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**The control of anti-apoptotic and antioxidant pathways
in neural cells**

by

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Abstract

Oxidative stress is a feature of many chronic neurodegenerative diseases as well as a contributing factor in acute disorders including stroke. Fork head class of transcription factors (Foxos) play a key role in promoting oxidative stress-induced apoptosis in neurons through the upregulation of a number of pro-apoptotic genes. Here I demonstrate that synaptic NMDA receptor activity not only promotes Foxo nuclear exclusion but also suppresses the expression of Foxo1 in a PI3K-dependent fashion. I also found that *Foxo1* is in fact, a Foxo target gene and that it is subject to a feed-forward inhibition by synaptic activity, which is thought to result in longer-term suppression of Foxo downstream gene expression than previously thought. The nuclear factor (erythroid 2-related) factor 2 (Nrf2) is another transcription factor involved in oxidative stress and the key regulator of many genes, whose products form important intrinsic antioxidant systems. In the CNS, artificial activation of Nrf2 in astrocytes has been shown to protect nearby neurons from oxidative insults. However, the extent to which Nrf2 in astrocytes could respond to *endogenous* signals such as mild oxidative stress is less clear. The data presented herein, demonstrate for the first time that endogenous Nrf2 could be activated by mild oxidative stress and that this activation is restricted to astrocytes. Contrary to the established dogma, I found that mild oxidative stress induces the astrocytic Nrf2 pathway in a manner distinct from the classical Keap1 antagonism employed by prototypical Nrf2 inducers. The mechanism was found to involve direct regulation of Nrf2's transactivation properties. Overall these results advance our knowledge of the molecular mechanism(s) associated with the control of endogenous antioxidant defences by physiological signals.

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Chapter 1
Introduction

Oxygen consumption through normal cell metabolism results in the generation of harmful reactive oxygen species (ROS). It normally would be assumed that, given its central role in physiology and survival, the brain would be enriched with protective mechanisms against oxidative stress. On the contrary, the brain is particularly vulnerable to the deleterious effects of ROS because of its high metabolic activity coupled with low levels of antioxidants and high abundance of heavy metal ions. (Reiter, 1995; Hardingham and Lipton; 2011 Gandhi and Abramov, 2012).

Oxidative stress is caused by an imbalance between the generation and the detoxification of ROS and it has been associated with acute and chronic neurological disorders. Cells' intrinsic antioxidant and neuroprotective pathways are subject to dynamic regulation, potentially allowing them to adapt to and survive various intrinsic and extrinsic stressors. Such pathways are essential, particularly in adult neurons, which exhibit a severely limited capacity for regeneration or *de novo* generation of neurons. However, the underlying molecular mechanisms, specifically in neural cells, are less understood.

The aim of this thesis is to gain a better understanding of the control of neural intrinsic antioxidant and neuroprotective pathways by N-methyl D-aspartate receptor (NMDAR) mediated synaptic activity or mild oxidative stress. This introduction is intended to summarize some of the present knowledge on oxidative stress, generation of ROS and antioxidant defense mechanisms specifically those regulated by the transcription factor nuclear factor (erythroid 2-related) factor 2 (Nrf2), as well as the role of the fork head box O family of transcription factors (Foxo) in oxidative stress induced neuronal death. In addition, the introduction will include an overview on the NMDAR composition and their role in neuronal death and survival.

1.1 Oxidative stress

It has been long recognised that oxidative stress plays an important role in the etiology of various pathological conditions including acute and chronic neurodegenerative disorders. Oxidative stress elicits its deleterious effect through inflicting damage to cellular biomolecules and it could occur as a secondary effect of a pre-existing pathological condition or could be the primary cause of the disease.

1.1.1 Mechanisms of Oxidative stress damage

Oxygen is required to sustain life, but paradoxically, highly toxic reactive oxygen species (ROS) are produced as a byproduct of its metabolism. Oxidative stress is a condition in which there is a mismatch between the production of ROS and the cell's ability to detoxify these species and repair the damage. However, this imbalance results in the accumulation of damaged biomolecules that can cause cellular dysfunction, and for post-mitotic cells with relatively restricted replenishment such as neurons, cell death (Simonian and Coyle, 1996; Wang and Michaelis, 2010). ROS react with cellular components resulting in deleterious effects on their function. They can damage both nuclear and mitochondrial DNA, through inducing strand breaks and chemical alterations in the deoxyribose and in the purine and pyrimidine bases (Simonian and Coyle, 1996; Reiter, 1995). In addition to their role in pathophysiology, ROS are beneficial to cells in certain physiological processes such as signal transduction, immune defence and oxygen homeostasis (Allen and Bayraktutan, 2009).

In terms of protein damage, ROS can oxidize the protein backbone, amino acid side chains and induce extensive protein-protein cross-linking at cysteine residues, carbonyl derivatives and other oxidized residues (Berlett and Stadtman, 1997; Kregel and Zhang, 2007). Oxidatively damaged proteins, many of which are critical in neuronal physiology or structure, lose their efficiency and eventually disturb cellular functions. For instance, oxidative damage can alter enzyme activity, modulate

transcription factor activity and lead to the formation of protein aggregates (Kregel and Zhang, 2007).

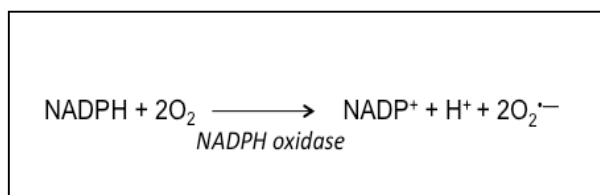
Polyunsaturated fatty acids (PUFA), which are abundant in the neural tissues, render lipids highly sensitive to oxidation by ROS (Kregel and Zhang, 2007). Oxidative lipid damage, termed lipid peroxidation, is initiated by the incorporation of oxygen into PUFA. This process compromises the membrane integrity by decreasing its fluidity, reducing membrane potential and thereby allowing ions such as Ca^{2+} to leak into the cell (Simonian and Coyle, 1996).

1.1.2 Generation of reactive oxygen species (ROS)

The term free radical refers to any species with an unpaired electron. The majority of free radicals have a high chemical reactivity, and can either react with each other or with non-radicals. Molecular oxygen O_2 qualifies as a radical because it possesses two unpaired electrons each on a different orbital and both spinning in the same direction, however, unlike other radicals O_2 reactivity is poor due to this parallel spin. Reactive oxygen species (ROS), is a collective descriptor that includes not only the oxygen radicals such as superoxide ($\text{O}_2^{\bullet-}$) and hydroxyl radical (OH^\bullet), but also some (non-radical) derivatives such as hydrogen peroxide H_2O_2 (Halliwell, 2006).

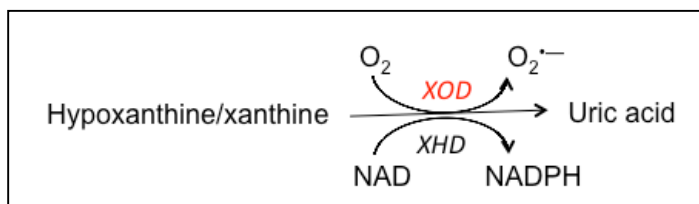
$\text{O}_2^{\bullet-}$ is produced via the activity of mitochondrial and microsomal electron transport chains (McCord and Omar, 1993). Most of the O_2 in aerobic organisms is reduced to water by the mitochondrial cytochrome *c* oxidase enzyme, which adds four electrons to O_2 in a step-wise fashion resulting in the formation of two molecules of water. Although the partially reduced oxygen intermediates are usually retained by the cytochrome *c* oxidase until full reduction is achieved, other sites in the respiratory chain namely complex I and III may directly leak electrons to O_2 resulting in the formation of $\text{O}_2^{\bullet-}$ (Turrens, 1997; Brookes, 2005).

The mitochondrion, though the prime site for $O_2^{\bullet-}$ generation, is not the only source of $O_2^{\bullet-}$ *in vivo*. In fact, various cytosolic and membrane-bound enzymes are involved in the generation of $O_2^{\bullet-}$, including nicotine amide adenine dinucleotide phosphate (NADPH) oxidase and Xanthine oxidase (Simonian and Coyle, 1996; Gandhi and Abramov, 2012). NADPH oxidase is a membrane enzyme and the first identified example of a system that generates ROS as a primary product rather than a byproduct (Bedard and Krause, 2007) It is a multi-subunit enzyme consisting of two membrane-bound components, several cytosolic proteins and a low-molecular-weight G protein (Babior, 1999). Activation of the oxidase involves the phosphorylation of the cytosolic subunits leading to their translocation to the membrane and eventually the complete assembly of the complex. This enzyme is present in phagocytes and in diverse brain cell types such as neurons, astrocytes (Noh and Koh, 2000) and microglia (Colton and Gilbert, 1987), in which it catalyses the production of $O_2^{\bullet-}$ from O_2 and NADPH according to the following reaction (Ago et al., 1999):



Xanthine oxidase (XOD) is another major cellular source of $O_2^{\bullet-}$. It originally exists in the form of xanthine dehydrogenase (XDH) and is converted to xanthine oxidase in cells under some pathological conditions either reversibly by the oxidation of sulfhydryl residues or irreversibly by partial cleavage of the xanthine dehydrogenase (Chung et al., 1997). However, both enzymes share the same general metabolic function, which is the conversion of hypoxanthine to xanthine and the subsequent production of uric acid. Xanthine oxidase is the $O_2^{\bullet-}$ producing form. It catalyses the conversion of hypoxanthine or xanthine to uric acid and superoxide using O_2 as a co-factor, while xanthine dehydrogenase utilizes the same substrates using NAD as a

cofactor to produce uric acid and NADPH instead of superoxide (Chung et al., 1997; Boueiz et al., 2008):



Owing to its low reactivity and poor membrane permeability, $O_2^{\bullet-}$ itself is not highly toxic to macromolecules, however, it is readily converted to more toxic species (Halliwell and Gutteridge, 1990; Starkov, 2010). Moreover, $O_2^{\bullet-}$ is converted spontaneously or enzymatically to H_2O_2 . Unlike $O_2^{\bullet-}$, however, H_2O_2 has a higher oxidant potential, and being uncharged, can easily diffuse through cell membranes. Although H_2O_2 is an oxidizing agent itself, it is not reactive. However, in the presence of transition metals such as Fe^{2+} , H_2O_2 is further reduced to the highly toxic radical OH^{\bullet} via the Fenton reaction (Fig. I.1) (Halliwell and Gutteridge, 1990; Hogg et al., 1992).

Superoxide dismutase (SOD) in its three forms (cytosolic, extracellular Cu, Zn-SOD and mitochondrial Mg-SOD) eliminate $O_2^{\bullet-}$ by catalysing its dismutation, one $O_2^{\bullet-}$ being reduced to H_2O_2 and the other oxidized to O_2 (Fig. 1.1) (Fridovich, 1995). Although SOD is classified as a component of the antioxidant defences, due to its ability to metabolize the superoxide free radical to non-radical species (H_2O_2), excessive SOD gene expression, such as in individuals with Down syndrome, has been linked with premature aging, and neurodegeneration (Lejeune, 1990). Moreover, missense mutations in the cytosolic Cu/Zn form of SOD have been associated with familial ALS (Rosen et al., 1993).

Besides SOD, a number of enzyme systems also generate H_2O_2 . These include L-amino acid oxidase, glycolate oxidase and monoamine oxidase (Reiter, 1995). The

mitochondrially located flavoenzyme, monoamine oxidase (MAO), catalyses the oxidative deamination of important neurotransmitters such as dopamine and serotonin. Two subtypes of monoamine oxidase have been identified: MAO-A and MAO-B. In the central nervous system (CNS), MAO-A is found in neurons while MAO-B is expressed in both neurons and astrocytes. MAO-B have been implicated in the etiology of Parkinson's disease, as MAO-B accelerates the oxidation of dopamine in dopaminergic nerve axons resulting in elevated H_2O_2 generation beyond the capacity of cells' antioxidant defence systems, eventually leading to neuronal destruction (Edmondson et al., 2009). In addition, superoxide is formed non-enzymatically through the auto-oxidation of small molecules such as catecholamines (Blake et al., 1987), haemoglobin and myoglobin (Allen and Bayraktutan, 2009).

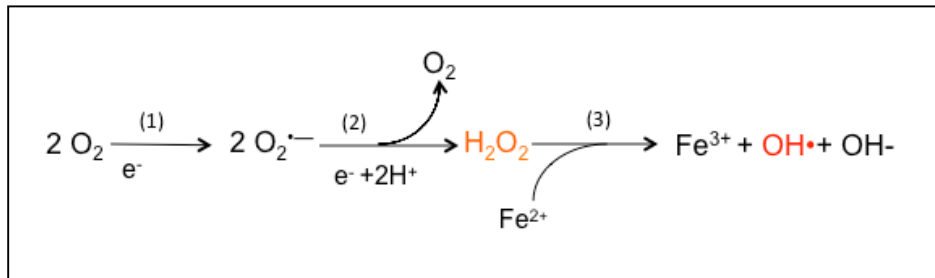


Figure 1.1. H_2O_2 generation by SOD and the conversion of H_2O_2 to the highly toxic hydroxyl radical via the Fenton reaction.

Molecular oxygen in the presence of electrons forms superoxide, which is readily converted to O_2 and H_2O_2 . H_2O_2 in return, can be further reduced in the presence of transition metals such as Fe^{2+} to the highly toxic hydroxyl radical (OH^{\cdot}): (1) The formation of superoxide by various sources such as mitochondrial oxidative metabolism and by the enzymatic activity of NADPH and xanthine oxidases among many other sources. (2) Superoxide conversion to H_2O_2 either spontaneously or enzymatically through the action of SOD. (3) The conversion of H_2O_2 to (OH^{\cdot}) via the Fenton reaction.

1.2. Oxidative stress and neuronal death

It is widely accepted that mammalian neurons are among the longest-living cell types. However, neuronal death can occur naturally during embryonic and early postnatal development to ensure proper and precise synaptic connection, or as a pathological process in disease (Yuan et al., 2003). Oxidative stress can trigger neuronal death in various pathological scenarios and in this section I shall discuss the two main forms of cell death associated with oxidative stress; apoptosis and necrosis.

1.2.1. Apoptosis

Cells undergoing apoptosis display distinct morphological hallmarks, which include nuclear pyknosis, chromatin condensation, DNA fragmentation and cell rounding (Kerr et al., 1972; Yuan et al., 2003). This ordered morphology is a result of ATP-requiring caspase-mediated cleavage. Unlike necrosis, apoptotic cells have intact membranes and are not associated with inflammation.

