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Induction of sulfiredoxin expression and reduction of peroxiredoxin hyperoxidation by the neuroprotective Nrf2 activator 3H-1,2-dithiole-3-thione

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Abstract

Peroxiredoxins are an important family of cysteine-based antioxidant enzymes that exert a neuroprotective effect in several models of neurodegeneration. However, under oxidative stress they are vulnerable to inactivation through hyperoxidation of their active site cysteine residues. We show that in cortical neurons, the chemopreventive inducer 3H-1,2-dithiole-3-thione (D3T), that activates the transcription factor Nrf2, inhibits the formation of inactivated, hyperoxidized peroxiredoxins following oxidative trauma, and protects neurons against oxidative stress. In both neurons and glia, Nrf2 expression and treatment with chemopreventive Nrf2 activators, including D3T and sulforaphane, up-regulates sulfiredoxin, an enzyme responsible for reducing hyperoxidized peroxiredoxins. Induction of sulfiredoxin expression is mediated by Nrf2, acting via a cis-acting antioxidant response element (ARE) in its promoter. The ARE element in Srxn1 contains an embedded AP-1 site which directs induction of Srxn1 by synaptic activity. Thus, raising Nrf2 activity in neurons prevents peroxiredoxin hyperoxidation and induces a new member of the ARE-gene family, whose enzymatic function of reducing hyperoxidized peroxiredoxins may contribute to the neuroprotective effects of Nrf2 activators.

Keywords

oxidative stress; neurodegeneration; phase II enzymes; chemoprevention; neuroprotection; thioredoxin

Introduction

Oxidative stress occurs due to an imbalance between production of reactive oxygen species (ROS) and the cell's capacity to neutralize them through its intrinsic antioxidant defences. Key among these is the thioredoxin-peroxiredoxin system which is an important reducer of oxidative stressors such as peroxides (Winyard et al. 2005). The thioredoxin system protects against H_2O_2-induced apoptosis, and its inhibition promotes oxidative stress and cell death (Yoshida et al. 2005). The thioredoxin-peroxiredoxin system detoxifies peroxides by transferring reducing equivalents from NADPH to peroxides via thioredoxin reductase, thioredoxin and finally peroxiredoxins (Prxs). Prxs are a ubiquitous family of peroxidases with cytoprotective and antioxidative effects (Immenschuh and Baumgart-Vogt 2005). The

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2-Cys Prxs is the predominant Prx subfamily, comprising Prx I-IV (Wood et al. 2003) and are implicated in protecting neuronal cells from Aβ toxicity (Yao et al. 2007), excitotoxicity (Hattori et al. 2003), oxygen-glucose deprivation (Boulos et al. 2007), peroxide (Sanchez-Font et al. 2003; Fang et al. 2007), and MPP^+ toxicity (Qu et al. 2007).

These Prxs contain a peroxidatic cysteine residue, oxidized by peroxides to cysteine sulfenic acid (−SOH), which then forms a disulfide bond with the resolving cysteine, which is in turn reduced by thioredoxin (Wood et al. 2003). Sometimes, Prx-SOH is further oxidized by peroxide to sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) acid, causing inactivation of peroxidase activity (Rhee et al. 2007). Prx-$SO_{2/3}H$ is not a substrate for the resolving cysteine and cannot be reduced by thioredoxin. Hyperoxidation takes place when there is not enough reduced Prx to deal with peroxides present, and is associated with oxidative neuronal death *in vitro*, and also ischemic brain damage *in vivo* (Papadia et al. 2008). Previously, hyperoxidation of Prx was thought to be irreversible. However, more recently it has been found that Prx-$SO_{2/3}H$ can be reduced back to the catalytically active thiol form in eukaryotic cells by the ATP-dependent reductase, sulfiredoxin (Biteau et al. 2003; Rhee et al. 2007; Jonsson et al. 2008). The activity of sulfiredoxin restores inactive Prxs back to the thioredoxin cycle and prevents permanent oxidative inactivation of Prxs by strong oxidative insults. Overexpression of sulfiredoxin has been shown to prevent Prx hyperoxidation in response to an oxidative insult (Woo et al. 2005). Conversely, knockdown of sulfiredoxin prevents reduction of Prx-SO_2H following a transient oxidative insult (Chang et al. 2004).

One known defence against oxidative insults is the induction of a group of genes encoding antioxidative and drug-metabolizing enzymes (also known as Phase II enzymes). These genes are induced by a variety of small thiol-active molecules including the potent chemopreventive agent 3H-1,2-dithiole-3-thione (D3T), as well as dietary phytochemicals such as Sulforaphane (Nguyen et al. 2004). Mild oxidative stress also induces these genes (Giudice and Montella 2006). Transcriptional regulation of this group of genes is mediated by a cis-acting promoter element termed the antioxidant response element (ARE), which recruits the transcription factor Nuclear factor erythroid 2-related factor (Nrf2) as a heterodimer with small Maf proteins (Zhang 2006). Nrf2 levels are constitutively low due to being targeted for degradation by Keap1. Under conditions of oxidative stress, Nrf2 degradation is slowed and Nrf2 accumulates in the nucleus and activates ARE-containing genes, with a net antioxidative effect (Nguyen et al. 2004). Small molecule activators of Nrf2 also act by interfering with Keap1-mediated degradation. Activation of Nrf2 and induction of ARE-driven defences is implicated in protecting against a variety of diseases in many organs and tissues, including autoimmune diseases, cancer, cardiovascular, neurodegenerative disease and ischemia (Lee et al. 2005; Shih et al. 2005; Giudice and Montella 2006; Zhang 2006). The ability of Nrf2 to exert a cytoprotective effect in neural cells has mainly been studied in the context of glia. In a mixed culture of neurons and glial cells, Nrf2 expression in glial cells can confer protection on a large number of normal non-overexpressing neurons (Shih et al. 2003; Kraft et al. 2004).

Here we find that oxidant-induced neuronal apoptosis can be prevented by expression of Nrf2 specifically in neurons, and also by the Nrf2 activator D3T. Furthermore, D3T strongly impairs oxidant-induced peroxiredoxin hyperoxidation in neurons. D3T induces expression of the enzyme responsible for reducing hyperoxidized peroxiredoxins, sulfiredoxin. We find that this induction is mediated at the transcriptional level and that sulfiredoxin is a new member of the Nrf2/ARE regulated gene family. Given its function, it may form part of the cytoprotective gene battery, the transcription of which is promoted by chemopreventive agents.

