEM on cellular GSH levels and ROS production in mature primary rat neurons, using a glutathione assay measuring total glutathione (oxidized and reduced forms). Fig. S2A shows that application of DEM to primary cortical neuronal cultures caused a concentration and time-dependent decrease in cellular glutathione. A 1 h and 4 h treatment of DEM at concentrations of 10 μM and 100 μM caused a rapid decrease in GSH (Fig. S2A). Lower concentrations of 1 μM DEM reduced cellular GSH content with less severity. Given the extent of GSH depletion by DEM, we investigated whether DEM induces mitochondrial toxicity by monitoring WST-1 absorbance (Water Soluble Tetrazolium Salts) as a measure of mitochondrial stress. Compared to vehicle treated neurons, DEM induced a small but significant decrease in mitochondrial capacity, however this was much less than treatment with equivalent concentrations of paraquat (PQ) and rotenone (RT) (Fig. S2B). To determine if DEM induces a ROS burden as it does in our fly model we measured H<sub>2</sub>O<sub>2</sub> levels in neurons treated with DEM using Amplex red reagent. The Amplex red assay revealed that DEM (100 μM) significantly increased in H<sub>2</sub>O<sub>2</sub> levels (Fig. S2C), after 24 h compared to ethanol treated controls (Fig. S2C). DEM-induced oxidative stress is significantly attenuated when neurons
are pre-incubated with catalase. The duration and concentrations of DEM that we employed deplete glutathione and induce an increase in oxidative stress with minimal toxicity to mitochondrial function.

To model chronic oxidative stress, we treated neurons for longer time points (48 h) with DEM (100 μM) and found that DEM causes a retraction of the dendritic arbor. This effect is rescued when neurons are pre-incubated with Catalase (100 nM, Fig. S2D) and when the components that produce glutathione, the catalytic and modifying subunits of glutamate cysteine ligase (GCL), are transduced into neurons (Fig. S3).

### 2.3. Oxidative stress activates JNK-dependent SRXN-1 expression, in primary mammalian neurons

JNK enzymes are key mediators of cellular responses to stress in neurons [21], including oxidative stress [22,23], and alterations in JNK activity have previously been linked to changes in dendritic complexity [24]. Phosphorylation of threonine 183 and tyrosine 185 increase JNK activity [21]. To characterize the effectiveness of both DEM and the JNK inhibitor SU 3327 (SU, 700 nM), we determined whether oxidative stress also induced transcription downstream of oxidative stress does not require c-Fos. JNK regulates SRXN-1 expression under oxidative stress but not during synaptic activity. Representative Western blot probed for SRXN-1, Pan-JNK, phospho-JNK, Pan-ERK, phospho-ERK and GAPDH antibodies. Neurons were treated with Bic/4AP (50/500 μM) or DEM (100 μM) in the presence or absence of either the JNK inhibitor SU 3327 (SU, 100 μM) alone or in the presence of Bic/4AP (50/500 μM) to increase synaptic activity (Bic/4AP) or DEM (100 μM) as a positive control (Bic/4AP).

Using DEM, we found that JNK activity regulates SRXN-1 transcription. DEM (10 μM) induced a 1.73-fold induction in SRXN-1 mRNA expression compared to the ethanol treated controls (Fig. 2C). Similar levels of SRXN-1 mRNA were observed at higher concentrations of DEM (Fig. S4C). When neurons were pre-treated with the JNK inhibitor SU 3327 for 24 h, DEM failed to induce SRXN-1 mRNA. Similar to DEM treatment, increasing neuronal activity with Bic/4AP increased SRXN-1 mRNA (1.32-fold), however this induction was unaffected by JNK inhibition. Synaptic activity is known to induce expression of c-Fos, an AP-1 component. We determined whether oxidative stress also induced c-Fos transcription. Fig. 2D shows that there was no change in c-Fos mRNA levels with DEM treatment whereas Bic/4AP treatment robustly induced c-Fos transcription. Similar to activity-induced SRXN-1 induction, c-Fos induction by synaptic activity was unaffected by inhibition of JNK (Fig. 2D) suggesting that synaptic activity and oxidative stress recruit signalling pathways differentially to activate potentially divergent gene transcription responses.