Apoptosis centres on proteolytic activation of caspases, a family of cysteine proteases, which target a wide range of proteins involved in cell survival, such as cell cycle control and DNA repair. Caspases also undergo autolytic activation and in turn cleave and activate other downstream caspases in the cascade leading to apoptosis. Based on the activation order in the cells, caspases are divided into initiator and effector groups, with the former activating the latter. Active initiator caspases (caspases 8-10) will cleave and activate effector caspases (caspase-3, -7 and -6) which in turn cleave specific substrates to produce the morphological and biochemical changes characteristic of apoptosis (Nijhawan et al., 2000).

To date, two major apoptotic pathways have been described: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The extrinsic pathway involves the engagement of extracellular ligands with cell death receptors, such as Fas and members of the tumour necrosis factor (TNF) family (Ashkenazi and Dixit, 1998). Fas ligand (FasL) and TNF bind their receptors and stimulate the recruitment of adaptor proteins such as Fas-associated death domain (FADD) and the TNF receptor 1-associated death domain protein (TRADD). The adaptor proteins in turn facilitate the recruitment of pro-caspase-8 (or -10) to the receptor complex and the formation of the death-inducing signalling complex (DISC), leading to the activation of the initiator caspases and subsequently the down stream effector caspases (Fig. 1.2) (Yang et al., 1998; Elmore, 2007).

The intrinsic apoptotic pathway is mediated by the mitochondria and is activated by a diverse array of non-receptor mediated extracellular and intracellular stimuli including toxins, oxidative stress, ischemia-reperfusion, DNA damage and loss of trophic/survival factors (Soriano et al., 2011; Foo et al., 2005). All these stimuli cause changes in the mitochondrial outer membrane, leading to increased membrane permeability and eventually the release of cytochrome *c*, which binds the cytosolic monomer apoptotic peptidase activating factor-1 (Apaf-1). The Apaf-1/cytochrome *c* complex then recruits and activates initiator caspase-9, which in turn activates the down stream effector caspases such as caspase-3, caspase-6 and caspase-7 (Fig. 1.2) (Nijhawan et al., 2000).

The regulation of cytochrome *c* release occurs through members of the Bcl-2 family of proteins, which govern the mitochondrial membrane permeability. They are characterized by the presence of Bcl-2 homology (BH1-4) domains, and are divided into pro-apoptotic (Bax, Bim, Puma, Bak, Bid etc.) and anti-apoptotic (Bcl-2, Bcl-xL, Bcl-xS, etc.) groups. Following apoptotic stimuli, Bax and Bak form oligomeric structures and translocate to the mitochondria where they promote outer membrane permeabilization and eventually lead to cytochrome *c* release (Kroemer et al., 2007). On the other hand, the Bcl-2 anti-apoptotic members localized to the mitochondria, sequester pro-apoptotic Bcl-2 members through binding to their BH3 domains,

preventing their activation/oligomerization and eventually inhibiting the release of cytochrome *c* (Lindsten et al., 2000).

In addition to its detrimental effect, apoptosis fulfils a beneficial role in organs and limbs remodelling during embryogenesis, in tumour formation prevention and in normal cell turnover (Meier et al., 2000). Apoptosis occurs during neurodevelopment not only in the progenitor cells, but also in neurons and glia (Nijhawan et al., 2000). Studies underscored the importance of apoptosis in patterning of the CNS, as mutant mice deficient in key pro-apoptotic genes *Casp3*, *Casp9* and *Apaf1* all exhibited brain abnormalities and increased embryonic lethality (Kuan et al., 2000; Buss et al., 2006). In addition to the physiological role of apoptosis, studies indicate that apoptosis plays an important role in neurodegeneration and in neuronal death in response to ischemic-hypoxia (Thompson, 1995).

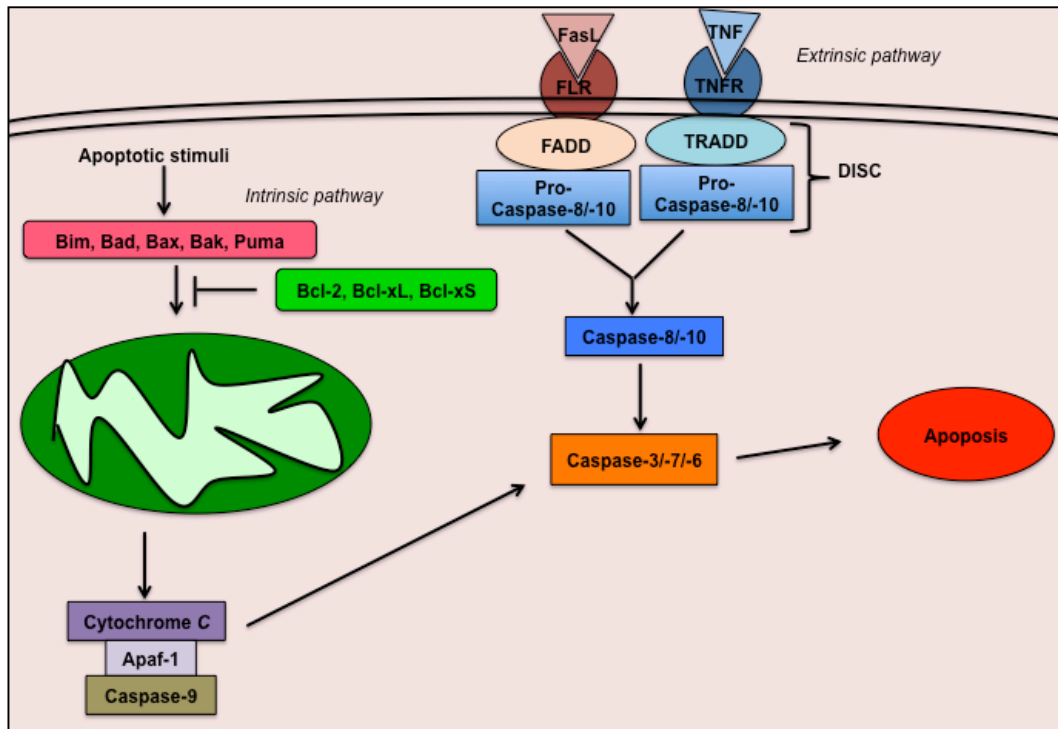


Figure 1.2. Apoptosis signalling.

The two main pathways of apoptosis are extrinsic and intrinsic. The extrinsic signalling pathway involves the binding of death receptors such as FasL receptors (FLR) and TNF receptors (TNFR) to their corresponding ligands and the subsequent recruitment of adaptor proteins such as FADD and TRADD. The adaptor proteins then associate with procaspase-8 or -10 forming DISC, which in turn leads to the activation of the initiator caspases-8, -10 and subsequently the down stream effector caspases-3, -7, -6. The intrinsic pathway is a mitochondrial-initiated event and is activated by a diverse array of non-receptor mediated stimuli, which cause changes to mitochondrial membrane permeability and the subsequent release of cytochrome *c*. The release of cytochrome *c* triggers effector caspases activation through formation of cytochrome *c*/Apaf-1/ caspase-9 complex. The release of cytochrome *c* is regulated by Bcl-2 family members (see above text).

1.2.2. Necrosis

Necrosis is usually elicited by exposure of cells to toxic substances or to severe mechanical or ischaemic/hypoxic insults, and is morphologically characterized by swelling of the intracellular organelles followed by swelling and rupture of both the plasma and nuclear membranes (Edinger and Thompson, 2004; Clarke, 1990). The loss of membrane integrity may be a consequence of metabolic failure, damage to membrane lipids, and/or dysfunction in ion pumps/channels, in addition to the release of excitatory amino acid neurotransmitters (in the case of neurons) (Werner and Engelhard, 2007; Zong and Thompson, 2006).

While necrosis has long been regarded as an uncontrolled form of death, accumulating studies have suggested that necrosis can be a regulated event that contributes to pathological and physiological conditions (Edinger and Thompson, 2004). Holler *et al.* and others have found receptor-interacting proteins kinase (RIP) to be crucial for programmed necrosis (Holler *et al.*, 2000; Chan *et al.*, 2003).

Although necrosis induces inflammation which may lead to the death of neighbouring cells through the release of cytokines, the necrosis-induced inflammation have a positive role in the protection against viral infections (Edinger and Thompson, 2004). In regard to the CNS, necrosis has been associated with various acute and chronic neurodegenerative conditions including Alzheimer's and Parkinson's diseases, ischemia and traumatic brain injury (Yuan *et al.*, 2003; Sattler and Tymianski, 2001; Werner and Engelhard, 2007).

1.2.3. Mechanisms of oxidative stress-induced cell death

Oxidative stress-induced cell death displays characteristics of both apoptosis and necrosis. However, the same insult can lead to apoptosis or necrosis depending on its intensity, cell type, and the species and organism involved. According to the hypothesis and observations of Duvall and Wyllie, severe non-physiological levels of oxidative stress cause necrosis, rather than apoptosis (Duvall and Wyllie, 1986). Excessive accumulation of lipid hydroperoxides, lipid peroxidation products, in response to oxidative stress induced by glutathione (GSH) depletion (an important antioxidant discussed in detail in section 1.4) leads to cell's demise by necrosis (Higuchi, 2004). However, several lines of evidence suggest the involvement of oxidative stress in apoptosis. For instance, H_2O_2 has been shown to induce apoptosis in B-cells (Takada et al., 2011) and in the CNS, chronic SOD inhibition results in the apoptotic degeneration of motor neurons (Rothstein et al., 1994) and PC12 neuronal cells (Troy and Shelanski, 1994). Moreover, cortical neurons undergoing GSH-deprivation-induced oxidative stress, either by growing them in low cysteine media or through treatments with the glutamate analog homocysteate (HCA), suffer from apoptotic degeneration, which can be prevented by co-application of antioxidants (Ratan et al., 1994).

Oxidative damage to DNA is believed to trigger apoptosis. Poly (ADP-ribose) polymerase, an enzyme involved in DNA damage repair, is activated under various conditions of DNA damage and is suggested to contribute to cell death by depleting the cell of NAD and ATP (Berger and Petzold, 1985; Schraufstatter et al., 1986).

In addition to DNA damage, membrane integrity is thought to be compromised by oxidative stress. Rapid loss of membrane integrity is observed in neurons with concomitant nuclear condensation and DNA fragmentation following treatment with H_2O_2 (Higgins et al., 2009a).

Furthermore, oxidative stress can trigger apoptosis through disturbing signalling pathways associated with growth factor receptor stimulation. Insulin-like growth factor I (IGF-I) is a classical neuroprotective factor, which upon binding to its receptor triggers the PI3K/Akt signalling pathway. Activation of the PI3K/Akt pathway in turn, inactivates Foxo transcription factors, responsible for transactivating a number of pro-death genes, through inhibitory phosphorylation. However, oxidative stress inactivates IGF-I receptor function through abnormal glycation and consequently induces neuronal death by stimulating Foxo3 (Davila and Torres-Aleman, 2008).

1.2.4. Foxos and apoptosis

The fork head box O family of transcription factors (Foxo) initially described in *Drosophila melanogaster*, are characterized by the presence of a conserved 100 amino acid DNA binding domain, or forkhead domain. Owing to the presence of the DNA-binding domain, Foxo proteins not only act as transcriptional activators but also as transcriptional repressors possibly through interacting with other transcription factors and modulating their activity, hence exerting positive and negative effects on gene expression. However, the net effect of Foxos on gene expression depends on the promoter context and extracellular conditions. Foxos also harbour nuclear export and nuclear localization signal sequences allowing for nucleocytoplasmic shuttling (Fu and Tindall, 2008). The subcellular localization and transcriptional functions of Foxos are tightly controlled by multiple post-translational modifications, such as phosphorylation, acetylation, and ubiquitination (Vogt et al., 2005). Depending on the nature of the stimuli, Foxos are subject to two types of phosphorylation: inhibitory phosphorylation by a number of protein kinases including Akt, serum- and glucocorticoid-inducible kinase (SGK), Cyclin-dependent kinases-2 (CDK2) and I kappa B kinase (IKK), and secondly activating phosphorylation mediated by other kinases such as c-Jun NH2-terminal kinase (JNK) and mammalian Sterile 20-like kinase 1 (MST1) (Brunet et al., 2004; Huang and Tindall, 2007). All Foxo proteins are negatively regulated by Akt-mediated phosphorylation at three specific sites in response to growth factor and insulin except

for Foxo6, which contains only two sites and is predominantly nuclear (van der Heide et al., 2005; Salih and Brunet,

2008). Phosphorylation of Foxo transcription factors by Akt promotes their nuclear export and subsequent ubiquitination and degradation. Direct phosphorylation of Foxos by Akt is initiated in response to growth factors, insulin and other cell stimuli (Obsil and Obsilova, 2008).

Emerging evidence suggests the involvement of Foxos in multiple biological functions including cell cycle arrest, differentiation, stress response and apoptosis (Obsil and Obsilova, 2008). Foxos regulate components of both the intrinsic and extrinsic apoptosis pathway. In lymphocytes, Foxo1 was reported to induce Bim expression upon growth factor withdrawal (Dijkers et al., 2000). Besides Bim, Foxo3 has been shown to upregulate *Puma* gene expression in response to cytokine or growth factor withdrawal (You et al., 2006). Furthermore, Foxo4 induces apoptosis in part by suppressing the levels of anti-apoptotic BCL-XL through the transcriptional repressor BCL-6 (Tang et al., 2002).

In addition, Foxos mediate the extrinsic apoptotic pathway through enhancing the transcription of pro-apoptotic factors such as FasL and tumour necrosis factor-related apoptosis inducing ligand (TRAIL) (Brunet et al., 1999; Modur et al., 2002). FasL was one of the early pro-apoptotic genes identified as a Foxo target gene. Previous studies demonstrated a critical role of FasL in mediating Foxo-induced apoptosis in cerebellar granule cells and Jurkat T cells (Brunet et al., 1999). Moreover, mutations in phosphatase and tensin homologue (*PTEN*) are very common in prostate cancers leading to constitutive activation of Akt pathway and eventually the loss of FOXO1 and FOXO3 activity. Reduce *TRAIL* expression in human prostate cancer was directly linked to the loss of FOXO activity. However, studies revealed that overexpression of FOXO1 and FOXO3 promoted apoptosis and increased *TRAIL* mRNA levels in prostate cancer cell lines (Modur et al., 2002). Thus, Foxos regulate

apoptosis through controlling the transcription of core components of both the intrinsic and extrinsic pathway.

In the CNS, apoptosis is the primary cellular output of Foxo activation in response to environmental stressors such as trophic factor deprivation (Gilley et al., 2003), oxidative stress (Lehtinen et al., 2006) or extrasynaptic NMDAR activation (Dick and Bading, 2010). Foxo activity was shown to mediate Bim-dependent apoptosis in sympathetic neurons undergoing neuronal growth factor withdrawal-induced apoptosis (Gilley et al., 2003). FasL promoter harbours three Foxo DNA binding sites, and activation of Foxo3 was shown to cause cell death in motor and cerebral neurons, this killing effect, however, was attenuated in neurons derived from FasL mutant mice (Brunet et al., 1999; Barthelemy et al., 2004). In neuroblastoma cells, Foxo1 induces apoptosis through activation of pro-death genes Noxa and Bim (Obexer et al., 2007). Also Foxos activation was associated with hippocampal injury following prolonged seizures (Shinoda et al., 2004). Conversely, Foxo inactivation may contribute to the neuroprotective effect of estradiol observed in rodent models of stroke (Won et al., 2006). In humans, *FOXO3* haplotype analysis revealed increased risk of stroke and increased mortality for carriers of certain *FOXO3* haplotypes (Kuningas et al., 2007; Maiese et al., 2007).