Materials and methods

Tissue culture and the induction of oxidative stress

Cortical rat neurons were cultured as described (Hardingham et al. 2002) from E21 rats except that growth medium contained B27 (Invitrogen). A single dose of anti-mitotic agent (AraC, 4.8 μM) was added to the cultures at DIV4 to minimize glial numbers. Experiments were carried out after being cultured for 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. Experiments were performed after transferring neurons at DIV8 into defined medium lacking trophic support "TMo" (Papadia et al. 2005): this is composed of 10% MEM (Invitrogen) and 90% Salt-Glucose-Glycine (SGG) medium (SGG: 114 mM NaCl, 0.219 % $NaHCO_3$, 5.292 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 1 mM Glycine, 30 mM Glucose, 0.5 mM sodium pyruvate, 0.1 % Phenol Red; osmolarity 325 mosm/l, hereafter TMo). D3T (10-25 μM) was applied to neurons 16 h prior to the application of an oxidative insult in the form of H_2O_2 (100 μM, stabilized solution: Sigma) . Neurons were fixed after a further 24 h and subjected to DAPI staining and cell death quantified by counting (blind) the number of apoptotic nuclei as a percentage of the total. Approximately 1500 cells were counted per treatment, across 4 independent experiments. Morphologically, peroxide-treated neurons show typical signs of apoptotic-like cell death (shrunken cell body and large round chromatin clumps).

Cultures of cortical glial cells were obtained by plating mixed neuronal/glial cultures at low density in 10% FBS /DMEM (no anti-mitotic). After 6 days the small number of neurons that remained were killed by 1 mM NMDA overnight. This procedure leaves >99% GFAP positive glial cells. HEK293 cells were maintained in 10% FBS/DMEM and passaged 1:5 every 4 days.

Transfection and following the fate of transfected cells

All transfections on all cell types were performed using Lipofectamine 2000 (Invitrogen). Neurons and glial cells were transfected at DIV8 in TMo (see above). Transfection efficiency was approximately 5 % for neuronal cultures. It was found that more than 99% of eGFP-expressing transfected neurons were NeuN-positive, and <1% were GFAP positive (see results) confirming their neuronal identity. For monitoring the fate of Nrf2-expressing neurons, peGFP was used to track the fate of transfected neurons expressing the plasmid of interest (Nrf2 vs. globin control). To ensure that GFP-positive neurons were also expressing the plasmid of interest, a favorable ratio was used (peGFP: plasmid of interest, 1:2). Coexpression at this ratio was confirmed in the case of pRFP (Papadia et al. 2008). Pictures of GFP-expressing neurons were taken using a Leica AF6000 LX imaging system, with a DFC350 FX digital camera. Neurons were then treated with 100 μM H_20_2 and images of the same cells were taken 24 h after H_2O_2 exposure. Cell death was assessed by counting the number of surviving GFP-positive neurons pre- and post- exposure to H_20_2. In the vast majority of cases, death was easily spotted as an absence of a healthy GFP-expressing cell where one once was. In place of the cell, there was in most cases (>90%) evidence of death in the form of fragmented neurites, fluorescent cell debris, and an apoptotic nucleus. This confirmed that the cells were genuinely dying as opposed to more unlikely scenario such as peroxide-induced quenching of eGFP fluorescence in a sub-population of neurons. This is also underlined by the fact that death measured by this technique is blocked by caspase inhibitors (Papadia et al. 2008). For each plasmid/condition, the fate of approximately 150 neurons was monitored over 3 independent experiments.

Plasmids and reporter assays

Srxn1-Luc (Papadia et al. 2008) was subjected to site-directed mutagenesis of its ARE with the QuikChange II XL site-directed mutagenesis kit (Stratagene), using the oligonucleotide 5′-acctgcaaactcaccctgggcccgcgacccgacgcgtc-3′ and its reverse-complementary sequence. The mutation was verified by sequencing. ARE-Luc (Numazawa et al. 2003), pcDNA3.1/V5HisBmNrf2 and pcDNA3.1/V5HisCmKeap1 (McMahon et al. 2003) and pEF-Nrf2 (Kotkow and Orkin 1995) have been described previously. Firefly luciferase-based reporter gene constructs (*Srxn1*-Luc and its ARE mutated variant were transfected along with a renilla expression vector (pTK-RL), and also, where relevant, other expression vectors. Luciferase assays were performed 30 h post-transfection using the Dual Glo assay kit (Promega) with Firefly luciferase-based reporter gene activity normalized to the Renilla control (pTK-RL plasmid) in all cases.

Immunocytochemistry, Western blotting and antibodies

Immunofluorescence was performed as described (Mckenzie et al. 2005). Antibodies (Sulfiredoxin-S17, 1:500, Santa Cruz; NeuN, 1:15 Chemicon; GFAP, 1:1000, Sigma) were incubated with fixed cells overnight and visualized using biotinylated secondary antibody/cy3-conjugated streptavidin. Nuclei were counter-stained with DAPI. Pictures were taken on a Leica AF6000 LX imaging system, with a DFC350 FX digital camera. For western blotting, 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 %; β-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 1 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-$SO_{2/3}$H (1:1000, Abcam), Sulfiredoxin (P16, 1:250, Santa Cruz), β-tubulin isotype III (1: 125000, Sigma). 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 analyses involved normalizing to a loading control (β-tubulin or Prx).

RNA isolation and qPCR

RNA was isolated using the Qiagen RNeasy kit (including DNAse treatment) following disruption of cells (QiaShredder column). cDNA was synthesized from 1-3 μg RNA using the Stratascript QPCR cDNA Synthesis kit. Dilutions of this cDNA were used for real-time PCR (cDNA equivalent to 6 ng of initial RNA per reaction for *Srxn1*; 3 ng equivalent for GAPDH). qPCR was performed in an Mx3000P QPCR System (Stratagene) using Brilliant SYBR Green QPCR Master Mix. No-template and no-RT negative controls were included. Primers used: *Srxn1*–F: 5′-GACGTCCTCTGGATCAAAG-3′ 200 nM, -R: 5′-GCAGGAATGGTCTCTCTCTG-3′ 200 nM; GAPDH –F: 5′-GGGTGTGAACCACGAGAAAT-3′ 200 nM, -R: 5′- CCTTCCACAATGCCAAAGTT-3′ 100 nM. 18s rRNA – F: 5′- GTGGAGCGATTTGTCTGGTT -3′, -R: 5′ – CAAGCTTATGACCCGCACTT – 3′. *Sesn2* F: 5′- GGATTATACCTGGGAAGACC -3′ 200 nM, -R: 5′- CGCAGTGGATGTAGTTCC-3′ 200 nM. *Hmox1* F: 5′-AGCACAGGGTGACAGAAGAG -3′ 200 nM, -R: 5′ GGAGCGGTGTCTGGGATG -3′. The data were analysed using the MxPro QPCR software (Stratagene). Expression of the *Srxn1* was normalized to GAPDH.