We next evaluated whether JNK activity regulates SRXN-1 protein
expression. Fig. 2E shows that treatment of neurons with DEM (100 μM) rapidly increased SRXN-1 protein expression within 30 min, with high levels detected at 4 h as assessed by Western blot using an antibody that we first validated by overexpressing human SRXN-1 in HEK293T cells (Fig S4A). DEM-induced SRXN-1 expression was attenuated when neurons were pre-treated with SU 3327 (700 nM, 1 h pre-incubation). As expected, and in agreement with previous work [26], synaptic activity also induced SRXN-1 expression after a 4 h treatment with Bic/4AP, which was unaffected by JNK inhibition. Moreover, synaptic activity but not DEM, induced ERK activation (Fig. 2E). Taken together, these data indicate that oxidative stress activates JNK to coordinate an antioxidant response in neurons, which is distinct from antioxidant responses coordinated by synaptic activity.

2.4. JNK-c-Jun signalling regulates antioxidant responses in mammalian neurons

To identify the cellular mediators that co-ordinate SRXN-1 expression under conditions of synaptic activity or oxidative stress, we assessed the expression and interaction of putative AP-1 components under the two conditions. Cortical neurons were treated with Bic/4AP or DEM for 1 or 4 h and c-Fos or c-Jun immunofluorescence was quantified in NeuN positive neurons. In neurons treated with DEM (Fig. 3A and B) c-Fos immunofluorescence was found to decrease. In contrast, DEM induced a significant increase in c-Jun immunofluorescence. As expected, in neurons stimulated with Bic/4AP for 1 or 4 h, both c-Fos and c-Jun immunofluorescence was found to increase (Figs. S4, B,D). These data suggest that oxidative stress relies on Jun proteins to mount an antioxidant response in the absence of c-Fos. To further investigate the differential involvement of specific AP-1 components we immunoprecipitated c-Jun from cortical neurons treated for 1 h with either DEM or Bic/4AP and assessed its phosphorylation and association with c-Fos protein. While both DEM and Bic/4AP induce c-Jun expression and phosphorylation, c-Jun co-immunoprecipitated with c-Fos following Bic/4AP-induced synaptic activity but not during oxidative stress mediated by DEM (Fig. 3C). In mammals, c-Jun can form heterodimers with other members of the Jun family to form the dimeric AP-1 transcription factor. To identify if c-Jun was associated with another Jun family member, we probed membranes for JunB and JunD and found no association between either protein with c-Jun in DEM-treated neurons (Fig. S4E). To determine whether c-Jun alone is sufficient to induce SRXN-1 expression, lentiviral particles encoding human c-Jun (Fig. 3D) were transduced into cortical neurons. Lentiviral c-Jun expression was sufficient to induce SRXN-1 expression in neurons (Fig. 3E), however activation of Nuclear Factor Erythroid 2-related factor 2 (Nrf2) using the well characterized agonist TBHQ (10 μM, 16 h) had no effect on neuronal expression of SRXN-1. Taken together, these data suggest that oxidative stress activates JNK, which selectively recruits c-Jun to induce
SRXN-1 expression in neurons (Fig. 3F).

2.5. SRXN-1 is localised at synaptic terminals and prevents dendrite loss

To understand how SRXN-1 contributes to neuronal antioxidant defenses we investigated SRXN-1 localization in neurons. Having previously identified that chronic treatment of cortical neurons with DEM causes a loss of dendrites, we transfected neurons with PSD95-GFP alone (left panels) or in combination with Flag-tagged human SRXN-1 (right panels) ± 100 μM DEM (48hr). Cells stained with anti-GFP (green), anti SRXN-1 (Flag antibody, red) and nuclear staining with DAPI (blue). Scale bar = 100 μm. B Overexpressed human SRXN-1 co-localises with PSD95-GFP in dendritic spines. Representative images show an overlay of GFP and Flag immunofluorescence. Magnified images of dendritic spines outlined in white boxes (PSD95-GFP + SRXN-1, scale bar = 5 μm). C Endogenous SRXN-1 protein is present at synapses. Representative Western blot of synaptosome fractions prepared from mouse brain, probed with antibodies for the nuclear protein FOXO3a, the pre-synaptic marker synapsin 1 (Syn1), the mitochondrial protein ATP5A1, JNK and SRXN-1 as indicated. D Schematic representation of likely signalling pathways mediating Srxn-1 transcriptional induction in response to synaptic activity and oxidative stress. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
is consistent with the co-localization with PSD95 we observed by fluorescence microscopy. Therefore, in neurons, antioxidant proteins such as SRXN-1 and sensors of oxidative stress such as JNK are located at synapses, placing them at functionally important sites to orchestrate the adaptive antioxidant response (Fig. 4D).