Outside the CNS, Foxos coordinate diverse cellular functions. These multi-tasking proteins play key roles in many biological process including, cell-cycle progression, differentiation, cell growth and development, immune system activation and metabolism (Dansen and Burgering, 2008; Maiese et al., 2008; Salih and Brunet, 2008). For instance Foxo1 plays a significant role in maintaining energy metabolism by increasing hepatic gluconeogenesis, reducing insulin secretion and regulating energy storage and expenditure through adipose tissue. Foxo1 exerts a further level of metabolism control by coordinating neuropeptide production in hypothalamic neurons, which regulates food intake and metabolite homeostasis and by regulating skeletal muscle atrophy in response to starvation or inadequate trophic support (Salih

and Brunet, 2008; Kousteni, 2012).

Studies in several animal models support a role for Foxo proteins as tumour suppressors, owing to their established involvement in cell-cycle arrest, apoptosis, DNA-damage repair and angiogenesis restriction. Moreover, spontaneous tumour formation in Foxo conditional knockout mice, confirms the tumour suppressing-effects of Foxos (Dansen and Burgering, 2008).

1.2.5. Foxos and oxidative stress

Foxos play a key role in promoting oxidative stress-induced apoptosis in neurons through the upregulation of a number of pro-apoptotic genes (Lehtinen et al., 2006; Davila and Torres-Aleman, 2008; Gilley et al., 2003; Brunet et al., 1999). Bonni and co-workers demonstrated that oxidative stress activates MST1, which in turn phosphorylates Foxo3 at ser 207, resulting in the disruption of Foxo3 interaction with 14-3-3 proteins (a family of conserved modulator proteins which regulate diverse cellular processes through binding to their target proteins and affecting their function by several means (Tzivion et al., 2011)), which leads to Foxo3 nuclear accumulation and thereby induces neuronal cell death (Lehtinen et al., 2006). Another study suggested that oxidative stress-induced neuronal death involves the activation of Foxo3 via recruiting two independently activated pathways. A rapid pathway involving the attenuation of Akt inhibition of Foxo3 through p38 MAPK-mediated inhibition of IGF-I stimulation of Akt and a subsequent delayed pathway involving the activation of Foxo3 by jun-kinase 2 (JNK2) (Davila and Torres-Aleman, 2008).

Although in neurons Foxo has been reported to mediate oxidative stress-induced apoptosis, Foxo activation in non-neuronal cells has been shown to reduce levels of

oxidative stress by upregulating the expression of two potent antioxidant enzymes; MnSOD and catalase (Storz, 2011). Another diversion of Foxo3 function from apoptosis to stress resistance has been reported in the context of Sirt1-mediated

deacetylation. In mammalian cells, the silencing information regulator 2 homolog, sirtuins (Sirt1) controls cellular responses to stress by deacetylating various proteins including Foxos. Brunet *et al.* reported an increased Foxo3 acetylation in response to H₂O₂-induced oxidative stress accompanied with increased interaction between Sirt1 and Foxo3. Interestingly, Sirt1 differentially affected Foxo3 function, potentiating Foxo3's ability to induce cell cycle arrest and resistance to oxidative stress but attenuating its ability to induce cell death in the presence of stress stimuli (Brunet *et al.*, 2004). However, the mechanism by which Foxos switch from apoptotic signalling to antioxidant signalling is unclear (Storz, 2011).

In the context of Foxo regulation, our group has previously shown that synaptic NMDAR signalling promotes sustained activation of the Akt pathway, leading to Foxo1 phosphorylation and nuclear exclusion and eventually the subsequent inactivation of Foxo downstream genes (Soriano *et al.*, 2006; Papadia *et al.*, 2008; Martel *et al.*, 2009). In addition synaptic activity protects against oxidative stress by triggering a number of changes to the thioredoxin-peroxiredoxin antioxidant system. These changes include the downregulation of thioredoxin endogenous inhibitor and the newly identified Foxo target gene, thioredoxin interacting protein (*Txnip*). Synaptic activity turns off *Txnip* transcription by promoting PI3K-directed nuclear exclusion of Foxos, and the subsequent dissociation from the *Txnip* promoter (Papadia *et al.*, 2008). Whether the effect of synaptic activity on Foxos nuclear distribution extends to the other major neuronal Foxo, Foxo3, or whether it induces changes to Foxos expression is going to be investigated in this thesis.

1.3. Oxidative stress and neurodegeneration

Ageing was the earliest degenerative condition to be associated with oxidative stress, (Harman, 1956; Muller et al., 2007). Most theories of ageing in general and aging of the CNS in particular, are centred on the idea that accumulation of ROS coupled with diminished antioxidant defences (as a consequence of normal aging process) lead to cellular damage involving mitochondrial dysfunction (Harman, 1972; Lin and Beal, 2006). More recently, this potential association between oxidative stress and ageing has been extended to many ageing-related diseases including Parkinson's disease (PD), Alzheimer's disease (AD) and Amyotrophic lateral sclerosis (ALS) (Bowling and Beal, 1995; Lin and Beal, 2006).

AD is the most common ageing-related neurodegenerative disorder, characterized by progressive decline in memory and cognitive abilities, which are accompanied by neuronal loss in the forebrain. Evidence of oxidative damage has been seen in AD brains as well as transgenic animal models of the disease, in which markers of lipid peroxidation, protein and DNA oxidation were increased (Sayre et al., 2008; Vargas and Johnson, 2009). The major neuropathological hallmarks of AD are the extracellular β -amyloid protein aggregates ($A\beta$) and the intracellular neurofibrillary tangles (NFT) (Simonian and Coyle, 1996; Selkoe, 2001). Oxidative stress is thought to contribute to $A\beta$ toxicity and thus antioxidants protect against $A\beta$ -induced neurotoxicity (Hensley et al., 1996; Quintanilla et al., 2005). It is also associated with the formation of NFTs, whereby oxidative stress triggers tau (the major protein in the NFT) phosphorylation leading to the formation of neurofibrillary lesions (Su et al., 2010).

PD is a common neurodegenerative movement disease associated with loss of dopaminergic neurons in the substantia nigra and is characterized by movement and postural dysfunction (Sayre et al., 2008; Shukla et al., 2011). Postmortem tissues from PD patients have shown evidence of mitochondrial complex I reduced activity in the substantia nigra (Dawson and Dawson, 2003). The loss of complex I activity is thought to occur due to oxidative damage to certain complex I subunits resulting in the disassembly and impairment of the complex (Keeney et al., 2006). Further evidence for oxidative stress in PD is obtained from examination of human PD brains revealing oxidative damage to DNA and protein within the nigro-striatal region (Beal, 2002; Seet et al., 2010; Kikuchi et al., 2002).

Besides being implicated in ageing-related neurological disease, oxidative stress plays a key role in the pathogenesis of acute neurological disorders such as ischemic stroke (Allen and Bayraktutan, 2009; Cherubini et al., 2005). Oxidative stress mediates ischemic injury in part through increasing cerebral vasodilation, blood-brain barrier permeability and formation of focal lesions (Wei et al., 1996). It also has profound damaging cellular effects, leading to cells death and tissue destruction; these include nucleic acid modifications, lipid peroxidation and Ca^{2+} release from intracellular stores (Allen and Bayraktutan, 2009).

Although increased oxidative damage has been reported in various human neurodegenerative conditions, it is not clear whether oxidative stress is the primary pathological mechanism resulting from either overproduction of ROS or a defect in cellular antioxidant defences or both, or whether it is a secondary manifestation of neurodegenerative process triggered by mitochondrial dysfunction or excitotoxicity. (Sayre et al., 2008; Vargas and Johnson, 2009). In addition to oxidative stress, neurodegenerative conditions involve a number of neuron-damaging events

including inflammation, excitotoxicity, mitochondrial impairment and calcium dysfunction (Andersen, 2004; Wang and Michaelis, 2010). However, the interconnection between these events is not necessarily a cascade but they may constitute a vicious cycle of which oxidative stress is a major component.

Regardless of whether oxidative stress is primary or secondary event, it has become evident that it is involved in at least the exacerbation of cellular injury that leads to neuropathology (Ischiropoulos and Beckman, 2003; Andersen, 2004). Therefore, gaining a better insight of the regulation of intrinsic cellular antioxidant defences can offer therapeutic strategies to alleviate the burden of oxidative stress or delay the progression of neurodegenerative conditions.

1.4. Cellular antioxidant defences

Cells are equipped with a wide range of endogenous antioxidant defence systems to cope with the damaging effects of ROS. These include enzymatic and non-enzymatic antioxidants that control the levels of ROS. The CNS contains multiple antioxidant defences such as the glutathione (GSH) system, thioredoxin/peroxiredoxin, and ascorbate.

1.4.1. Glutathione system

The tripeptide glutathione (GSH; γ -L-glutamyl-L-cysteinylglycine) is the most abundant non-protein thiol in mammalian cells, consisting of glutamate, cysteine and glycine. Within cells GSH is synthesized in consecutive ATP-requiring reactions of two enzymes. The initial reaction is rate limiting and catalysed by glutamate-cysteine ligase (GCL), a heterodimeric enzyme consisting of a catalytic subunit (GCLC) and a modifier subunit (GCLM), generating the dipeptide γ -glutamyl cysteine; the second one is the addition of glycine to the dipeptide by GSH synthetase (Fig. 1. 4) (Meister and Anderson, 1983).

The importance of the glutathione system for the detoxification of ROS in brain cells is becoming more evident (Meister and Anderson, 1983; Cooper and Kristal, 1997; Fernandez-Fernandez et al., 2012). Glutathione deficiency induced by the application of the GCL inhibitor BSO was shown to enhance the toxic effects of ROS generating insults such as ischemia (Mizui et al., 1992) and to induce mitochondrial damage in newborn rats brains (Jain et al., 1991). Furthermore, significant loss of GSH has been reported in human brains from patients with AD (Gu et al., 1998), PD (Sofic et al., 1992), and schizophrenia (Do et al., 2000), as well as in rat models for Huntington's disease (Cruz-Aguado et al., 2000).

The detoxification of ROS by GSH is achieved either non-enzymatically through the direct reaction with radicals such as superoxide or hydroxyl (Saez et al., 1990; Winterbourn and Metodiewa, 1994) or through its role as an electron donor in the enzymatic reduction of peroxides catalysed by Glutathione peroxidases (GPx) (Chance et al., 1979). GPx are selenium-dependent enzymes that play a major role in disposing of H₂O₂ through catalysing the reduction of H₂O₂ to water utilizing reduced GSH as an electron donor. During the GPx reaction, GSH is oxidized to GSSG then recycled back to GSH in an NADPH-dependent reaction catalyzed by glutathione reductase (GSR) (Fig. 1.3) (Kosower and Kosower, 1978; Reed, 1986).

In addition to H₂O₂, glutathione peroxidases catalyse GSH-dependent reduction of fatty acid hydroperoxides to alcohol (Reiter, 1995; Gandhi and Abramov, 2012). At least five GPx isozymes have been identified in mammalian tissues; with GPx1 being the most abundant form (de Haan et al., 1998). The importance of GPx1 in protecting against oxidative stress is highlighted by the increased neuronal susceptibility to oxidative stress-induced death in GPx1 KO cells/animals challenged with H₂O₂ or paraquat (Haan et al., 1998; Taylor et al., 2005).

Whilst GSH has been found, as seen by histochemical and immunohistochemical techniques, to be present in neurons and glia (Dringen, 2000), the efficiency of the GSH-dependent peroxide detoxification appears to be lower in neurons than in astrocytes (Dringen et al., 1999a). This was attributed, at least partially, to the GSH concentration being half of that found in astrocytes (Sagara et al., 1993; Makar et al., 1994) as a result of significantly lower protein levels and enzymatic activity of neuronal GCL in comparison with astrocytes (Makar et al., 1994). Consequently, cultured neurons are more vulnerable to damaging compounds such as H₂O₂ than cultured astrocytes (Dringen et al., 1999b) while astrocytic-GSH synthesis protects neurons from oxidative stress (Shih et al., 2003).

Neurons rely on astrocytes for the provision of the necessary precursors for GSH synthesis (Dringen, 2000; Vargas and Johnson, 2009). GSH is released from astrocytes into the extracellular space via the multidrug-resistance-associated protein 1 (MRP1) (Dringen and Hirrlinger, 2003) and subsequently hydrolysed to its precursors; first its broken down by γ - glutamyl transpeptidase to the dipeptide cysteinyl-glycine (Cys-Gly) (Dringen et al., 1999b), which can then be hydrolysed by aminopeptidase N to release cysteine and glycine (Dringen et al., 2001). The availability of cysteine, which is extremely unstable extracellularly and rapidly auto-oxidized to cystine, is the rate-limiting factor in glutathione synthesis (Chen and Swanson, 2003; Vargas and Johnson, 2009). Neurons reportedly utilize cysteine but not cystine for GSH synthesis, while glia can utilize both (Kranich et al., 1996; Sagara et al., 1993). The cystine/glutamate exchange transporter (xCT) imports cystine into the cells in exchange with glutamate (Bannai, 1986) and is localized in both neurons and astrocytes of the cerebral cortex (Shih et al., 2006). However, xCT expression and activity was shown to be higher in astrocytes compared to neurons (Jackman et al., 2012). Immature neurons exclusively uptake cystine via xCT (Murphy et al., 1990), whereas mature neurons primarily uptake cysteine via the excitatory amino acid transporters (EAATs), also known as cysteine-permeable Na⁺-dependent glutamate transporter (X_{AG}), which are widely expressed by neurons in

mature brain (Shanker et al., 2001; Chen and Swanson, 2003). In addition to supplying cysteine, astrocytes release glutamine, thus providing all the necessary precursors for neuronal GSH synthesis (Fig. 1.4) (Hertz et al., 1999).

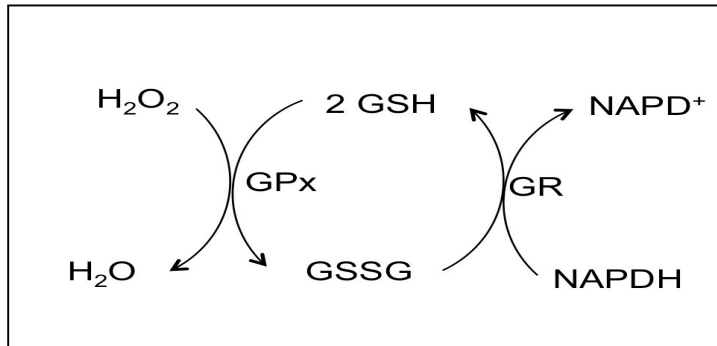


Figure 1.3. Detoxification of H₂O₂ by glutathione peroxidases.