Results

Neuronal Nrf2 expression, and the Nrf2 activator D3T, prevent oxidative neuronal death

NRF2 overexpression in glial cells can confer protection on a large number of normal non-overexpressing neurons (Shih et al. 2003; Kraft et al. 2004). It is not clear whether NRF2 can act solely in neurons to confer neuroprotection against oxidative insults. We analyzed the neuroprotective effect of boosting Nrf2 activity specifically in neurons, using a neuron-specific transfection protocol previously employed (Papadia et al. 2008) and briefly described below. Cortical 'neuronal' cultures naturally contain a small percentage of glial cells (5-10%). Transfection of these cultures results in tiny numbers of transfected glial cells, fewer than the 5-10% expected if both cell types were equally amenable to transfection (Papadia et al. 2008). To quantify this, mixed cultures were transfected with eGFP and subjected to NeuN immunofluorescence. Of 189 eGFP-expressing cells, 186 were NeuN-positive. We then transfected cultures again with eGFP but this time stained for GFAP. Of 271 eGFP-expressing cells, 0 (zero) were GFAP-positive (see example pictures in Fig. 1a). Thus, nearly all transfected cells in neuronal cultures are neurons, potentially due the fact that quiescent (AraC-treated) glial cells are not amenable to transfection (NB. Non-quiescent glial cells are amenable to transfection-see below).

We transfected the Nrf2-expressing constructs into neurons, along with an eGFP co-transfection marker to enable us to monitor the fate of the neurons (a technique previously described (Papadia et al. 2008)). Pictures of eGFP-expressing neurons were taken 24 h post-transfection, after which neurons were treated with an oxidative insult (100 μM H_2O_2). After a further 24 h pictures of the same cells were taken, before the cultures were fixed and nuclear integrity assessed by DAPI staining. By monitoring the neurons before and after H_2O_2 treatment, we found that neurons expressing Nrf2 were completely resistant to cell death following H_2O_2 treatment (100 μM, Fig. 1b, see example pictures in 1d). We also studied the fate of neurons within a 150 μm radius of Nrf2-transfected neurons and found that susceptibility of neurons to death by H_2O_2 treatment was not changed by being in close proximity to a Nrf2-expressing neuron (Fig. 1c), in contrast to the reported non-autonomous effects of Nrf2-expressing glial cells (Shih et al. 2003).

We next investigated the influence of known activators of Nrf2 activity on cortical neuronal vulnerability to an oxidative insult (H_2O_2) *in vitro*. We tested 3H-1,2-dithiole-3-thione (D3T), sulforaphane and *tert*-butylhydroquinone (tBHQ). The drugs were applied at a wide range of concentrations for 16 h prior to the application of H_2O_2 (for further 24 h). The protection that was achieved by these inducing agents varied: D3T conferred significant and strong neuroprotection (Fig. 1e), whereas tBHQ and sulforaphane were significantly less potent at the range of doses tested (data not shown). Further studies using D3T revealed that increased neuroprotection at a lower dose (10 μM) could be achieved by applying a second identical dose of the compound immediately prior to the oxidative insult (Fig. 1e).

D3T prevents the thioredoxin-peroxiredoxin system from becoming overwhelmed by oxidative stress

The existence of hyperoxidized Prx-$SO_{2/3}$H in neurons indicates an overwhelmed thioredoxin-peroxiredoxin system, and its formation is associated with oxidative neuronal death (Papadia et al. 2008). We therefore investigated whether the neuroprotective effects of D3T were associated with changes in Prx-$SO_{2/3}$H levels. Western analysis using a Prx-$SO_{2/3}$H-specific antibody revealed that H_2O_2 caused Prx hyperoxidation in vehicle (control)-treated neurons, while D3T-treated neurons displayed far less hyperoxidation (Fig. 1f). The strong Prx-$SO_{2/3}$H band represents hyperoxidized PrxII (Papadia et al. 2008), although a higher exposure of the blot reveals hyperoxidation of the upper band identified as

Prx III (Papadia et al. 2008) which also appears to be weaker in D3T-treated neurons (Fig. 1f). Therefore, D3T pre-treatment renders neurons resistant to oxidative stress and inhibits the appearance of hyperoxidized Prx protein.

Sulfiredoxin expression is induced in glia and neurons by activators of Nrf2

Because D3T-treated neurons displayed lower levels of Prx-$SO_{2/3}H$ in response to H_2O_2 treatment (Fig. 1f), we studied the transcriptional regulation of the two genes whose products mediate reduction of Prx-$SO_{2/3}H$: *sulfiredoxin* (*Srxn1*, (Rhee et al. 2007)) and *sestrin2* (*Sesn2* (Budanov et al. 2004)). We found that D3T induced transcription of *Srxn1*, producing an increase at both the mRNA and protein level in cortical neurons (Fig. 2a, 2b). In contrast, D3T did not induce expression of Sesn2. Other Nrf2 activators (tBHQ and sulforaphane) also promoted expression of Srxn1 but not Sesn2 (Fig. 2a) strongly suggesting that *Srxn1* is a Nrf2 target gene.

Since our cortical neuronal cultures contain 5-10% of glial cells (Papadia et al. 2008) we wanted to know whether these Nrf2 activators are promoting Srxn1 expression in neurons or glia or both. We performed experiments comparing Srxn1 expression in pure glial cultures (>98% GFAP-positive, Fig. 2c) with that of our mixed cultures. Expression of *Srxn1* within the neuronal cultures was not significantly different to that in glial cultures (normalizing to 18S rRNA levels, data not shown). Therefore, in our neuronal cultures, the 5-10% of glial cells by number represent a similar percentage of total *Srxn1* mRNA. We found that D3T induced *Srxn1* expression in pure glial cultures at 4 h by around 6-fold (Fig. 2d), similar to the level of induction of *Hmox1* (a known Nrf2-regulated gene, Fig. 2d). Assuming that the glial cells within the neuronal cultures respond similarly, induction of *Srxn1* in glial cells alone cannot account for the 3.5-fold induction of *Srxn1* by D3T in the neuronal cultures. The 3.5-fold induction observed can only be explained if D3T can induce *Srxn1* in the neurons as well as glial cells (possibly by around 3.2-fold).