**3. Discussion**

Here we have uncovered a role for JNK signalling in the regulation of neuronal antioxidant capacity using *Drosophila* and mammalian neurons in culture. We and others have shown previously that JNK regulates changes in synaptic morphology independently of any stimuli [11] or in response to ROS [5,12,14]. We now show that JNK activation drives an antioxidant response, which shapes cellular reductive capacity and synaptic morphology.

We show that *Drosophila* mutants that have enhanced JNK activity have low levels of ROS and furthermore, are resistant to chemically induced oxidative stress. These reduced ROS levels in mutant flies can be reinstated back to levels observed in wild type flies by inhibiting the JNK-AP-1 pathway. In addition to changes in ROS levels, we show that activation of the JNK-AP-1 pathway leads to overgrown synapses at the *Drosophila* NMJ suggesting that the status of JNK signalling has implications for structural synaptic plasticity. In mammalian cortical neurons we identified an ‘adaptive’ JNK-dependent antioxidant response when neurons are challenged with oxidative stress. This adaptive response relies on JNK’s downstream effector c-Jun, and results in increased abundance of the antioxidant protein SRXN-1. In contrast, JNK-independent SRXN-1 expression is triggered with increased synaptic activity of cortical neurons. We therefore implicate JNK signalling in the regulation of neuronal antioxidant defenses and identify distinct differences in neuronal responses to ROS generated in conditions of synaptic activity and oxidative stress.

**3.1. Constitutive JNK activation drives an antioxidant response in *Drosophila***

Genetic and pharmacological activation of JNK changes neuronal morphology and this structural change is dependent on AP-1 [15,28]. JNK-AP-1 signalling has long been known to positively regulate growth and strength at the larval NMJ [15]. JNK has also been shown to mediate the effect of ROS generated during oxidative stress associated with excitotoxicity [14] and lysosomal storage disease [12]. Combined with reports that activation of the JNK pathway after neuronal injury coordinates a regenerative response [16] and that AP-1 signalling regulates dendrite growth during both development and during conditions of synaptic activity; JNK-AP-1 signalling has been demonstrated as a crucial regulator of neuronal homeostasis. Both *puc*<sup>609/+</sup> heterozygotes and *hiw* mutants, which have increased JNK activity [11,19], showed reduced total ROS levels and rendered flies resistant to chemically induced oxidative stress. The reduced ROS levels in *puc*<sup>609/+</sup> heterozygotes and *hiw* mutants flies could be reversed by inhibition of neuronal JNK-AP-1, suggesting that the altered ROS originates from neurons. The reduced total ROS levels, arising from protective JNK signalling, in *hiw* mutants might explain the recently reported resistance of neurons in *highwire* null flies to the damaging effects of physical blows, which mimic traumatic brain injury [29].

A key question raised by our work is whether the JNK-dependent changes in synaptic morphology are driven by the increased antioxidant defenses. We have previously identified DEM as a regulator of neuronal morphology at the NMJ [5] and here we show that this DEM-induced change in synaptic morphology is regulated by JNK. Furthermore, we show that *puc*<sup>609/+</sup> larvae have overgrown NMJ’s which are not influenced by oxidative stress induced by DEM. Given that *puc*<sup>609/+</sup> and *hiw* larvae have elevated JNK activity, reduced ROS, elaborate NMJ’s and are resistant to chemically induced oxidative stress, our data suggests that changes in neuronal plasticity in these models is at least, in part, driven by an antioxidant response.

**3.2. JNK coordinates antioxidant responses in mammalian neurons**

It is known that synaptic activity can regulate the expression of neuronal antioxidant genes [26]. Furthermore, we have recently shown that ROS generated from synaptic activity acts as a signalling molecule to regulate neuronal plasticity, in a PI3K/DJ-1-dependent manner [5] implicating synaptic activity and ROS in the regulation of synaptic plasticity. What is currently unknown is how neurons recognize and respond to differences in physiological and pathological ROS. Our data shows that neurons recruit different signalling pathways to mediate ROS generated by synaptic activity or oxidative stress, but converge to regulate the same antioxidant gene, SRXN-1.