Glutathione peroxidases (GPx) remove H₂O₂ by coupling its reduction to H₂O with the oxidation of two molecules of glutathione (GSH) forming glutathione disulfide (GSSG) that subsequently can be reduced by glutathione reductase (GR) under the consumption of NADPH.

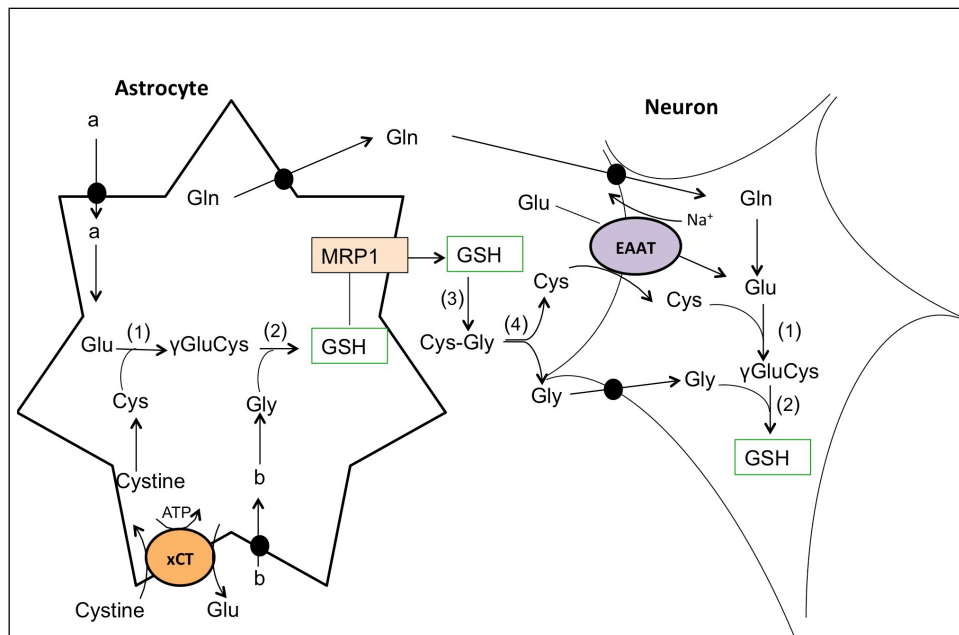


Figure 1. 4. Glutathione synthesis in neurons and astrocytes.

Within cells GSH is synthesized in consecutive ATP-requiring reactions of two enzymes GCL (1) and GSH synthetase (2). The intracellular substrates for GSH synthesis; glutamate, glycine and cysteine, may be derived from the extracellular precursors a, b and cystine (or other precursors for review see (Dringen et al, 1999a and 1999b)) respectively. Astrocytes have more efficient GSH synthesis system and higher GSH content in comparison with neurons. MRP1 transporter proteins mediate GSH export from astrocytes. In the extracellular space, GSH is hydrolysed by γ -glutamyl transpeptidase (3) to the dipeptide Cys-Gly, which then can be further hydrolysed to Cys and Gly by aminopeptidase N (4). Astrocytes import cystine via the glutamate/cystine exchange transporter (xCT) for utilization in GSH synthesis. Neurons, on the other hand, import cysteine but not cystine via the excitatory amino acid transporter (EAAT) and other transporters (refer to section 1.4.1). In addition, astrocytes release glutamine, which is used by neurons as a precursor for the glutamate, therefore providing all the necessary precursors for GSH synthesis in neurons.

1.4.2. Thioredoxins and peroxiredoxins

Thioredoxins (Trxs) are key players in protection against oxidative stress and are characterized by a conserved active site (Cys-Gly-Pro-Cys), which forms a disulfide bond between its two cysteine residues when reducing oxidized proteins (Patenaude et al., 2005). This oxidized form is then reversibly reduced to its active form by the action of thioredoxin reductase (TrxR) and NADPH (Fig. 1.5) (Holmgren, 1985).

Trxs exist in several distinct isoforms, which are localized in specific cell compartments; cytoplasmic Trx1 and mitochondrial Trx2, in addition to the more recently identified microtubule-associated Trx (Trx1-2) (Sadek et al., 2003) and a transmembrane isoform (Tmx) (Matsuo et al., 2001). The cytoplasmic (Trx1) and mitochondrial (Trx2) isoforms are widely expressed in rat brains, especially in regions with high-energy demands (Lippoldt et al., 1995; Rybnikova et al., 2000). The expression of these isoforms is subject to modulation by changes in the cellular and molecular redox-status, and also by various stress factors.

Trxs are considered to be crucial antioxidant proteins, due to their capacity to quench singlet oxygen and scavenge hydroxyl radicals. Inhibition of Trx has been shown to sensitize cells to oxidative stress (Yoshida et al., 2005), while Trx overexpression renders the cells more resistant to H₂O₂-induced cell death (Berggren et al., 2001; Bell and Hardingham, 2011). Additionally, Trx overexpressing transgenic mice can achieve extended life span and are more resistant to focal brain ischemia or kainite-mediated excitotoxicity than their wild type littermates (Patenaude et al., 2005).

Besides its antioxidant potentials, Trx is involved in various redox-regulated signalling events and plays a role in the control of many physiological processes

such as apoptosis, cell growth and differentiation. For instance, several transcription factors are targets for regulation by Trx these include; AP-1, P53 and nuclear factor kappa-B (NF κ B). It is also reported to act as potent survival factor through an inhibitory binding to the apoptosis signal-regulating kinase-1 (ASK1) (Nordberg and Arner, 2001; Powis and Montfort, 2001).

Despite its capacity to directly quench singlet oxygen and scavenge hydroxyl radicals independently of its redox state (Das and Das, 2000), Trx exerts most of its antioxidant properties through its cooperation with the peroxide scavengers peroxiredoxins (Prx) (Powis and Montfort, 2001).

Peroxiredoxins are a conserved family of proteins, which catalyse the reduction of H₂O₂ and organic peroxides, using thiol-based reducing equivalents, to water and alcohol respectively (Chae et al., 1994). The most predominant class of peroxiredoxins possesses two cysteine residues within its catalytic site, a peroxidatic residue and a resolving one. Upon the reduction of peroxides, the peroxidatic cysteine undergoes oxidation followed by the formation of disulfide bond with the resolving cysteine, which is then reduced by thioredoxin to restore the active peroxidase (Fig. 1.5) (Halliwell, 2006; Bell and Hardingham, 2011). The peroxidatic and resolving cysteines are on different molecules and so 2-Cys peroxiredoxins exist as functional homodimers. However, Prxs are susceptible to inactivation by H₂O₂-induced hyperoxidation, whereby the peroxidatic cysteine is oxidized to sulfinic derivatives (Fig. 1.5). Sulfinic derivatives are resistant to reduction by thioredoxin, therefore hyperoxidation of Prx catalytic cysteine residue to sulfinic acid (Prx-SO₂H) was thought to be irreversible. Studies on the fate of the hyperoxidized Prx have shown that it can be reduced back to its catalytically active form by the action of two enzymes sulfiredoxin (Biteau et al., 2003; Chang et al., 2004) and sestrin 2 (Budanov et al., 2004) in an ATP-dependent manner (Fig. 1.5), however, the role of the latter is controversial (Essler et al., 2009; Wood et al., 2003).

Sulfiredoxin (Srxn1) was initially identified by its robust upregulation in response to H₂O₂ and by the reduced H₂O₂ tolerance when genetically ablated (Biteau et al., 2003). The Srxn protein family is present in lower and higher eukaryotes, whose members sharing a conserved cysteine residue required for the reduction of Prx in addition to ATP-hydrolysis, Mg²⁺ and thiol as a reducing equivalent. Sulfiredoxin reduces the overoxidized Prx through catalysing the formation of a sulfinic acid phosphoric ester on Prx, which then can be reduced by thioredoxin (Rhee et al., 2007). Although Srxn prevents bursts of ROS from permanently inactivating Prx, Srxn expression, however, is tightly regulated, suggesting that Prx inactivation is desired in some circumstances to accommodate for the intracellular messenger function of H₂O₂ (Wood et al., 2003; Biteau et al., 2003).

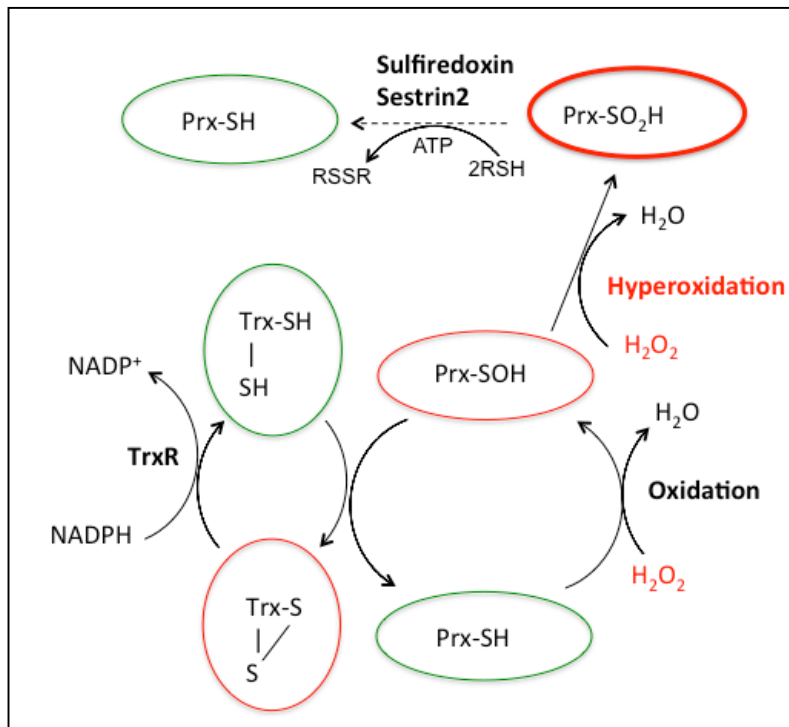


Figure 1.5. The thioredoxin-peroxidase system.

Schematic illustrating the detoxification of H₂O₂ by the classical 2-Cys peroxidases (Prx-SH) by the oxidation of peroxidatic Cys residue to cysteine sulfenic acid (Prx-SOH) and the subsequent formation of disulfide bond between the peroxidatic and resolving cysteine residues. This intermolecular disulfide bond is in turn reduced by thioredoxin resulting in the conversion of thioredoxin's 2 cysteine active site into a disulfide bond. This oxidized form is then reversibly reduced to its active form by the action of thioredoxin reductase (TrxR) and NADPH. Under increased oxidative stress, Prx-SOH can sometimes undergo further oxidation by H₂O₂ resulting in the generation of the inactive cysteine sulfinic acid (Prx-SO₂H) form, which cannot be reduced by thioredoxin. However, hyperoxidized peroxidase can be restored back to its catalytically active form by the action of two enzymes; sulfiredoxin and sestrin2.

1.4.3. Ascorbate

In addition to GSH, the brain is enriched in several low molecular mass non-enzymatic antioxidants, especially ascorbate. Ascorbate (vitamin C) is a water-soluble vitamin, present in human CSF at levels exceeding those in the plasma (Spector and Eells, 1984). In the CNS, neurons and glia are able to concentrate ascorbate further via the sodium-vitamin C transporter 2 (SVCT2) (Tsukaguchi et al., 1999; Rice, 2000). Intracellular ascorbate contributes to numerous functions in the CNS, including antioxidant protection, collagen synthesis, formation of myelin sheath (Passage et al., 2004), and protection against glutamate toxicity (May, 2012). The primary function of the vitamin is scavenging of free radicals to prevent damage of macromolecules particularly lipids (Blake et al., 1987). The antioxidant function of ascorbate is due to its electron donor properties. Given its low redox potential, ascorbate acts as broad-spectrum free radical scavenger (Rice, 2000). Due to its antioxidant activity, ascorbate is oxidized to semi-dehydroascorbate and dehydroascorbate, which then can be reduced and recycled by GSH-dependent reactions (Rose, 1993; Meister, 1994) or NADPH-dependent enzymes including thioredoxin reductase (May et al., 1997). GSH can either directly reduce ascorbate or act as an electron donor in the enzyme-dependent reduction of ascorbate (May, 2012). However, at low concentrations and in the presence of metal ions (an event which occurs during brain damage), ascorbate can be a strong prooxidant, stimulating the hydroxyl radical generation and accelerating lipid peroxidation (Halliwell and Gutteridge, 1990). However, *in vivo* studies highlight the importance of ascorbate for the CNS and for perinatal survival. For instance, mice lacking SVCT2 die within the first day of birth with cerebral haemorrhage and respiratory failure (Sotiriou et al., 2002). Moreover, supplements with dehydroascorbate, the oxidized and blood-brain barrier transportable form of vitamin C, markedly decreased infarct size, mortality and neurological deficits in mouse models of stroke (Huang et al., 2001).

1.5. The Nrf2/ARE control of antioxidant defences

Exposure of mammalian cells to elevated levels of ROS, causes damage to macromolecules, and consequently leads to various pathological conditions such as cancer and neurodegenerative diseases. Higher animals have developed adaptive, dynamic defence programs to protect against oxidative stress; central among them are the phase II detoxification proteins (involved in the detoxification of xenobiotics) and antioxidant enzymes (Ishii et al., 2002). Biochemical analysis of the promoter region of phase II genes revealed a central role for the regulatory DNA sequence element referred to as the antioxidant response element (ARE) (Rushmore et al., 1991). Studies conducted in many laboratories strongly suggest the intimate involvement of the Nrf2 protein in ARE-driven gene expression (Zhang, 2006; Nguyen et al., 2000; Itoh et al., 1997).

1.5.1. The antioxidant response element (ARE)

The ARE is a *cis*-acting regulatory element, which was originally identified in the promoters of rodent genes coding for two important antioxidant enzymes. Initially it was identified within a 41-bp section from the 5' upstream region of the rat glutathione S-transferase A2 subunit (*Gsta2*) (Rushmore and Pickett, 1990) and shortly after it was discovered in the promoter of mouse NADPH: quinone oxidoreductase 1 (*Nqo1*) (Favreau and Pickett, 1991). This enhancer sequence was designated the ARE, due to its response to phenolic antioxidants and metabolizable planar aromatic compounds such as *tert*-butylhydroquinone (tBHQ) and β -naphthoflavone respectively and was later found to respond to reactive oxygen species including H₂O₂ (Rushmore et al., 1991). The ARE consensus sequence, determined through deletion and mutational analysis studies is defined as 5'-

gagTcACaGTgAGtCggCAaaatt-3' (with the essential nucleotides shown in capitals) (Nioi et al., 2003).