Nrf2 directly regulates *Srxn1* expression via a cis-acting ARE

We next investigated whether *Srxn1* is directly regulated by Nrf2. Nrf2 acts by binding the cis-acting antioxidant response element (ARE) in the promoters of target genes. Analysis of the rat *Srxn1* promoter revealed a sequence at −188 relative to the transcription start site (TCACCCTGAGTCAGCG) which resembled an ARE (Nioi et al. 2003), and was conserved in the gene from various mammalian species (Fig. 3a). To test whether this putative element is functional we created a luciferase reporter of the *Srxn1* promoter containing 585 nt of 5′ promoter sequence (from −577 to +8). We then analysed the effect of Nrf2 expression on activity of this reporter. We first used primary cortical neuronal cultures and found that driven Nrf2 expression induced *Srxn1*-Luc reporter gene activity (Fig. 3b). Furthermore, mutation of the core of the putative ARE (to TCACCCTG**G**G**C**C**C**GCG, *Srxn1*(mut)-Luc) abolished Nrf2-responsiveness (Fig. 3b). The capacity of Nrf2 to induce *Srxn1*-Luc was similar to its capacity to induce an artificial ARE-Luc reporter (Numazawa et al. 2003), generated by placing AREs from the Heme oxygenase-1 (*Hmox1*) promoter upstream of the luciferase open reading frame (Fig. 3c). This indicates that the *Srxn1* putative ARE is a bona fide Nrf2-responsive element, and is similarly responsive to Nrf2 as the *Hmox1* AREs.

To test whether endogenous Srxn1 was also induced by Nrf2, we overexpressed Nrf2 in neurons and performed immunocytochemistry with an appropriate antibody. The transfected neurons exhibited elevated levels of Srxn1 (Fig. 3d), confirming that the endogenous gene is also Nrf2-responsive. We next investigated whether the *Srxn1* ARE was responsive to Nrf2 in other cell types, since *Srxn1* expression is induced in glial cells by D3T as well as neurons (Fig. 2). Nrf2 induced *Srxn1* promoter activity in glial cells (Fig. 3e) to a similar

degree as it activated ARE-Luc (Fig. 3f). We also observed Nrf2-responsiveness of the *Srxn1* ARE in HEK293 cells (Fig. 3g) confirming the ARE's functionality in several cell types.

Nrf2 controls the basal expression of *Srxn1* in glia but not in neurons

To determine whether Nrf2 controls basal levels of *Srxn1* expression in neurons and glia, we first looked at the relative basal activity of *Srxn1*-Luc and *Srxn1*(mut)-Luc in both neurons and glia (Fig. 3b and 3f). Mutation of the ARE sequence within the construct had only a modest effect on basal Srxn1 promoter activity in neurons (Fig. 3b) but reduced basal promoter activity in glia by around 80% (Fig. 3f), suggesting that glial *Srxn1* mRNA levels rely on Nrf2 activity. However, the *Srxn1* ARE contains an embedded AP-1 like sequence, so basal activity could be due to AP-1 activity rather than Nrf2. To investigate this further, we examined the effect of suppressing basal Nrf2 activity by expressing Keap1, an endogenous repressor of Nrf2 which targets Nrf2 for proteosomal degradation (McMahon et al. 2003; Zhang 2006). Keap1 overexpression suppressed basal ARE-Luc expression in glial cells but not in neurons (Fig. 4a,b). In both glia and neurons, Keap1 impaired ARE activity induced by Nrf2 expression (Fig. 4a,b), confirming that Keap1 was functioning properly in neurons. These experiments indicate that basal Nrf2 activity is relatively low in neurons compared to glia consistent with other studies (Shih et al. 2003). Analysis of *Srxn1* reporter activity revealed that Keap1 effectively suppressed basal glial *Srxn1* expression but failed to affect neuronal basal *Srxn1* reporter activity (Fig. 4c,d), consistent with normal constitutive levels of Nrf2 activity in neurons being low. This indicates that factors other than Nrf2 are responsible for maintaining basal levels of sulfiredoxin in neurons.

Activity-dependent induction of *Srxn1* is largely independent of Nrf2

We reported previously that synaptic activity strongly induces Srxn1 via the two AP-1 sites at −188 and −239 (Papadia et al. 2008). The proximal AP-1 site identified in this earlier study is in fact contained within the core of the ARE identified here. This raises the possibility that some of the activity-dependent induction of Srxn1 is actually due to Nrf2. We used an established method of network disinhibition to enhance synaptic activity, applying the $GABA_A$ receptor antagonist bicuculline to induce bursting, and the K^+ channel antagonist 4-aminopyridine (which enhances burst frequency, hereafter BiC/4-AP (Hardingham et al. 2001)). We analyzed the effect of Keap1 on activity-dependent induction of the *Srxn1* promoter and found a small but significant reduction after 8 h stimulation (Fig. 5a), but not at 24 h stimulation (the time-point used in our previous study (Papadia et al. 2008)). By comparison we found that induction of a reporter of Sesn2, which is also induced by synaptic activity, was not inhibited by Keap1 at either time-point (Fig. 5a). To test this further, we investigated the degree to which activity-dependent induction of the composite ARE/AP-1 site is dependent on AP-1. We utilized expression of TAM67, a dominant negative form of c-jun which interferes with AP-1 mediated gene expression. Activity-dependent induction of a reporter containing 5 tandem AP-1 sites is inhibited by 65% by TAM67 (Fig. 5b). Induction of the mutant *Srxn1* reporter dependent on the distal AP-1 site (i.e. with the composite ARE/AP-1 site mutated) was inhibited by 55% (Fig. 5b), similar to inhibition of a pure AP-1 reporter. In contrast, induction of the mutant *Srxn1* reporter dependent on the composite ARE/AP-1 site (i.e. with the distal AP-1 site mutated) was inhibited by only 30% (Fig. 5b). Thus, our data indicate that at early time-points the activity-dependent induction of the composite ARE/AP-1 site is partly dependent on AP-1, and is also partly dependent on Nrf2.