Increasing synaptic activity activates ERK1/2 [30,31] that in turn phosphorylates AP-1 components [32,33]. Consistent with this, we found that increasing neuronal activity using Bicuculline and 4-AP regulates Srxn-1 mRNA and protein expression [26]. Conversely, inducing oxidative stress in neurons with DEM did not affect ERK phosphorylation of AP-1 but did activate JNK and SRXN-1 expression. JNK inhibition abolished increased SRXN-1 in conditions of oxidative stress but not during synaptic activity. This suggests that neurons use distinct kinases to upregulate SRXN-1 expression in response to synaptic activity or oxidative stress.

Synaptic activity has been demonstrated to boost glutathione synthesis, providing a positive feedback loop, where accumulating levels of ROS from increased synaptic activity are neutralized by increases in cellular glutathione [26]. JNK activity is also regulated by cellular glutathione levels. In non-stressed cells, JNK is tethered to monomeric glutathione S-transferase Pi (GST-Pi) and increased H<sub>2</sub>O<sub>2</sub> can trigger the detachment and oligomerization of GST-Pi, conjugating ROS to glutathione [34]. This suggests that JNK activity is tuned to the redox thresholds of the cell [35]. This is important given that glutathione homeostasis is altered in many neurodegenerative diseases [60,61] and decreased glutathione levels have been observed in mouse models of Alzheimer’s Disease [62] and in AD patients [63].

Both ERK1/2 and JNK kinases regulate AP-1 [21,32], raising the possibility that the mechanisms converge at the level of this dimeric transcription factor. The AP-1 heterodimer of c-Fos and c-Jun [36] is well characterized but the conditions driving differential heterodimer composition are not well understood. In our work, a notable difference between synaptic activity and oxidative stress conditions was the lack of c-Fos transcriptional/translational induction by DEM and reduced dimerization with c-Jun. This indicates that DEM-induced AP-1 activity and Srxn-1 transcription is c-Fos independent (Fig. 4C and D). The mammalian AP-1 complex can contain Fos, Jun, Fra, and ATF components and multiple hetero- and homo-dimeric partners are capable of binding to AP-1 DNA binding sites [37,38]. Our immunoprecipitation of c-Jun showed a clear association with c-Fos during conditions of increased neuronal activity, but this association was absent during conditions of oxidative stress. Moreover, total c-Jun increased during oxidative stress and we did not detect JunB or JunD in immunoprecipitates suggesting that c-Jun may homodimerise to generate an antioxidant response in neurons. Our data identify a clear divergence between physiological and stress-dependent signalling in neurons, where activation of JNK and lack of c-Fos defines the stress response.

**3.3. SRXN-1 is present at the synapse**

SRXN-1 is an oxidoreductase that reduces sulfinylated proteins such as 2-cys peroxiredoxins (PRDX), small redox sensitive proteins widely

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expressed in mammalian neurons [39]. Both cysteine residues in PRDX can undergo reversible hyper-oxidation by peroxides to form sulfenic acid (Cys-SO\(_2\)H) [40]. Hyper-oxidation of PRDX proteins inactivates their peroxidase activity. This inactivation is known to occur in neurons where it can be seen to follow a circadian time course [41], and occur during glutamate induced excitoxicity [42] in addition to oxidative stress conditions [43]. SRXN-1 catalyses the reduction of Cys-SO\(_2\)H in an ATP dependent manner, restoring PRDX activity [44]. Both PRDX and SRXN-1 are regulated by synaptic activity [26]. Our data indicate that the cellular levels of SRXN-1 can also be tuned by the intracellular concentration of GSH and H\(_2\)O\(_2\) levels.

We additionally show that SRXN-1 can be found at cellular locations that are particularly vulnerable to ROS - synaptic terminals and dendrites [45]. Overexpressed human SRXN-1 localised to dendrites and dendritic spines and endogenous SRXN-1 protein was particularly enriched in synaptosomal membrane fractions. We also found that JNK kinases were present in synaptosomes. The localization of SRXN-1, a cytosolic protein, in a synaptic membrane fraction is intriguing. However, in non-neuronal cells, SRXN-1 has been reported to translocate from the cytosol to a ‘heavy membrane’ fraction enriched in mitochondria in response to oxidative stress [46]. Mitochondrial SRXN-1 has a role in re-activation of oxidized peroxiredoxins [40,47] and our data indicate that SRXN-1 might reside constitutively with neuronal mitochondria, consistent with a greater basal ROS burden in neurons.