Some AREs harbour two or more 12-*O*-tetradecanoylphorbol-13-acetate (TPA) - response elements (TRE) within their core sequences that are recognized by members of the activator protein-1 (AP-1) family of transcription factors (Xie et al., 1995; Presteria et al., 1995). The AP-1 transcription factor belongs to the bZip class of transcription factor, and is formed by a dimeric association between the Jun and Fos family of proteins or other proteins including activation transcription factor- 4 (ATF4) (Hai and Curran, 1991). Because of the resemblance between the AP-1 binding sequence and the ARE, AP-1 was initially believed to play a role in the ARE activation. However, despite this resemblance, studies have indicated that the transcription factors that activate the ARE sequences are quite distinct from those that activate the TRE (Nguyen et al., 1994; Lee and Johnson, 2004). More recently, data obtained from *in vivo* and *in vitro* studies has demonstrated a crucial role for the Nrf2 transcription factor in regulating ARE-dependent transcription (Itoh et al., 1997; Chanas et al., 2002; Lee et al., 2003).

1.5.2. The Nrf2 protein

Nrf2 was initially identified during attempts to screen for factors that can interact with the nuclear factor erythroid-2 (NF-E2), which regulates globin gene expression in erythroid cells (Moi et al., 1994). Unlike NF-E2, which is solely expressed in developing erythroid cells (Igarashi et al., 1994), Nrf2 is present in a wide number of tissues, particularly those that are exposed to the external environment, including lung and skin and those involved in the detoxification processes such as kidney and liver (Motohashi et al., 2002). Nrf2 belongs to the CNC (“cap and collar”) subset of the basic lucine zipper (bZip) family of transcription factors (Moi et al., 1994). This family of transcription factors also includes Nrf1 and Nrf3.

Nrf2, like the rest of the bZip proteins, functions as a heterodimeric transcription factor by pairing with other members of the family including small-Mafs (Itoh et al., 1997) and AP-1 (Venugopal and Jaiswal, 1998). While, Nrf2 binds the ARE with high affinity and specificity upon heterodimerization with small-Maf proteins (Itoh et al., 1997), overexpression of Maf proteins, which lack intrinsic transactivation activity (Motohashi et al., 2002), serves to suppress Nrf2-mediated ARE activation (Nguyen et al., 2000). One plausible explanation for this observation is that Maf proteins are capable of binding DNA as homodimers thereby restricting Nrf2 access to the ARE promoter (Dhakshinamoorthy and Jaiswal, 2000).

In addition to small-Mafs, other members of the bZip family have also been reported to form heterodimers with Nrf2 serving to modulate its transcriptional activity. Jun and Fos have been shown to interact with Nrf2 and differentially regulate the transcriptional activity of Nrf2. Co-expression of Nrf2 and Jun was reported to activate ARE-mediated transcription in human hepatoma cells (Venugopal and Jaiswal, 1998), whereas co-expression of Nrf2 and Fos had the opposite effect on ARE activity (Venugopal and Jaiswal, 1996).

A cross-species comparison of Nrf2 amino acid sequence enabled the identification of six well-conserved domains (Itoh et al., 1995), termed Nrf2-ECH homology (Neh) domains (Itoh et al., 1997) (Fig. 1.6). The Neh1 (residues 427-560) domain is located in the C-terminal half of the protein and contains the conserved CNC and bZip motifs responsible for DNA binding and dimerization with small-Maf proteins (Itoh et al., 1999a). The Neh2 (residues 1-96) is a redox-sensitive degron located in the proximal N terminus and contains the interaction points with the E3 ubiquitin ligase adaptor Keap1 (Itoh et al., 1999b). Adjacent to Neh2 are the main transactivation domains (TADs); Neh4 (residues 111-141) and Neh5 (residues 172-201) (Kato et al., 2001). Within the central part of Nrf2 lies the Neh6 (residues 330-380) degron, which is responsible for the interaction with the newly identified E3 ubiquitin ligase adaptor β -transducin repeat-containing protein (β -TrCP) (Rada et al., 2011). In contrast to the other domains, the Neh3 (residues 561-597) located at the C-terminus

of Nrf2, has not been well characterized. However, a study suggests that Neh3 may act as a transactivation domain (Nioi et al., 2005).

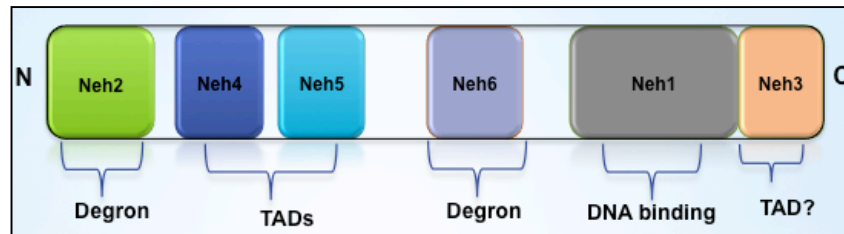


Figure 1. 6. Nrf2 functional domains.

Schematic representation of the six conserved functional domains in the mouse Nrf2 protein. Refer to above text for details.

Although growing evidence emphasizes the central role of Nrf2 in the regulation of ARE-driven gene expression (Copples et al., 2010). Nrf1 also appears to have an overlapping function with Nrf2 in the regulation of ARE. However, Nrf1 is less potent than Nrf2 at transactivating ARE-dependent gene expression as demonstrated by reporter genes expression driven by ARE-containing sequences from the promoters of mouse *Nqo1* and *Gclm* as well as the human *PBGD* (porphobilinogen deaminase), whereby Nrf1 exhibited less transactivation activity in comparison to Nrf2 (Zhang et al., 2006). Furthermore, Nrf1- knockout mice embryos die during mid gestation as a result of foetal liver abnormalities and subsequent anaemia (Chan et al., 1998).

In contrast to Nrf1 KO mice, Nrf2 is dispensable for normal growth and development (Chan et al., 1996), however, Nrf2 KO mice have lower basal and inducible expression of phase II enzymes, and display enhanced susceptibility to a variety of diseases, including cancer (Fahey et al., 2002), neurodegeneration (Johnson et al., 2008), and inflammation (Rangasamy et al., 2005). Moreover, aged Nrf2 KO mice develop lupus-like autoimmune symptoms (Yoh et al., 2001; Ma et al., 2006) and

vacuolar leukoencephalopathy (Hubbs et al., 2007). Contrary to the extensively studied homolog Nrf2, our current knowledge on the function and role of Nrf3 in the protection against cellular stress is less advanced. Furthermore, Nrf3 null mice develop normally and exhibit no obvious phenotype (Derjuga et al., 2004).

1.5.3. Keap1

In the cytoplasm, Nrf2 associates with the kelch-like ECH-associated protein 1 (Keap1) forming a regulatory system, which serves as a sensor of oxidative and electrophilic stress and controls the transcriptional activity of Nrf2 (Giudice and Montella, 2006). Keap1 is an actin cytoskeleton binding protein homologous to the *Drosophila* Kelch protein. It consists of three distinctive domains; the BTB domain at the N-terminal region involved in the homodimerization of the protein (Zipper and Mulcahy, 2002), a cysteine-rich region known as the linker or intervening region (IVR) crucial for the activity of Keap1 (Zhang and Hannink, 2003) and a C-terminal double glycine (DGR or Kelch) region comprising six conserved Kelch motif repeats forming the Nrf2/Keap1 binding site (Fig. 1.7) (Li et al., 2004). According to previous studies, the amino acid sequence of Keap1 is highly conserved among human, mouse and rat (Giudice and Montella, 2006).

Keap1 was initially described as a negative regulator of Nrf2. Under normal physiological conditions, Keap1 sequesters Nrf2 in the cytoplasm, preventing it from traveling to the nucleus and activating ARE-dependent gene expression. However, when cells are exposed to oxidative or electrophilic stimuli Nrf2 evades Keap1-mediated suppression, translocates to the nucleus, and activates ARE-driven phase II detoxifying and antioxidant genes (Zhang, 2006). Studies using functional ARE reporter assays, revealed that overexpression of Keap1 in quail fibroblast cells (Itoh et al., 1999b), rodent neuronal and glial primary cultures (Soriano et al., 2008a) and zebrafish (Kobayashi et al., 2002), reduces Nrf2-mediated transactivation of the ARE-reporter gene.

Furthermore Keap1 has an inhibitory effect on the Nrf2/ARE signalling pathway not only by anchoring Nrf2 in the cytoplasm but also by targeting Nrf2 for ubiquitination and subsequent proteasomal degradation. In unstressed cells, Nrf2 exhibits a short half-life of 10-30 mins (Alam et al., 2003; He et al., 2006). The rapid degradation of Nrf2 protein under basal conditions has been attributed to constitutive ubiquitin-proteasomal degradation (Zhang and Hannink, 2003; Cullinan et al., 2004). Keap1 was shown to mediate Nrf2 ubiquitination and proteasomal degradation, through its function as a substrate adaptor for a Cullin-dependent E3 ubiquitin ligase complex (Cullinan et al., 2004; Kobayashi et al., 2004; Zhang et al., 2004; Furukawa and Xiong, 2005).

Several models have been proposed for the Nrf2-Keap1 interaction (Fig. 1.8). The widely accepted model was first proposed by Dinkova-Kostova *et al.*, in which modifications of specific Keap1 cysteine residues by ARE-inducers lead to the liberation of Nrf2 (Dinkova-Kostova et al., 2002). Other studies have proposed Nrf2 phosphorylation as a mechanism for the release of Nrf2 from Keap1-mediated repression (Bloom and Jaiswal, 2003; Huang et al., 2002), however, this model is debatable. Accumulating evidence from various laboratories, suggests that ARE inducers such as tBHQ don't evoke complete dissociation of Nrf2 from Keap1. Conversely, such molecules may actually increase the association of Nrf2 with Keap1, probably via disrupting Keap1-mediated degradation. In support of this, Nrf2 translocation into the nucleus following the application of Nrf2 inducers such diethylmaleate (Itoh et al., 2003) or tBHQ, (Kobayashi et al., 2006) was inhibited by cycloheximide. This observation was first documented by Itoh *et al.* (Itoh et al., 2003) and later by others (Eggler et al., 2005; Zhang, 2006), forming the initial basis for the "hinge and latch" or the two-site substrate recognition model. In this model Nrf2 is proposed to interact with the Keap1 homodimer via two distinct sites within the Neh2 domain; the low-affinity DLG and the high-affinity ETGE motifs (Tong et al., 2007). Under conditions of oxidative/chemical stress, conformational changes occur in the Keap1 IVR domain provoking the loss of the DLG motif binding, whilst Nrf2 association with Keap1 is still maintained through the high-

affinity ETGE binding motif. Consequently, Nrf2 is no longer positioned in the correct orientation to accept ubiquitin and thus Nrf2 proteasomal degradation is ceased. As a result, Keap1 becomes saturated with Nrf2 that cannot be degraded efficiently, allowing newly synthesized Nrf2 to translocate into the nucleus and activate ARE-dependent transcription (McMahon et al., 2006). Of note, a very recent study provided evidence suggesting that inhibition of Nrf2 ubiquitination by prototypical Nrf2 inducers (sulforaphane and 5,6-dihydrocyclopenta-1,2-dithiole-3-thione (CPDT)) doesn't result from Nrf2 release from Keap1, as Nrf2-Keap1 complex were detected in both the nucleus and cytoplasm following drug application (Li et al., 2012). Finally, an alternative model proposes Cul3 dissociation from Keap1 as a mechanism of Nrf2 activation (Rachakonda et al., 2008).

Additionally, the Cuadrado group recently reported a Keap1- independent Nrf2 degradation mechanism, in which phosphorylation of the Neh6 domain of Nrf2 by glycogen synthase kinase (GSK), leads to ubiquitination via a β -TrCP /Cul1 E3 ligase complex (Rada et al., 2011).

In addition to Keap1, other proteins have been implicated in the control of Nrf2 activity. Wang and Zhang recently reported a novel Nrf2- repressor termed the ectodermal neural cortex-1 (ENC1), which belongs to the same family as Keap1 and decreases the rate of Nrf2 synthesis (Wang and Zhang, 2009). Another group described the involvement of cancer and Parkinson's disease associated protein DJ1/PARK7 in stabilizing Nrf2 protein thereby conveying protection against stressors (Clements et al., 2006). More recently Maruyama *et al.* has identified [KRAB (Krüppel-associated box)-associated protein 1] KAP1 as a novel Nrf2-NT-interacting protein facilitating Nrf2 transactivation activity (Maruyama et al., 2011).

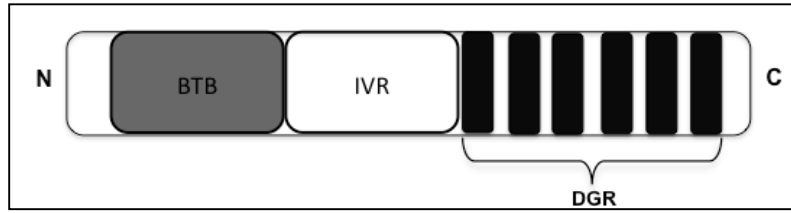


Figure 1. 7. Keap1 functional domains.

Schematic representation of the three major functional domains in the mouse Keap1 protein. Refer to above text for details.

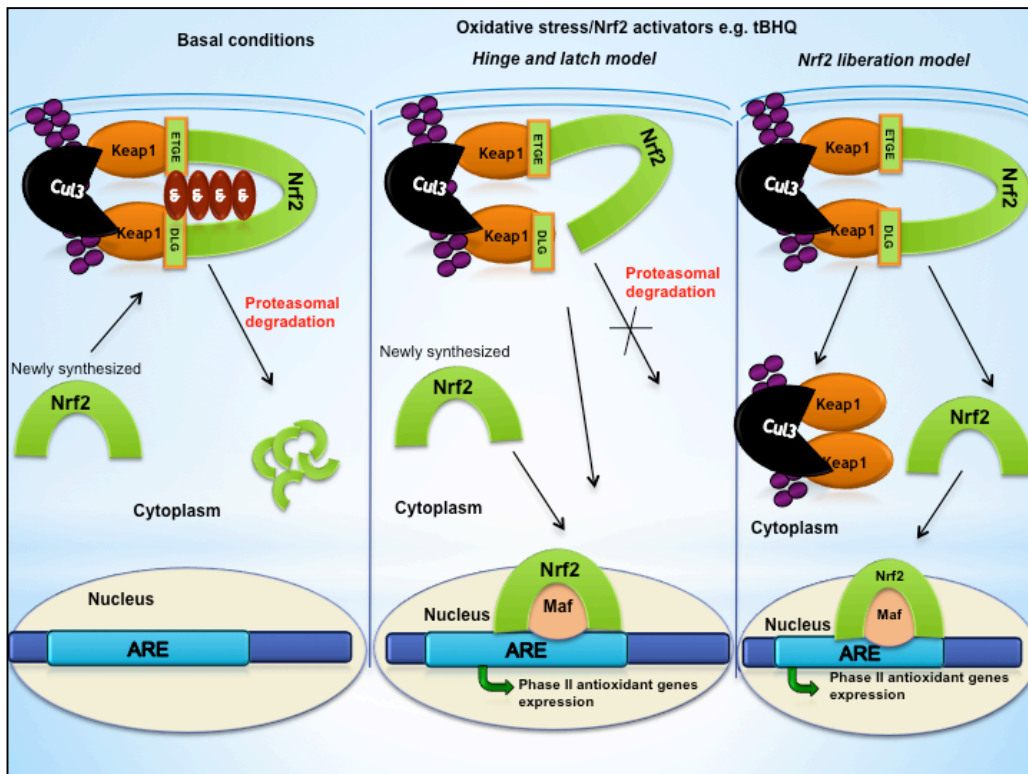


Figure 1. 8. Proposed models of Nrf2 activation.