Discussion

This study provides evidence that activation of the Nrf2 pathway specifically within neurons can antagonize H_2O_2-stimulated apoptosis. We also show that activation of this pathway can prevent hyperoxidation of peroxiredoxins that occurs in neurons upon exposure to an oxidative insult. Also we have found that expression of sulfiredoxin, an enzyme that reduces hyperoxidized peroxiredoxins, is regulated by Nrf2 in neurons and other cell types. Thus, expression of sulfiredoxin may contribute to the antioxidative effects of chemopreventive agents that activate Nrf2.

Reactivation of peroxiredoxins by sulfiredoxin

Prxs are a family of thiol-based antioxidants that protect against damage caused by oxidative stressors. PrxII protects cortical neurons against Aβ toxicity (Yao et al. 2007) and oxygen-glucose deprivation (Boulos et al. 2007). Interfering with PrxII expression renders neuroblastoma cells vulnerable to oxidative stress (Sanchez-Font et al. 2003), and renders cortical neurons vulnerable to MPP^+ (Qu et al. 2007). PrxIII protects hippocampal neurons against excitotoxicity (Hattori et al. 2003). Thus, molecular events that render Prxs inactive *in vivo* in pathological scenarios may contribute to disease progression. We have previously observed Prx-inactivating Prx-$SO_{2/3}$H formation following cerebral ischemia *in vivo* (Papadia et al. 2008). It remains to be seen whether Prx hyperoxidation occurs in more chronic neurodegenerative diseases associated with oxidative stress.

Sulfiredoxin was initially characterized in yeast (Biteau et al. 2003) and then in mammalian cells (Rhee et al. 2007). It acts by catalysing the ATP-dependent formation of a sulfinic acid phosphoric ester on Prx (Rhee et al. 2007) which is then reduced by thiol equivalents such as thioredoxin. Interfering with Srxn1 expression impairs the ability of cells to reduce hyperoxidized Prxs, while overexpression of Srxn1 enhances it (Chang et al. 2004; Woo et al. 2005). Our finding that Srxn1 expression can be induced by small thiol-active molecules that activate Nrf2 suggests that exposure to such inducers may increase the level of peroxides that a cell can tolerate before Prx is inactivated by hyperoxidation. Given the antioxidant and cytoprotective effects of Prxs, induction of sulfiredoxin expression may be a significant contributor to the net effects of Nrf2 activation. It will be of considerable interest to see whether Nrf2 activators are able to reduce Prx-$SO_{2/3}$H formation following cerebral ischemia *in vivo*. This seems possible, since pre-administration of the Nrf2 activators tBHQ and sulforaphane can protect the brain from cerebral ischemia *in vivo* (Shih et al. 2005; Zhao et al. 2006). According to our observations, D3T is a superior neuroprotectant, so it will be of interest to know whether it is an efficient protector against ischemic injury.

Nrf2 is a therapeutic target for many disorders

Up-regulation of Nrf2 activity in Drosphila increases lifespan and resistance to oxidative stress (Sykiotis and Bohmann 2008). In mammals, gene expression programs induced by Nrf2 can protect many different organs against a variety of traumas. For example, Nrf2 protects lung against hyperoxic injury (Cho et al. 2002), and the liver against paracetamol-induced hepatotoxicity (Enomoto et al. 2001). Nrf2 is the primary molecular target of cancer chemopreventive blocking agents (Giudice and Montella 2006) which in general act by preventing carcinogens from forming adducts with DNA that lead to mutations. The boosting of intrinsic antioxidant defences of the cell by activation of Nrf2 is believed to be an important mediator of these protective effects, as is the co-ordinated induction of detoxification enzymes such as glutathione S-transferases and NAD(P)H:quinone oxidoreductase 1. The program of gene expression induced by NRF2 is well-placed to combat the actions and the production of a variety of free radicals. For example, the NRF2 target gene Ferritin sequesters Fe(II) and can thus restrict hydroxyl radical-generating

Fenton chemistry. Also, the NRF2 target gene Hmox1 degrades the pro-oxidant heme molecule, generating bilirubin as a breakdown product. Bilirubin can react directly with, and neutralize, superoxide, hydroxyl and peroxynitrite radicals (Stocker 2004). The actions of these, and other, gene products, in concert with those involved in the thiol-based antioxidant systems result in a powerful and general upregulation of antioxidant defenses (Lee et al. 2005).

In the central nervous system, Nrf2 has been suggested to be a therapeutic target in excitotoxic disorders such as stroke and seizure, as well as neurodegenerative diseases (Lee et al. 2005; Shih et al. 2005). While Nrf2 over-expressing glial cells strongly protect surrounding untransfected neurons (Shih et al. 2003; Kraft et al. 2004; Jakel et al. 2007), it was not clear whether Nrf2 can act solely in neurons to confer neuroprotection against oxidative insults. The electrophile NEPP11 induces Nrf2 preferentially but not exclusively in neurons (Satoh et al. 2006) and protects against models of NMDA receptor-dependent excitotoxicity. This suggested that Nrf2 can act in neurons, but contributions from glial cells could not be ruled out. Our results show that Nrf2 is strongly neuroprotective in neurons and that neuroprotection need not rely on glial Nrf2 expression.

The neuroprotective effect of Nrf2 places the many known activators of Nrf2 as potential lead compounds. We have found that the ability of different agonists of Nrf2 to induce *Srxn1* and *Hmox1* expression in neurons is not matched by their neuroprotective effects. In our hands, D3T is a far superior neuroprotectant than sulforaphane or tBHQ (GEH unpublished). Oltipraz, a structural relative of D3T, has also found to be protective (GEH unpublished). We hypothesize that in our particular system, tBHQ and sulforaphane induce Nrf2 target genes only at a dose that may exert significant toxicity, hence their relative lack of efficacy. This toxicity is likely due to unspecified off-target effects. Thus, while activators of Nrf2 remain attractive candidates for acute and chronic neurodegenerative disorders associated with oxidative stress, the therapeutic window of each molecule is of paramount importance with regard to its direct efficacy, even before pharmacodynamic and pharmacokinetic factors are considered.

The AP-1/ARE composite site in the Srxn1 promoter can integrate multiple signals

Our observations here and elsewhere (Papadia et al. 2008) identify *Srxn1* as a gene regulated by both AP-1 (via two phorbol 12-O-tetradecanoate 13-acetate (TPA)-response element (TRE) sites) and Nrf2 (one ARE site). Moreover, the proximal AP-1 site is contained within the ARE sequence. Several AREs such as those in the promoters of human *NQO1* and *HMOX1* contain AP-1 like sequences and can respond to AP-1-activating stimuli, as well as Nrf2 (Nguyen et al. 2004). The composite ARE/AP-1 site on the Srxn1 promoter enables it to respond both to small molecule Nrf2 inducers such as D3T and tBHQ, as well as to synaptic activity (mainly via AP-1). Induction of Srxn1 by synaptic activity contributes to the protection of cortical neurons by synaptic activity in the face of an oxidative insult (Papadia et al. 2008). It is an intriguing possibility that the protective effects of synaptic activity and Nrf2 inducers may have a partially overlapping mechanism: induction of certain genes containing ARE/AP-1 composite sites.