Taken together, our data from both fly and mammalian models identify a fundamental conserved role of JNK signalling in regulating neuronal redox homeostasis through an adaptive antioxidant response. JNK signalling is activated specifically by oxidative stress and activates c-Jun containing AP-1 transcription factors independently of c-Fos. In this manner, JNK imparts mechanistic specificity to activate AP-1 under conditions that have relevance to a number of neurodegenerative diseases.

4. Materials and methods

4.1. Culture of neurons and cell lines

Timed-mated female Wistar rats (Charles River UK) (RRID: RGID,737929) were maintained in accordance with the UK Animals (Scientific Procedures) Act (1986). Cortices were dissected from postnatal day 1 (P1) mixed sex rat pups. Animals were euthanised using pentobarbital injection followed by cervical dislocation, according to Home Office guidelines. Cortical cell suspensions were obtained as previously described and cytosine arabinoside (AraC, 2.4 \(\mu\)M final concentration) was added to the growth medium at 1 day in vitro (DIV) [48].

Neurons were transfected at 12 DIV with PSD95-GFP (a kind gift from David Breidt [49] and FLAG-tagged Human SRXN-1 (purchased from Origene: RC207654) for 5 h using Lipofectamine 2000 (11668019, Thermo Scientific). Experiments were performed in a defined culture medium (Transfection medium (TM) contains: 90 ml SGG; 114 mM NaCl, 26.1 mM NaHCO\(_3\), 5.3 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, 1 mM glycine, 30 mM glucose, 0.5 mM C\(_6\)H\(_{12}\)NaO\(_6\) and 10 ml MEM (51200046, Thermo Scientific), penicillin (50 U/ml) and streptomycin (50 \(\mu\)g/ml)). After 24 h incubation in TM, cells were treated with DEM, bicuculline, catalase, paraquat, rotenone, 4-Aminopyridine or SU 3327 at concentrations and times as previously described [53]. Briefly, seven mixed sex mouse forebrains were dissected and transferred into cold homogenization buffer (320 mM sucrose, 1 mM EDTA, 5 mM Tris, pH 7.4, 4 °C), homogenized in a glass Teflon homogenizer, and centrifuged in an SS-34 rotor (1000g, 10 min, 4 °C). The supernatant (S1) was collected and the pellet re-suspended in 15 ml of homogenization buffer and centrifuged again (1000g for 10 min). S1 supernatants were combined, and pellets (P1) collected. S1
lysates were further centrifuged at 13000g for 20 min at 4 °C. Supernatant (S2) was collected and the pellet (P2) re-suspended in 21 ml homogenization buffer. The P2 pellet was further centrifuged at 13000g for 20 min at 4 °C. P2 was re-suspended in 320 mM sucrose (300 μL/forebrain), transferred into a glass-Teflon homogenizer with 9 vol of ice-cold distilled water and immediately homogenized for 3 up/down strokes at 2000 rpm. Homogenates were incubated on ice for 30 min with 0.1 volume of 1 M HEPES-NaOH (pH 7.4) and centrifuged at 25000g for 20 min at 4 °C. Pellets (LP1, synaptosomal membranes) were collected and the supernatant (LS2) further centrifuged in a 70 Ti rotor at 165,000g for 2 h at 4 °C. The resulting supernatant (LS2) was collected and the pellet (LP2, synaptic vesicles) re-suspended in 40 mM sucrose (2 ml). Protein concentration was assayed using Bradford reagent and 20 μg of each fraction was prepared in 1x Laemmli buffer for western blotting.

4.8. Western blotting

Cells were lysed in RIPA containing phosSTOP phosphatase inhibitors (4906845001, Roche) and complete EDTA-free protease inhibitors (04693132001, Roche) as previously described [54]. Lysates were run on Novex pre-cast mini gels (NuPAGE 4-12% Bis-Tris Gels, NP0322BOX, Thermo Scientific) in either 1 x MES or 1 x MOPS buffer. Antibodies used for immunoblotting are detailed in Table 1.