Under basal homeostatic conditions, Keap1 anchors Nrf2 in the cytoplasm via two binding sites in the Neh2 domain, the high affinity ETGE motif and the low affinity DLG motif, and functions as an adaptor protein for the Cul3-based E3 ligase system, facilitating the rapid ubiquitination and proteasomal degradation of Nrf2. Two main models of Nrf2 activation (in response to oxidative stress or Nrf2 inducers) have been proposed; the hinge and latch and the Nrf2 liberation model. In the hinge and latch model, oxidative stress or Nrf2 inducers cause conformational changes within Keap1 resulting in the detachment of the weakly binding DLG motif (latch) from Keap1, whilst the high-affinity ETGE (hinge) is maintained. Consequently, Nrf2 ubiquitination and the subsequent proteasomal degradation are abolished. Newly synthesized Nrf2 translocates into the nucleus, where it transactivates ARE-driven

phase II genes. In the liberation model, oxidative stress/Nrf2 activators induce chemical modifications in either Keap1 or Nrf2 or both, resulting in the release of Nrf2 from the Keap1/Cul3 complex.

1.6. Nrf2 and neurodegeneration

A growing body of evidence demonstrates the neuroprotective ability of Nrf2 and its downstream genes against a variety of insults in both cell culture and animal models of neurodegeneration. The presence of Nrf2 has been shown to contribute to neuroprotection, or conversely, to increase sensitivity when Nrf2 is lacking. The activation of the Nrf2 pathway has been shown to increase neuronal resistance to oxidative insults triggered by H₂O₂ (Li et al., 2002) and glutamate (Murphy et al., 1991) in neuronal cell lines, as well as in primary cortical cultures (Kraft et al., 2004).

In recent years, a great deal of research has been focused on understanding the status of the Nrf2-ARE pathway in different models of neurodegenerative diseases. Nrf2 status was mainly inferred from the subcellular distribution of Nrf2 and the expression level of ARE-driven genes.

Studies performed on *in vitro* models of amyotrophic lateral sclerosis (ALS), revealed a reduction of Nrf2 mRNA and protein expression in the primary motor cortex and in the spinal cord neurons derived from ALS postmortem tissues (Sarlette et al., 2008). A similar decrease in mRNA encoding Nrf2 was observed in embryonic motor neurons isolated from rat models of ALS (Pehar et al., 2007). On the contrary, laser-capture microdissected motor neurons from mouse models of ALS show no change in Nrf2 expression or in ARE-driven genes (Ferraiuolo et al., 2007). Despite these apparent discrepancies, changes in the expression levels of Nrf2 and its target gene heme oxygenase-1 (Hmox1) (Alam et al., 2003) were reported in SOD1 G93A mouse model of ALS (Vargas et al., 2005). Hmox1 is an oxidative stress inducible cytoprotective enzyme that catalyses the conversion of haem to bilirubin, CO and

free iron (Ryter and Choi, 2002). Elevated Hmox1 expression has been reported in neurodegenerative diseases including ALS. Increased Nrf2 and Hmox1 expression was found in the spinal cord of early- stage symptomatic SOD1 G93A rats, which may represent an endogenous neuroprotective response in early stages of the disease that could be overwhelmed with the disease progression by other mechanism(s) leading to neuronal loss (Vargas et al., 2005). Moreover, pharmacological or genetic activation of Nrf2 rescues motor neurons from toxicity mediated by astrocytes expressing mutated human SOD1 *in vitro* and delays onset and increases life span in ALS mouse models when over expressed under the control of an astrocyte specific promoter (Nagai et al., 2007; Vargas et al., 2006; Vargas et al., 2008). This was one of the earliest *in vivo* studies to demonstrate the beneficial effect of Nrf2 activation in astrocytes on neighbouring neurons in chronic neurodegenerative conditions.

In the case of PD, postmortem PD brain tissue analysis revealed an increase in HMOX-1 (Schipper, 2004) and NQO1 expression (van Muiswinkel et al., 2004) in glial cells and induced nuclear localization of Nrf2 in the substantia nigra, however, this response appears to be insufficient to confer neuroprotection against degeneration (Ramsey et al., 2007). *In vitro* Nrf2 activation has been shown to limit the extent of neurotoxicity resultant from the exposure to dopamine analogue 6-hydroxydopamine or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Yamamoto et al., 2007; Jakel et al., 2007; Wruck et al., 2007). In addition Nrf2 KO mice display increased MPTP sensitivity, whereas astrocyte-specific Nrf2 overexpression in a Nrf2-KO background completely reverses MPTP toxicity (Chen et al., 2009). Furthermore, activation of Nrf2 *in vitro* by tBHQ or *in vivo* via Nrf2-overexpressing astrocyte grafts, resulted in protection against 6-hydroxydopamine (Jakel et al., 2007).

Similar to PD, data obtained from immunohistochemistry experiments performed in post-mortem temporal cortex and hippocampus of AD patients showed a significant increase in astrocytic HMOX-1 expression compared to the non-demented group

(Schipper et al., 2006). In addition to HMOX-1, NQO1 expression and activity was reported to be elevated both in neurons and astrocytes from individuals with AD (Raina et al., 1999; Wang et al., 2000; Santa-Cruz et al., 2004). In contrast to the nuclear localization of Nrf2 in substantia nigra neurons from PD cases, Nrf2 staining in hippocampal neurons of AD cases was predominantly cytoplasmic. The aberrant distribution of Nrf2 in AD could be due to differential regulation of Nrf2 localization in various neuronal subpopulations (Ramsey et al., 2007). Moreover, the expression of Nrf2 and its target genes including Nqo1 was reduced in mouse models of AD (APP/PS1) upon accumulation of A β deposits. However, Nrf2 induction either by tBHQ or through adenovirus-mediated overexpression protected cultured neurons from A β toxicity (Kanninen et al., 2008).

The status of the Nrf2-ARE pathway is less clear in Huntington's disease (HD). However, Nrf2 activation was shown to protect neurons against mitochondrial toxins (e.g. 3-nitropropionic acid (3-NP) and malonate), which produce striatal degeneration similar to that observed in HD. Nrf2 deficiency in primary neurons potentiated 3-NP toxicity and rendered mice more vulnerable to striatal lesions caused by administration of 3-NP or malonate (Calkins et al., 2005; Calkins et al., 2009). Dietary administration of tBHQ or adenoviral overexpression of Nrf2 attenuated 3-NP-induced striatal lesioning (Shih et al., 2005). In addition, Nrf2-overexpressing astrocytes grafted into the striatum provided remarkable protection to the surrounding neurons against malonate toxicity (Calkins et al., 2009).

Besides its beneficial role in neurodegenerative diseases, Nrf2 activation was reported to protect in acute neurodegenerative scenarios such as ischemia and intracerebral haemorrhage. Work from the Murphy laboratory has demonstrated the neuroprotective effect of Nrf2 activation in ischemic injury, whereby intracerebral or intraperitoneal tBHQ administration, significantly reduced cortical damage and sensorimotor deficit at 24 h and up to 1 month after ischemic-reperfusion in rats. In contrast, loss of Nrf2 function exacerbated cortical damage after permanent ischemia

and abolished the protective effect of tBHQ (Shih et al., 2005). In line with this, systemic administration of the Nrf2 inducer sulforaphane has been shown to decrease cerebral infarct volume following focal ischemia (Zhao et al., 2006) and sulforaphane treatments significantly reduced neurologic deficit produced by intracerebral haemorrhage in Nrf2 WT not KO mice (Zhao et al., 2007).

1.7. The locus of Nrf2 activation in the CNS

The locus of Nrf2 activation in brain tissues is a controversial aspect of the Nrf2 pathway. A large number of studies have shown that activation of the Nrf2 pathway preferentially occurs in astrocytes not neurons. For instance, in rodent primary cerebellar neuronal cultures, basal transcriptional levels and enzymatic activity of the ARE-driven antioxidant enzymes NAD(P)H:quinone oxidoreductase (QR) and glutathione-S-transferases (GSTs), are remarkably greater in astrocytes than in neurons. Moreover, inducibility following the application of tBHQ or hydroquinone was only visible in astrocytes (Ahlgren-Beckendorf et al., 1999). Analysis of the ARE-driven gene expression in rat brain slices and primary cortical cultures, using a heat-stable human placental alkaline phosphatase reporter (hPAP), revealed that ARE-mediated gene expression was almost exclusive to the astrocyte subpopulation (Murphy et al., 2001). In line with this, a study using primary cortical cultures derived from hPAP-ARE transgenic mice reported that the increase in hPAP activity post-tBHQ or sulforaphane application was predominantly associated with GFAP-positive astrocytes as seen by immunofluorescence staining using an antibody against hPAP in conjunction with anti-GFAP. They also combined the use of cell sorting and gene chip technology in an effort to determine the cell-specific contribution to tBHQ-mediated ARE activation and found that the majority of the ARE-driven genes up-regulated by tBHQ were observed in astrocyte enriched populations (Kraft et al., 2004).

On the contrary, some studies have documented a functional Nrf2 pathway in neurons. Johnson *et al.* reported an increased histochemical staining for hPAP and

NQO1 activity after tBHQ treatment in both glia and neurons in primary cortical neuronal cultures derived from transgenic reporter mice for ARE core sequence coupled to hPAP (Johnson et al., 2002). They also showed in a separate study, cellular staining of ARE-hPAP in motor neuron cell bodies and astrocyte processes of 90 day old mice expressing mutant *SOD1* (Kraft et al., 2007). In line with this notion, a more recent study suggesting that Nrf2 plays a role in the transcriptional regulation of excitatory amino acid transporter 3 (EAAT3) in the context of oxidative stress, has shown increased EAAT3 expression and GSH levels in neurons of mice that have received striatal injections of either tBHQ or in neurons overexpressing Nrf2 under a neuron-specific promoter (Escartin et al., 2011). In addition, treatment with sulforaphane was shown to activate the ARE/Nrf2 pathway in hippocampal neurons conferring protection against oxygen glucose deprivation (OGD)-induced cell death via Nrf2-dependent gene expression (Soane et al., 2010).

1.8. The role of astrocytes in neurodegenerative diseases

Decades of neuropathological studies have been neuron-centric in addressing the causes of these disorders, but there is growing evidence pointing towards the important role of astrocytes in determining neuronal survival and demise. Until recently, the general thinking has been that the main function of astrocytes along with other cells of glial lineage is to hold neurons together. However, it is becoming clear that astrocytes serve many housekeeping functions, including amino acid metabolism, nutrient transport, ion homeostasis and modulation of excitatory synaptic transmission (Maragakis and Rothstein, 2006).

Studies performed on human tissue and transgenic models of ALS have provided evidence that astrocytic abnormalities and dysfunction precede clinical disease. For instance, markers of astrocytosis were detected months prior to the clinical onset in the spinal cord of G85R SOD1 mice (Bruijn et al., 1997). Similarly, Howland and co-workers reported loss of glutamate transporter EAAT2 (GLT-1) with concomitant astrocytosis preceding motor neuron degeneration and the clinical onset of the disease (Howland et al., 2002). However, EAAT2 overexpression in astrocytes of mutant SOD1 mouse model increased motor neuron survival and delayed disease onset, which indicate that astrocytic EAAT2 influences the timing of disease onset and motor neuron survival (Howland et al., 2002; Guo et al., 2003). Mutant SOD1 expression in neurons or motor neurons was insufficient to lead to neuronal death except when neurons were surrounded by mutant SOD1 expressing astrocytes (Clement et al., 2003).

The association of reactive astrocytes with neuritic plaques was first observed by Alois Alzheimer and has been subsequently confirmed to be a morphological characteristic of plaque-infested AD (Wisniewski and Wegiel, 1991). While activated astrocytes may mediate the cytotoxic effects of A β by releasing interleukins, cytokines and nitric oxide among other potentially cytotoxic molecules (Gitter et al., 1995; Griffin et al., 1998; Hu et al., 1998; Wallace et al., 1997), accumulating evidence demonstrates the involvement of astrocytes in the clearance of A β (DeWitt et al., 1998; Koistinaho et al., 2004; Matsunaga et al., 2003). However, A β internalization appears to induce detrimental changes in astrocytes including oxidative stress, mitochondrial dysfunction, accumulation of intracellular calcium and depletion of glutathione (Abramov et al., 2003; Abramov et al., 2004).

Astrocytes have a dual role in PD, as they can be injurious or protective depending on the context. They can confer neuroprotection to dopaminergic neurons either through the clearance of extracellular α -synuclein or through the release of

antioxidants and trophic factors. α -synuclein is a small protein abundantly available in the presynaptic terminals, which has the tendency to form insoluble neurotoxic aggregates under various pathological conditions (Rappold and Tieu, 2010). Under pathological conditions, astrocytes appear to induce neurodegeneration by releasing cytotoxic molecules including the active toxic cation MPP^+ , a product of MPTP metabolism by MAO-B, which is readily taken up by neighbouring dopaminergic neurons and terminals where it induces neurotoxicity. In addition, proinflammatory cytokines are also released from astrocytes as a result of α -synuclein accumulation in astrocytes beyond their degradation capacity (Rappold and Tieu, 2010).

1.9. The NMDAR control of pro-death and pro-survival pathways

1.9.1. NMDA receptors in the CNS

NMDARs are cation channels gated by glutamate, the predominant excitatory neurotransmitter in the CNS, and are permeable to Na^+ , K^+ and Ca^{2+} ions. They play a key role in synaptic transmission and in mediating synaptic plasticity, learning and memory. Besides NMDARs, α -amino-3-hydroxy 5-methyl 4-isoxazolepropionic acid receptors (AMPA receptors) and Kainate receptors are also glutamate gated ion channels that act in concert with NMDARs to mediate glutamatergic neurotransmission. However, most of the neurochemical foundations of perception, learning and memory are set by synaptic activity via the NMDARs (Bliss and Collingridge, 1993; Aamodt and Constantine-Paton, 1999). NMDARs are also present on oligodendrocytes (Karadottir et al., 2005) and astrocytes (Schipke et al., 2001), as well as non-neural cells such as osteoclasts (Szczesniak et al., 2005) and lymphocytes (Tuneva et al., 2003), however, NMDARs function in these types of cells is not well-defined.