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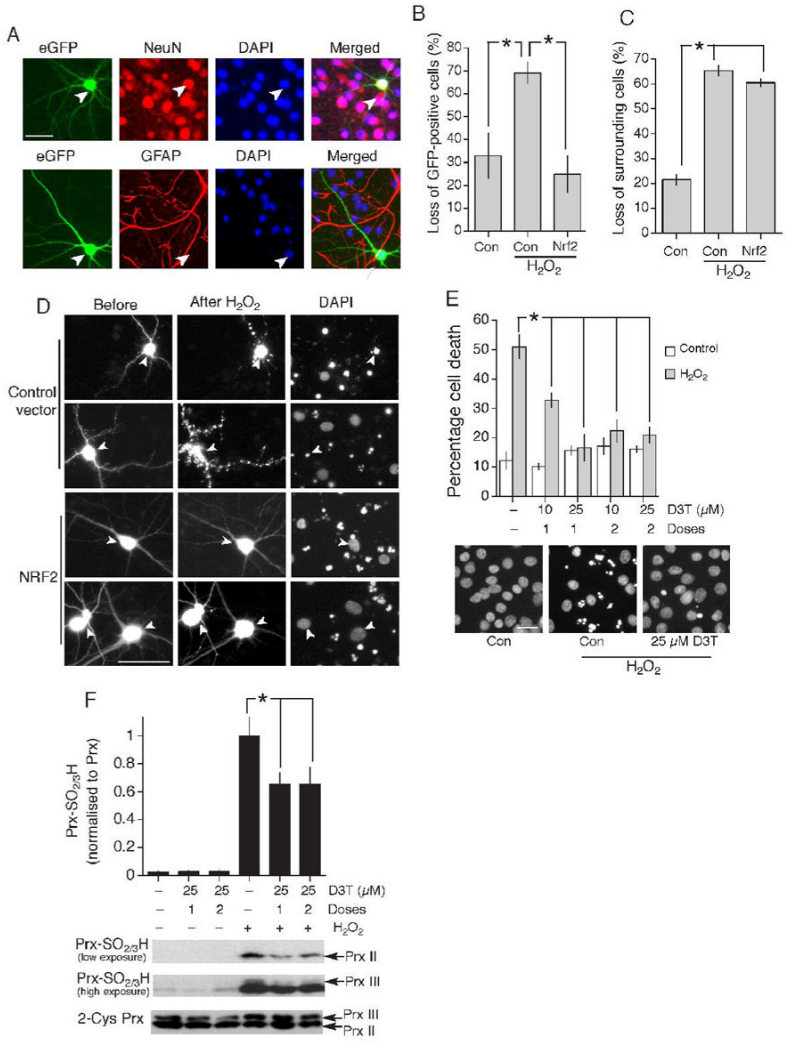

Fig. 1. Neuronal Nrf2 expression and the Nrf2 activator D3T protects neurons against oxidative stress; D3T inhibits the formation of inactivated, hyperoxidized peroxiredoxins following oxidative trauma

A) Upper: example of an eGFP expressing cell immuno-positive for NeuN. Lower: example of an e-GFP expressing cell immuno-negative for GFAP. Scale bar = 30 μm. B) Effect of expressing Nrf2 in cortical neurons on H_2O_2-induced neuronal death. The neurons were transfected with control vector or pNrf2, plus an eGFP expression vector to monitor cell fate (see methods). 24 h post transfection neurons were treated where indicated with H_2O_2 (100 μM here and throughout the study) and cell fate monitored after a further 24 h. For each vector/treatment, approx. 150 cells were studied across 3 independent experiments.*$p<0.05$ Bonferonni two-tailed paired T-test. C) Effect of expressing Nrf2 in cortical neurons on survival of nearby cells in response to H_2O_2-treatment. Survival/death of neurons within a 150 μm radius of Nrf2-expressing neurons was analyzed by assessing nuclear morphology of DAPI stained fixed cells. As can be seen in Fig. 1d, healthy neurons have large nuclei with diffuse DAPI staining. Apoptotic nuclei are characterised by pyknotic brightly stained clumps of condensed chromatin. Survival/death of over 1000 cells was scored across 3 independent experiments. *$p<0.05$ Bonferonni two-tailed paired T-test (n=3). D) Example pictures relating to the data shown in 1b. Arrows point to the transfected cells identified by co-expression of eGFP. Pictures before and after H_2O_2 treatment are taken of the cell, and

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DAPI stained images are also taken post-treatment. Scale bar = 30 μm E) Cell death due to 24 h H_2O_2 insult in the face of the indicated treatments. (Lower) Examples pictures, scale bar=25 μm. *p<0.05 Bonferonni two-tailed paired T-test (compared to control H_2O_2 treated, n=4), mean ± s.e.m. shown in this and all cases. F) Western analysis of Prx hyperoxidation using an anti-Prx$SO_{2/3}$H specific antibody. Two exposures are shown for optimal visibility of hyperoxidized Prx II and Prx III respectively. Analysis of Prx$SO_{2/3}$H levels is restricted to Prx II, and is normalized to total Prx II expression. *p<0.05 Bonferonni two-tailed paired T-test (compared to control, H_2O_2-treated neurons, n=7).