4.9. Co-immunoprecipitation

Neurons in 10 cm dishes were lysed as described. Lysates were incubated with primary antibodies for c-Jun at a 1:50 dilution and rotated overnight at 4 °C. Lysates were incubated with 15 μl (30 μl bead slurry) Protein G, Sepharose (GE Healthcare, 17-0618-01) washed 3 times with lysis buffer and the beads finally incubated with 4 x Laemmli (containing mercaptoethanol) (Sigma, CLS8163-100 EA), washed 3 times with lysis buffer and the slurry) Protein G, Sepharose (GE Healthcare, 17-0618-01) for 2 h at 4 °C. Lysates were incubated with 15 μl (30 μl bead slurry) Protein G, Sepharose (GE Healthcare, 17-0618-01) for 2 h at 4 °C, centrifuged at 10,000 g for 20 min at 4 °C and the supernatant analysed by western blotting. Proteins were detected using a Fast SYBR Green Master mix (Applied Biosystems, 4385612) and GAPDH for normalization. Primers used were:

4.10. Glutathione assay

Total levels of glutathione were assayed using a colorimetric Glutathione Assay Kit (CS0260, Sigma). Briefly, at 14 DIV, primary neurons in 35 mm dishes were treated with DEM in transfection medium for 1, 4 and 24 h. Cells were then washed with PBS (4 °C), lysed with 5% sulfosalicylic acid and snap frozen in liquid nitrogen. Lysates were then defrosted at 37 °C, centrifuged at 10,000g for 10 min and 10 μl samples were used for the glutathione assay in accordance with the manufacturer’s instructions.

4.11. Quantitative PCR

Real-time quantitative PCR on the resulting cDNA was performed using a Fast SYBR Green Master mix (Applied Biosystems, 4385612) and gene specific primers. Relative expression of genes was determined by the 2-ΔΔCT method and Gapdh for normalization. Primers used were: Gapdh forward: 5′-AAACCCATCATCCATCTCCA-3′ and Gapdh reverse: 5′-GTTGGTCACACCACACTCACA-3′; c-Fos forward: 5′-AGAACCTCCGCAAGGGAAAGGAA-3′ and c-Fos reverse: 5′-ATGGGAGGA-GAGCCAGGTGTA-3′; Srxl1 forward: 5′-GAGCTCCTCTGGATCAAAG-3′ and Srxl1 reverse: 5′-GAGCGAATGTCCTCTCTG-3′.

4.12. Drosophila stocks and husbandry

Drosophila were raised on 4–24% instant Drosophila medium (Carolina Biological Supply Company, USA) supplemented with a yeast

| Table 1 | Antibodies used in this study. |
|---------|-------------------------------|
| Name    | Supplier                      | Dilution | Species | RRID          |
| Anti- GFP| Synaptic Systems              | 1:1000   | Guinea Pig | AB_11042617  |
| Anti- Synapsin 1 | MERCK           | 1:1000   | Rabbit | AB_2200400    |
| Anti- GCLC| SCBT                          | 1:500    | Mouse | AB_2736837    |
| Anti- GCLM| SCBT                          | 1:500    | Mouse | AB_831789     |
| Anti- Phospho p44/42 MAPK (Erk1/2) | Cell Signalling Technology | 1:1000   | Mouse | AB_2903212    |
| Anti- Phospho-c-Jun | Technology | 1:1000   | Rabbit | AB_2130162    |
| Anti- NeuN | Millipore                     | 1:2000   | Guinea Pig | AB_11205592   |
| Anti- c-Fos | Cell Signalling Technology (WB) | 1:2000   | Rabbit | AB_2106617    |
| Anti- GAPDH | Millipore                     | 1:10,000 | Mouse | AB_2107445    |
| Anti- JunB | SCBT                          | 1:1000   | Mouse | AB_2130203    |
| Anti- JunD | SCBT                          | 1:1000   | Mouse | AB_10650101   |
| Anti- SrxN-1 | SCBT                         | 1:250    | Mouse | AB_2286615    |
| Anti- ATP5A1 | ProteinTech                  | 1:1000   | Rabbit | AB_2061761    |
| Anti- p44/42 MAPK (Erk1/2) | Cell Signalling Technology (WB) | 1:1000   | Rabbit | AB_330744     |
| Anti- Phospho-p44/42 MAPK (Erk1/2) | Cell Signalling Technology | 1:1000   | Mouse | AB_331768     |
| Anti-Rabbit IgG | Life Technologies | 1:500    | Goat | AB_2576217    |
| Anti-Syraptotagmin (Anti-STT91) | Life Technologies | 1:500    | Goat | AB_10563566   |
| Anti-Horseradish peroxidase- Cy3 (HRP-Cy3) | Sweeney Lab | 1:2000 | Rabbit | AB_2713991    |
| Secondary Ab’s Anti-Guinea Pig IgG (H + L) | Immunoresearch Labs | 1:200    | Goat | AB_2307391    |
| Anti-affiniPure Goat | AffinityLab | 1:10,000 | Goat | AB_2337402    |