The majority of mammalian NMDARs in the CNS occur as hetero-tetramers and are typically comprised of a pair of glycine-binding GluN1 subunits and another pair of glutamate-binding GluN2 subunit. Four types of GluN2 subunits (GluN2A-D) in

addition to 2 types of GluN3 subunits (GluN3A and GluN3B) provide NMDARs variety (Kohr, 2006). Studies of NMDAR subunit expression in the developing rat CNS revealed that GluN2B and GluN2D subunits occur prenatally, whereas GluN2A and GluN2C start to appear near birth. GluN1 however, is ubiquitously expressed throughout development and in the adult brain (Monyer et al., 1994).

The NMDAR receptor display several characteristic features that distinguish it from other types of glutamate receptors, those include; relatively high permeability to Ca^{2+} compared to AMPAR and Kainate receptors, voltage dependent block by extracellular Mg^{2+} and modulation by glycine (Masu et al., 1993; Cull-Candy et al., 2001). Normally, at negative resting membrane potential, the NMDAR pore is directly blocked by Mg^{2+} ions preventing other ions (Ca^{2+} , Na^+ and K^+) from passing through freely. This block is lifted following membrane depolarization, which occurs when presynaptic glutamate release causes Na^+ influx through AMPAR in the postsynaptic cell. Thus, allowing the NMDARs to function as “coincidence detectors”, whereby glutamate release must synchronize with membrane depolarization before the channel will pass current.

Ca^{2+} entry through the NMDARs can lead to a range of alterations in neuronal function through regulation of channel opening, neurotransmitter release and also by influencing gene expression (Burgoyne, 2007). Excessive Ca^{2+} influx can trigger cell death. The differing outcomes of NMDAR-mediated Ca^{2+} influx are dependent on the magnitude of the stimulus, be it intensity or duration, and the location of the receptors (synaptic vs. extrasynaptic) (Soriano and Hardingham, 2007).

At the synapse NMDAR are organized in multiprotein signalling complexes embedded within a dense protein matrix forming a distinct specialization known as the postsynaptic density (PSD) (Sheng, 2001). NMDARs are anchored in the PSD through interaction between cytoplasmic C-termini of GluN2 subunits, and the PDZ

domains of PSD-95 (Collins et al., 2006). PSD-95, is a prominent organizing protein in the PSD and a member of the membrane-associated guanylate kinase (MAGUK)

superfamily of proteins, which couples NMDARs to intracellular proteins and signaling enzymes such as nitric oxide synthase (nNOS), protein phosphatase 2b (calcineurin) and calmodulin dependent protein kinase II (CamKII) (Waxman and Lynch, 2005). As such, PSD facilitates NMDAR activation and the subsequent signal transduction.

1.9.2. Death and survival signalling from the NMDAR

Excessive NMDAR activity can contribute to neuronal loss in acute events such as traumatic brain injury and seizure, as well as certain chronic neurodegenerative diseases such as AD. However, physiological levels of synaptic NMDAR activation can promote neuroprotection (Papadia and Hardingham, 2007). Thus, responses to NMDAR activity follow a bell shaped curve: both excessive and poor activity can be harmful (Lipton and Nakanishi, 1999). Moreover, the nature of response to an episode of NMDAR activity is influenced by the stimulus intensity, NMDAR location and subunit composition (Martel et al., 2012). Recent studies show that stimulation of synaptic NMDAR can promote neuroprotection, whereas activation of extrasynaptic NMDAR promotes cell death (Hardingham and Bading, 2010).

1.9.2.1. Pro-survival signalling from the NMDAR

Neuronal health is more complex than that offered by the dichotomous description of neurons as either dead or alive. According to the dynamic equilibrium theory for neuronal health, neurons exist in a spectrum of states ranging between protected and fully functional to vulnerable and dysfunctional. A neuron's status within this spectrum is constantly influenced by internal and external stimuli (Isacson, 1993),

but is known to be shifted towards health and robustness by NMDAR-mediated synaptic activity (Papadia and Hardingham, 2007).

The notion that activity can be survival promoting stemmed from previous studies in which activity blockade caused death in the disconnected neurons (Mennerick and Zorumski, 2000). Analysis of brain sections from rat pups treated with NMDAR antagonist (MK-801) during the first postnatal week, displayed increased morphological changes associated with cell death within the dentate gyrus (Gould et al., 1994). In the developing brain, elimination of NMDAR activity triggers widespread apoptotic neurodegeneration in the rat forebrain (Ikonomidou et al., 1999), increases the rate of apoptotic elimination of granule neurons (Monti and Contestabile, 2000), causes a large increase in cell death in the somatosensory thalamus (Adams et al., 2004) and also enhances trauma-induced injury (Pohl et al., 1999). In addition, NMDAR blockade exacerbates neuronal loss in mature brain already undergoing neurodegeneration or subjected to traumatic injury (Ikonomidou et al., 2000), and prevents the survival of newly generated neurons in the adult dentate gyrus (Tashiro et al., 2006).

The activity-dependent neuroprotection has been recapitulated *in vitro* in neuronal cultures where enhanced synaptic NMDAR activity has been shown to render neurons more resistant to various death-inducing stimulus including trophic deprivation, retinoic acid (Papadia et al., 2005) and H₂O₂ (Papadia et al., 2008). Also, in mouse cortical cultures, the protective effect of oxygen glucose deprivation (OGD) preconditioning, in which transient exposure to non-lethal episode of OGD increases tolerance to a subsequent more lethal episode of OGD, was shown to be sensitive to NMDAR antagonists (Grabb and Choi, 1999). The protective role of endogenous NMDAR activity in injured brain is one of the contributing factors to the failure of clinical trials for stroke utilizing NMDAR antagonists (Ikonomidou and Turski, 2002).

1.9.2.1.1. Anti-apoptotic effect of synaptic NMDAR signalling

Physiological levels of synaptic NMDAR activation maintain neuronal health, longevity and boosts endogenous antioxidant defences (Papadia et al., 2008). Synaptic NMDAR activity confers neuroprotection via triggering posttranslational modification of existing proteins or changes in gene expression. The PI3K/Akt pathway is a key signalling pathway through which synaptic NMDAR activity exerts a neuroprotective effect. PI3K/Akt activity causes inhibitory phosphorylation of the pro-apoptotic Bcl-2 family member BAD (Brunet et al., 2001), as well as glycogen synthase kinase-3 beta (GSK3 β); a kinase closely involved in neuronal apoptosis (Hetman et al., 2000) and implicated in various neurodegenerative diseases (Kaytor and Orr, 2002). In addition, Akt controls the activity of two pro-death transcription factors; p53 and FOXO. P53 has the ability to induce the transcription of various pro-death genes, including those encoding Bax, Noxa and Puma (Amaral et al., 2010). Activated Akt was shown to promote survival in hippocampal neurons by inhibiting the activity of p53 (Yamaguchi et al., 2001). The underlying mechanism, however, doesn't appear to involve direct phosphorylation of p53 by Akt, but rather the phosphorylation /activation of the p53 negative regulator Mdm2 (murine double minute-2) by Akt (Ogawara et al., 2002). On the other hand, Akt directly phosphorylates Foxos, triggering their nuclear export and inhibiting their ability to induce the expression of pro-death genes (Leveille et al., 2010). Finally, stimulation of synaptic NMDAR activity promotes the nuclear exclusion of both Foxo and GSK3 β in a PI3K-dependent fashion (Soriano et al., 2006).

Besides pro-death gene suppression, synaptic activity also promotes neuroprotection by inducing the expression of survival genes. One important mediator of activity-dependent gene expression is the transcription factor cyclic-AMP response element binding protein (CREB), which binds to the cAMP response element and transactivates a number of pro-survival genes. CREB has a well-documented role in neurosurvival. For instance, CREB-dependent gene expression was found to be

required for the long-lasting activity-dependent neuroprotection against apoptotic (Papadia et al., 2005) and excitotoxic insults (Lee et al., 2005). More recently, studies by Zhang *et al.* identified a nuclear calcium-regulated genomic program that contribute to synaptic NMDAR-mediated acquired neuroprotection. This program consists of about dozen genes, termed activity-regulated inhibitors of death (*AID*), which have been shown to confer neuroprotection both in cell culture and in animal models of neurodegeneration (Zhang et al., 2009). Some of these genes, which include B-cell translocation gene 2 (*Btg2*) and B-cell lymphoma 6 (*Bcl6*), appear to be CREB targets and may provide neuroprotection through enhancing mitochondrial resistance to cellular stress or toxic insults. Brain derived neurotrophic factor (BDNF), which has a well-characterized neuroprotective effect, is another target for synaptic NMDAR and nuclear Ca^{2+} -CREB signalling (Hardingham and Bading, 2010).

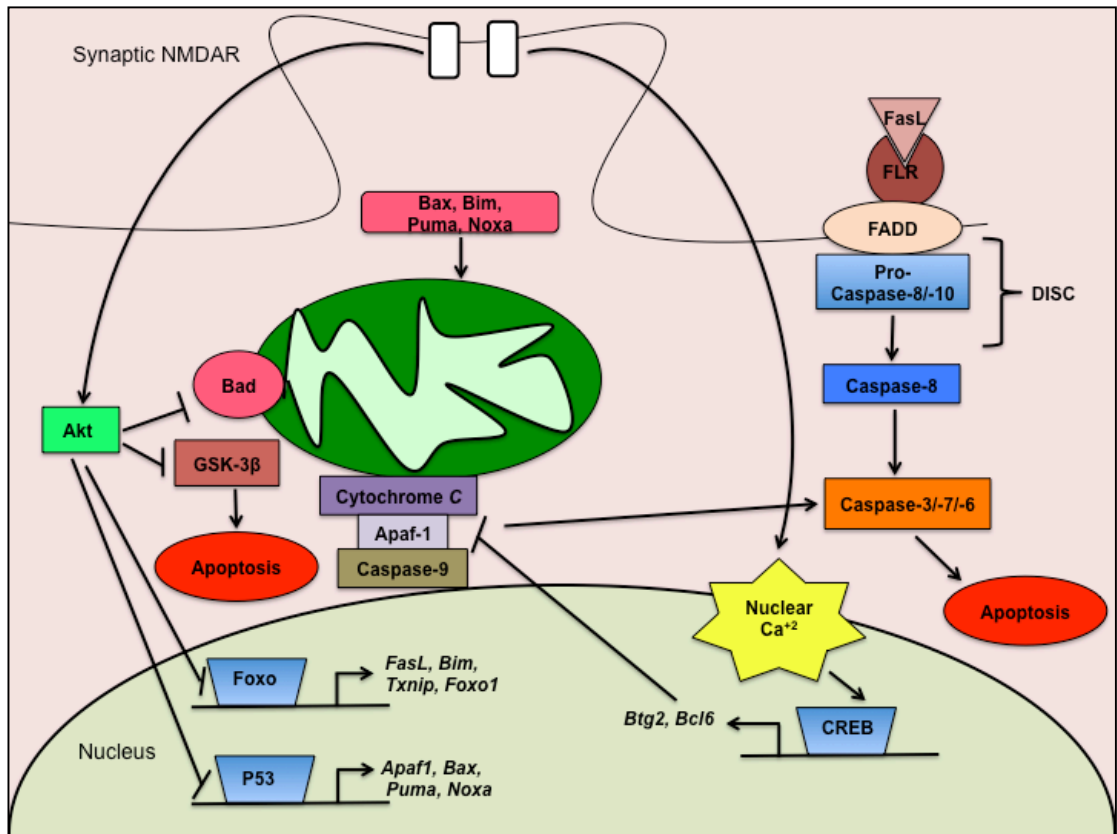


Figure 1.9. Anti-apoptotic effect of synaptic NMDAR activity.

Synaptic NMDAR signalling suppresses the intrinsic apoptosis cascade at multiple levels. PI3K/Akt pathway is a key signalling pathway through which synaptic NMDAR activity inactivates pro-death transcription factors such as Foxos and p53 and results in the subsequent suppression of their target genes expression. Activation of PI3K/Akt also leads to the inhibitory phosphorylation of Bad and GSK3 β . Synaptic activity also suppresses the extrinsic pathway via the downregulation of Foxo-mediated FasL expression. In addition, synaptic activity induces the expression of pro-survival genes (as shown) via a number of transcription factors including CREB (for review see (Hardingham and Bading, 2010)).

1.9.2.1.2. Antioxidant effect of synaptic NMDAR signalling

In addition to their well-established anti-apoptotic effects, physiological patterns of synaptic NMDAR activity can also confer protection against oxidative insults by enhancing neuronal intrinsic antioxidant defences. In an early study conducted in our laboratory, we showed that complete NMDAR blockade increases neuronal sensitivity to H₂O₂-induced cell death, while enhancing synaptic activity protects against it (Papadia et al., 2008). Moreover, suppression of physiological NMDAR activity promotes neuronal apoptosis associated with oxidative damage in P6 mice cortex (Papadia et al., 2008). Investigation into the molecular basis for this protection revealed a mechanism that at least in part, involves changes within the thioredoxin-peroxiredoxin antioxidant system. Synaptic activity prevented the oxidative insult-induced overoxidation of Prx (an antioxidant enzyme described earlier in section 1.4.2) by enhancing the expression of the two genes, *Srxn1* and *Sesn2*, whose products are involved in resorting the hyperoxidized Prx to its active form (Papadia et al., 2008). The activity-dependent up-regulation of *Srxn1* was mediated mainly by two AP-1 sites (Papadia et al., 2008; Soriano et al., 2008). Interestingly, synaptic activity not only boosts the expression of these genes but also suppresses the expression of the thioredoxin inhibitor *Txnip*, a Foxo target gene, through promoting the nuclear export of Foxo1 in a PI3K-dependent manner (Soriano et al., 2006; Papadia et al., 2008). However, whether synaptic activity induces long-term changes to Foxo1 activity beyond its acute nuclear exclusion is unknown and is investigated in this thesis.