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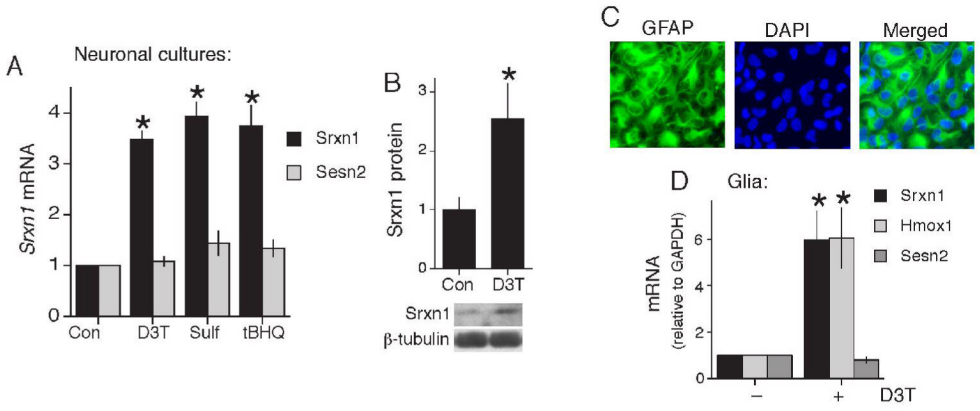

Fig. 2. Chemopreventive inducers of endogenous Nrf2 induce *Srxn1* expression in neurons and glia

A) Neuronal cultures were treated with D3T (25 μM), tBHQ (10 μM, Aldrich) or sulforaphane (5 μM, Merck Biosciences) for 4 h followed by RNA extraction and q-RT-PCR analysis of *Srxn1* and *Sesn2* (normalized to GAPDH). *p<0.05 (Bonferonni two-tailed T-test, n=3-5). B) Western analysis of Srxn1 protein expression in extracts taken from neuronal cultures treated for 24 h with D3T (25 μM). *p<0.05 (n=6). C) Glial cultures were treated with D3T (25 μM) for 4 h followed by RNA extraction and q-RT-PCR analysis of *Srxn1*, *Sesn2* and *Hmox1* (normalized to GAPDH, *p<0.05, *two-tailed T-test in this and subsequent experiments unless otherwise stated*, n=6).

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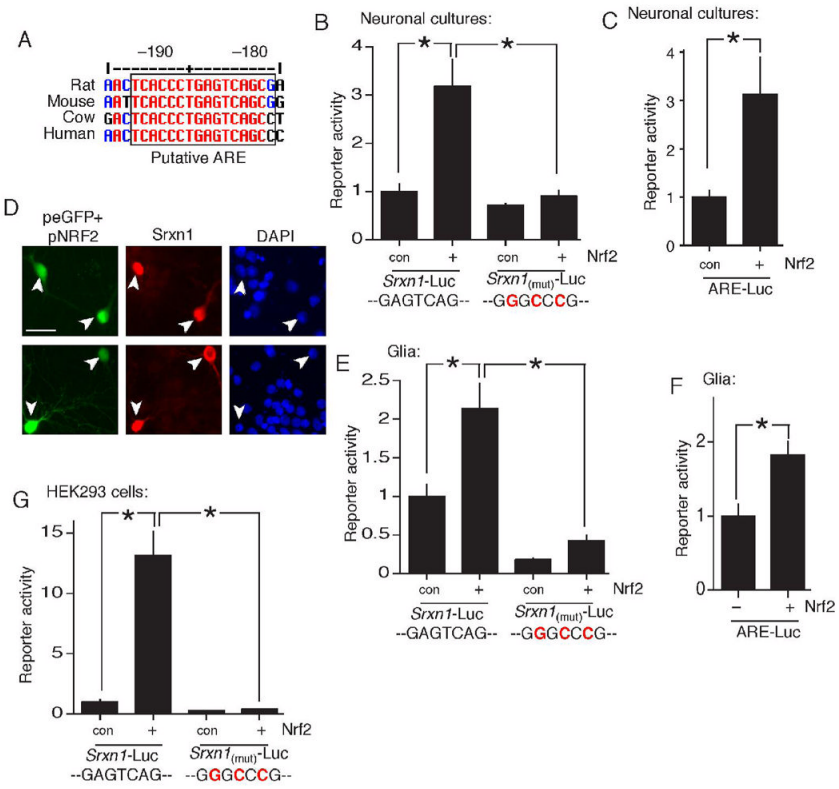

Fig. 3. The *Srxn1* promoter contains an ARE which is induced by Nrf2 expression in cortical neurons, as is the endogenous gene

A) Schematic showing the putative ARE within mammalian *Srxn1* promoters. B) Effect of Nrf2 expression on wild-type and mutant *Srxn1*-Luc reporters (normalized to Renilla control-see methods). Neurons were transfected with *Srxn1* reporter plus pcDNA3.1-Nrf2 or pGlobin control plasmid. *NB. In this and all experiments "con" denotes pGlobin control vector.* *p<0.05 (2-tailed paired T-test, n=3-5). C) Effect of Nrf2 expression on ARE-Luc activity. *p<0.05 (n=5). D) Effect of transfecting pEF-Nrf2 on endogenous Srxn1 expression. Neurons were transfected with the indicated vectors and after 24 h subjected to immunocytochemical analysis of sulfiredoxin expression. Immunofluorescence performed as described (McKenzie et al. 2006). Scale bar = 40 μm. E) Effect of Nrf2 expression on WT and mutant *Srxn1*-Luc reporters in glial cells *p<0.05 (n=7). F) Effect of Nrf2 expression on ARE-Luc activity in glial cells. *p<0.05 (n=4). G) Effect of Nrf2 expression on WT and mutant *Srxn1*-Luc reporters in HEK293cells. *p<0.05 (n=3).