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survive solution (5% w/v inactivated yeast, 10% w/v sucrose in ddH2O, 100 g/500 ml) and maintained at 25 °C on a 12 h light/dark cycle. Prior to mixing with 4–24® instant media, vehicle (Ethanol) and DEM were added at the desired concentration (Ethanol 0.16%, DEM 0–10 mM). The following stocks were obtained from Bloomington Drosophila stock center: Canton-S (CS), w1118, UAS-βgalK53R (#9311), UAS-fasDN (#7214), UAS-junDN (#7217), Act5C-Gal4 (Actin-Gal4), nSyh-Gal4. UAS-ask1K618M (ask1DN) was a kind gift from Masayuki Miura [55]. UAS-wmdDN (wmdGD1) and hisDN9 were kind gifts from Aaron DiAntonio [11, 56], Iu260F/TM6b flies were obtained from Alfonso Martinez-Arias [57]. SpnGal4/TM6b was obtained from Daisuke Yamamoto [58]. The JNK dominant negative (bskK53R) [59] and the highwire mutant (hisDN9) have been described previously [18]. All analyses were performed in males. hisDN9 females were crosses to Canton-S males to produce male hisDN9 animals in an outcrossed background. For hisDN9 crosses with dominant negative transgenes, hisDN9, nSyh-Gal4 females were crossed with males carrying the UAS transgenes. All wild types were an outcross of Canton S to w1118.

### 4.13. Survival analysis

Hatched 1st instar larvae were collected and raised in standard food with different DEM concentrations (0 mM, 1 mM, 5 mM and 10 mM). For each survival experiment, at least 2 vials, each containing 50 larvae, were maintained until fly eclosion. The number of eclosed flies were recorded.

### 4.14. Immunohistochemistry and NMJ analysis

Third instar wandering larvae were dissected, fixed, antibody stained, imaged and analysed as described previously [13]. All NMJ analysis was performed double-blind. Primary antibodies detailed in Table 1. Confocal microscopy was performed using a Zeiss LSM 880 on an Axio Observer.Z1 invert confocal microscope (Zeiss). Z-stacked projections of NMJ's and VNCs were obtained using a Plan Neofluar 40x/0.75 NA oil objective. NMJ lengths were measured from stacked NMJ images using the ImageJ plugin for ImageJ (National Institutes of Health) as described previously [13, 54].

### 4.15. Antibodies

#### Author contributions

C.U., S.C., and S.T.S. designed research; C.U., N.G., S.C., and L.F. performed research; G.I.O.E. contributed reagents/analytic tools; C.U., S.C., and L.F. analysed data; and C.U., S.C., and S.T.S. wrote the paper.

#### Table 1 (continued)

| Name                  | Supplier          | Dilution | Species | RRID          |
|-----------------------|-------------------|----------|---------|---------------|
| Anti-Rabbit IgG (H + L) | Cell Signalling Technology | 1:5000 Mouse | Mouse | AB_1549606   |
| IP Ab's               |                   |          |         |               |
| Mouse Anti-rabbit IgG (Conformation Specific) | Cell Signalling Technology | 1:10,000 Horse | Horse | AB_320924   |
| (L27A9) mAb #3678     |                   |          |         |               |

#### Declaration of competing interest

The authors declare no competing interests.

#### Acknowledgments

This work was supported by BB SRC Project Grants (BB/1012273/1) awarded to STS and (BB/M002322/1) awarded to STS and SC, and was part-funded by The Wellcome Trust [204829] awarded to CU, through the Centre for Future Health (CFH) at the University of York. We thank Addgene (Cambridge, MA, USA.) for providing plasmid stocks. We thank Lucy Rudd for technical assistance and the Bioscience Technology Facility at the University of York for providing access to confocal microscopes.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101712.

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