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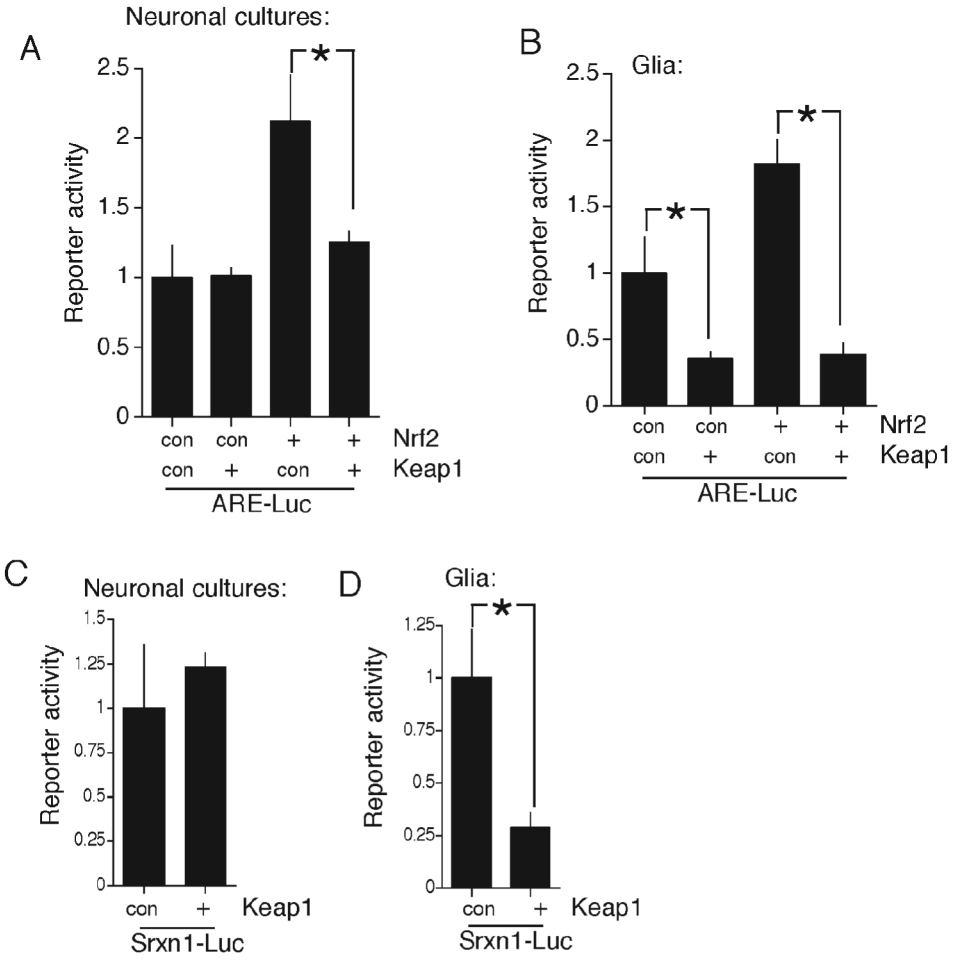

Fig. 4. Basal levels of *Srxn1* promoter activity are sensitive to Keap1 expression in glia but not neurons

A,B) Effect of Keap1 expression on basal and Nrf2-driven ARE-Luc reporter activity in neurons (A) and glia (B). Cells were transfected with ARE-Luc plus pcDNA3.1-Nrf2 (or pGlobin), plus pcDNA3.1-Keap1 (or pGlobin). *p<0.05 (n=4-6). C,D) Effect of Keap1 expression on basal *Srxn1*-Luc reporter activity in neurons (C) and glia (D). *p<0.05 (n=4).

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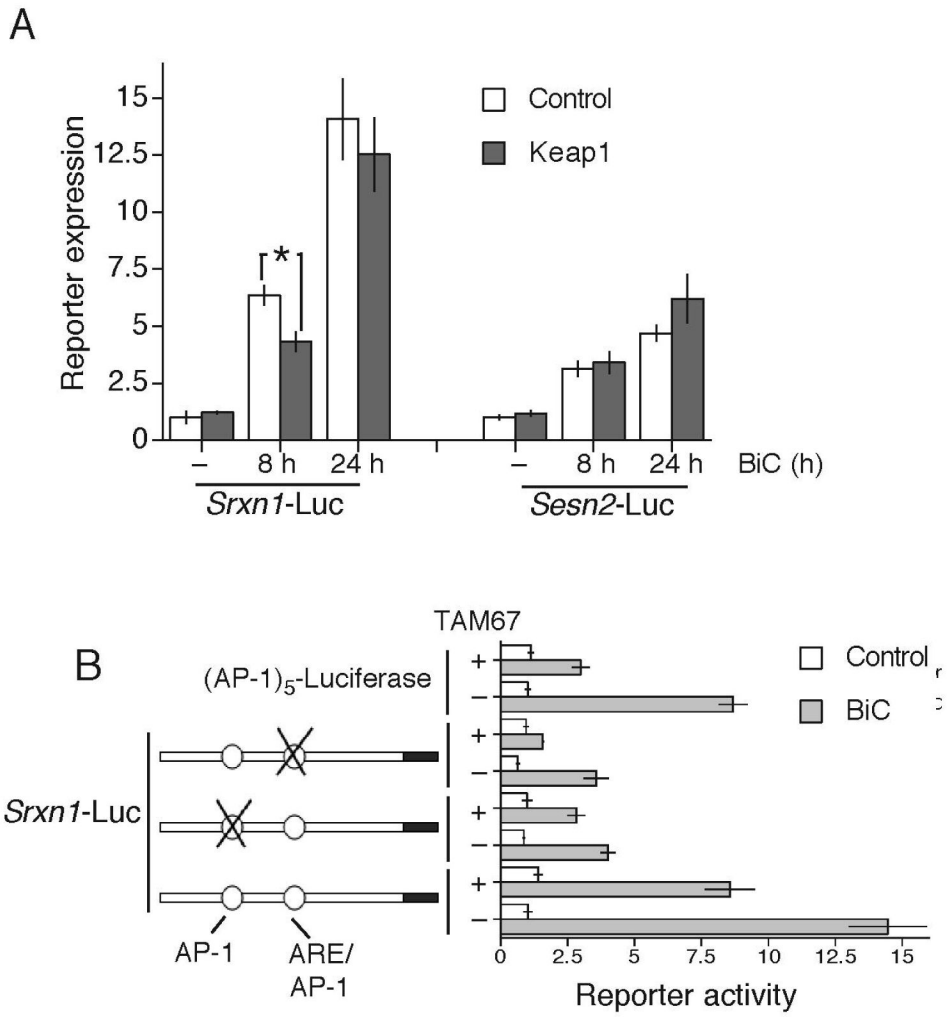

Fig. 5. Activity-dependent induction of Srxn1 via the ARE/AP-1 site is primarily mediated by AP-1

A) Effect of Keap1 expression on activity-dependent induction of reporters of Srxn1 and Sesn2. Neurons were transfected with the reporter plus TK-driven Renilla control vector. 24 h post-transfection, bursts of action potential firing were induced by treatment of neurons with 50 μM bicuculline, and burst frequency was enhanced by addition of 250 μM 4-amino pyridine (Hardingham et al. 2001). Reporter activity was assayed after the indicated time periods. *$p<0.05$ Bonferonni two-tailed paired T-test (compared to control, BiC/4-AP-stimulated level, n=6). B) Effect of an interfering mutant of AP-1 (TAM67) on activity-dependent induction of the putative ARE/AP-1 composite site, compared to an upstream AP-1 site. Neurons were transfected with the indicated reporter constructs, plus pTK-RL and either control vector or expression vector for TAM67. 24 h post-transfection neurons were stimulated with BiC/4-AP overnight prior to assay of reporter activity (n=5).