As well as boosting the antioxidant defences centred on the thioredoxin-peroxiredoxin system, synaptic activity also enhances the activity of the transcriptional co-activator peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α), which has been implicated in many cellular processes including energy homeostasis, β -oxidation of fatty acids, as well as glucose and ROS

metabolism (St-Pierre et al., 2006). PGC-1 α suppresses ROS via mediating the induction of ROS-detoxifying enzymes such as SOD1, SOD2, catalase and GPx1 in response to oxidative stress (St.-Pierre et al., 2006). PGC-1 α overexpression protects neurons from excitotoxic and oxidative stress in a cell autonomous manner, while knockdown rendered the neurons more vulnerable to insults. The mechanism by which synaptic activity enhances PGC-1 α activity, involves triggering the nuclear export of the transcriptional co-repressor silencing mediator of retinoic acid and thyroid hormone receptors (SMRT), which specifically antagonizes PGC-1 α -mediated antioxidant responses. Furthermore, synaptic activity boosts PGC-1 α transcription *in vitro* and *in vivo* and enhances its transcriptional activity *in vitro* (Soriano et al., 2010). Interestingly, PGC-1 α under expression has been associated with disease progression in models of HD (Cui et al., 2006; Weydt et al., 2006; Soriano et al., 2011), PD (Shin et al., 2011) and AD (Qin et al., 2009), however, PGC-1 α overexpression exhibits protective effects in these disease models.

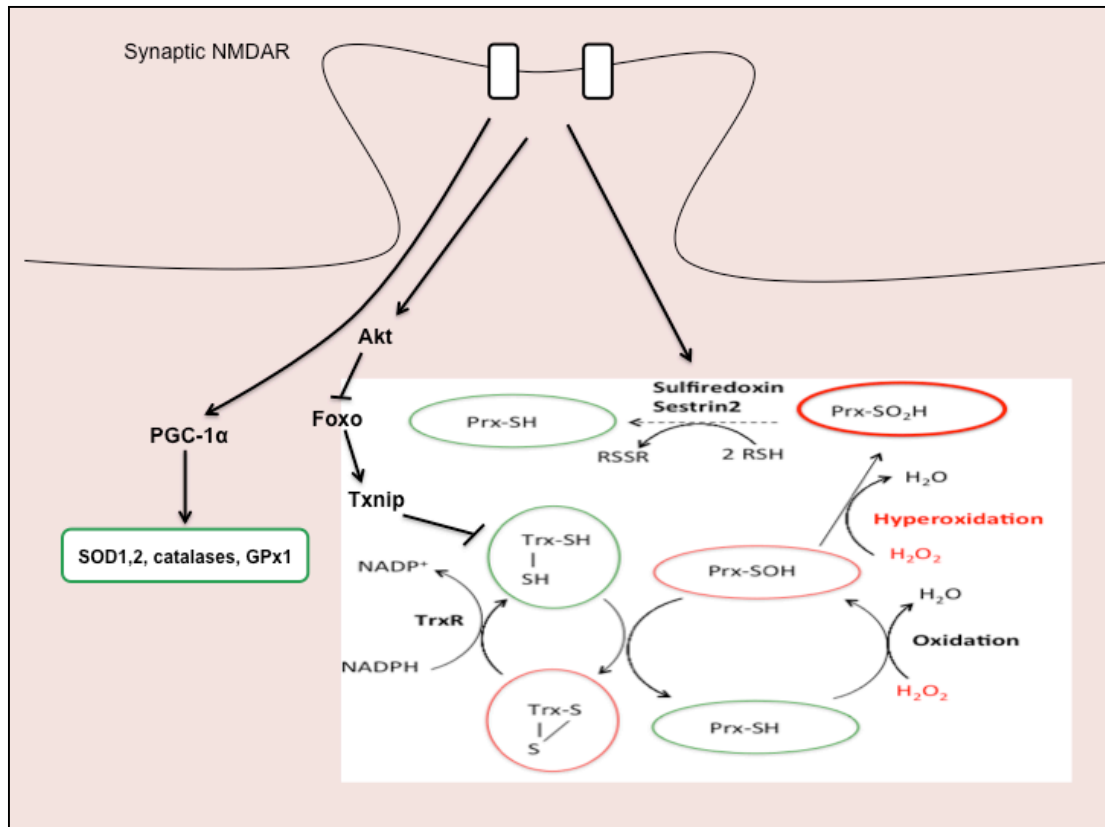


Figure 1.10. Antioxidant effect of synaptic NMDAR activity.

Schematic representation showing the antioxidant effect of synaptic NMDAR activity. Synaptic activity enhances the thioredoxin-peroxiredoxin pathway via *Txnip* suppression and the upregulation of *Srxn1* and *Sesn2*. Synaptic activity also enhances the activity of PGC-1 α .

1.9.2.2. NMDAR-mediated cell death

Excitotoxicity is a pathological process in which excessive activation of glutamate receptors (particularly NMDARs) results in neuronal death. Excitotoxicity is a major cause of neuronal death following acute insults such as hypoxia, ischemia and trauma. It is also implicated in the etiology of a number of chronic neurodegenerative diseases including AD, PD and ALS (Papadia and Hardingham, 2007). The build up of extracellular glutamate during pathological scenarios such as ischemia (Rossi et al., 2000; Camacho and Massieu, 2006) induces excessive activation of NMDAR, which results in Ca^{+2} -dependent cell death (Arundine and Tymianski, 2004). Strong Ca^{+2} influx through the NMDAR triggers cell death via diverse mechanisms. In severe excitotoxicity, simple overload of Ca^{+2} may mediate fast necrotic cell death. However, in many cases, active mechanisms could be involved such as mitochondrial dysfunction.

Excessive uptake of Ca^{+2} by the mitochondria through the potential-driven uniporter causes membrane depolarization and the subsequent reversal of mitochondrial ATPase. The reversal of mitochondrial ATPase inhibits ATP production and can even cause depletion of cytosolic ATP. This loss of ATP further limits the neuron's ability to maintain ion homeostasis and eventually leads to cell's demise (Papadia and Hardingham, 2007). Furthermore, Ca^{+2} uptake can trigger apoptosis through the release of cytochrome *c*.

While it had been generally accepted that Ca^{+2} influx promotes the generation of ROS (Nicholls, 2004), recent studies have made the case that ROS production following severe excitotoxic episodes is non-mitochondrial in origin (Nicholls, 2008). However, this does not rule out a role for oxidative stress in mediating NMDAR-dependent cell death, as a recent study demonstrated that activation of NADPH oxidase is the primary source of ROS generation by excessive NMDAR activation and therefore blocking this event was neuroprotective (Brennan et al., 2009).

In addition to impairing the mitochondrial function, NMDAR over-activation perturbs Ca^{+2} efflux. In neurons, the plasma membrane Ca^{+2} ATPase pump (PMCA) and the plasma membrane $\text{Na}^{+}/\text{Ca}^{+2}$ exchangers (NCXs) are the two routes through which Ca^{+2} can exit the cell. Excessive NMDAR-mediated Ca^{+2} influx activates calpains, Ca^{+2} -dependent proteases, which cleave a major isoform of the plasma membrane $\text{Na}^{+}/\text{Ca}^{+2}$ exchanger (NCX3), impairing its function in cerebellar granule neurons (Bano et al., 2005). Moreover, excitotoxic insults suppress PMCA activity via mechanisms attributed to both calpains (Pottorf et al., 2006) and caspases (Schwab et al., 2002). Striatal enriched tyrosine phosphatase (STEP), is another important substrate for NMDAR-mediated calpain cleavage. STEP cleaved from is unable to interact with its normal substrates such as p38 mitogen-activated protein (MAP) kinase, which is known to contribute to neuronal death in response to chronic NMDA or glutamate exposure, is negatively regulated by STEP and therefore inhibition of calpain-mediated STEP cleavage is neuroprotective (Hardingham and Bading, 2010).

Furthermore, NMDAR-dependent Ca^{+2} influx stimulates neuronal nitric oxide synthase (nNOS) activity, which triggers toxic down stream responses, including mitochondrial dysfunction, p38 mitogen activated protein-kinase signalling, and activation of the transient receptor potential melastatin (TRPM) channel (Papadia and Hardingham, 2007). nNOS is coupled to NMDAR receptors via the post-synaptic density protein PSD95 and in an excitotoxic context produces nitric oxide (NO), which in excess can be toxic both on its own or when it interacts with other ROSs such as superoxide resulting in the formation of peroxynitrite (ONOO^{-}) (Hardingham, 2009). NO/ ONOO^{-} has been shown to cause damage to cellular components, inhibit mitochondrial respiratory chain enzymes and promote depolarization. In addition, both NO and ONOO^{-} can damage DNA, leading to overactivation of the DNA repair enzyme, poly (ADP ribose) polymerase 1 (PARP-1). In the CNS, PARP-1 overactivation can promote neuronal death through mechanisms involving the depletion of cellular NAD(+) levels and the mitochondrial

release of the apoptosis activating factor (AIF) (Kauppinen and Swanson, 2007; Andrabi et al., 2008). Moreover, ONOO⁻ activates the cation channel TRPM7, which itself passes Ca²⁺, resulting in a positive feedback loop on nitric oxide activity (Aarts et al., 2003).

Activation of the stress-activated protein kinases (SAPKs) such as p38 and c-Jun N-terminal kinases (JNKs) represents another mechanism for NMDAR-dependent cell death. For instance, the activation of p38 leads to caspase-independent cell death in both cerebellar granule and cortical neurons (Kawasaki et al., 1997; Cao et al., 2004; Soriano et al., 2008b). JNK activation has been shown to contribute to NMDAR-dependent cell death in cortical neurons, both *in vivo* and *in vitro* (Borsello et al., 2003). In addition to its role in excitotoxicity, p38 was reported to promote neuroprotection through the activation of myocyte enhancer factor 2 (MEF2) (Okamoto et al., 2000) and to be activated by neuroprotective synaptic NMDAR activity (Soriano et al., 2008b; Soriano et al., 2010). Further studies have revealed new components of the excitotoxic cell-death pathway such as Rho, a member of the Rho-family of GTPases, which was associated with glutamate-induced p38 α -dependent excitotoxic neuronal death (Semenova et al., 2007).

Besides the stimulation of SAPKs, Foxo activation has been implicated in NMDAR-dependent cell death. Unlike synaptic NMDAR activity, which inactivates proapoptotic Foxo, extrasynaptic NMDAR-evoked signals fail to trigger Foxo nuclear export and instead increase Foxo nuclear localization, which subsequently contributes to NMDAR-dependent neuronal death (Dick and Bading, 2010).

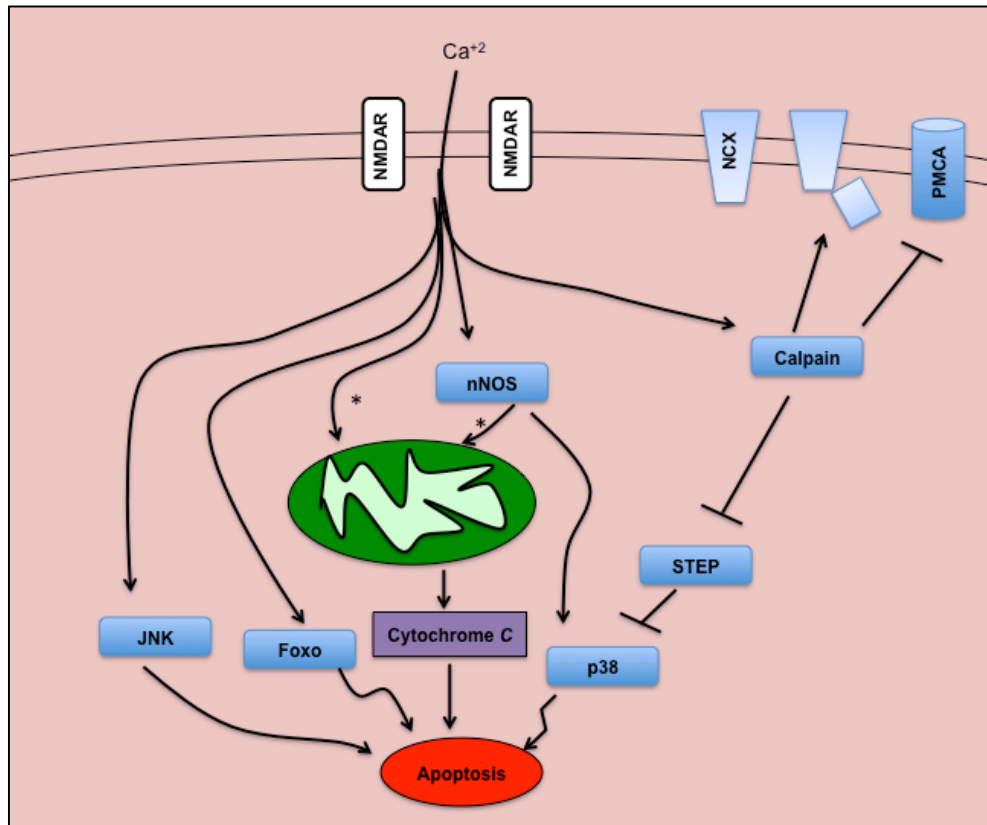


Figure 1.11. Pro-death signalling from NMDARs.

Excessive activation of NMDAR results in Ca^{+2} -dependent cell death via various mechanisms including the activation of Foxos, calpains, nNOS, JNK, as well as mitochondrial dysfunction (*) (refer to above text).

1.10. Aims

In previous studies, Nrf2 activation was achieved artificially either by astrocyte-specific over expression of Nrf2 (Shih et al., 2003; Shih et al., 2005; Nagai et al. 2007; Vargas et al. 2008; Kanninen et al., 2008), or by treatment with well-characterized small molecule activators of the pathway such as tBHQ and sulforaphane (Shih et al., 2005; Zhao et al., 2006; Zhao et al., 2007; Jakel et al., 2007; Kanninen et al., 2008), or by transplantation of Nrf2-overexpressing astrocytes (Jakel et al., 2007; Calkins et al., 2009). However, the extent to which endogenous Nrf2 could respond to intrinsic signals such as mild oxidative stress and subsequently mediate a physiologically relevant endogenous neuroprotective response is less clear.

Another issue is the locus of Nrf2 activation in brain tissues, which represent a controversial aspect of the Nrf2 pathway. While a large number of studies have shown that activation of the Nrf2 pathway preferentially occurs in astrocytes not neurons (Ahlgren-Beckendorf et al., 1999; Murphy et al., 2001; Kraft et al., 2004), this is controversial (Johnson et al., 2002; Kraft et al., 2007; Escartin et al., 2011). These studies whether supporting the astrocyte specific or non-specific activation of the ARE-Nrf2 pathway don't, however, establish the locus of endogenous Nrf2 activity in response to endogenous signals such as oxidative stress. Thus in this thesis I wish to investigate (1) the extent to which the endogenous Nrf2 pathway can respond to intrinsic signals such as mild oxidative stress by triggering antioxidant genes up-regulation, and (2) the locus of endogenous Nrf2 activation *in vitro*.

In line with the previously reported activity-dependent suppression of Foxo1 (Papadia et al., 2008; Leveille et al., 2010) I wished to investigate whether synaptic NMDAR activity: (1) promotes the nuclear exclusion of the other major neuronal Foxo, Foxo3, and (2) influences the expression of Foxo1.

The aims of the research presented here are summarized in the points below:

- To determine whether synaptic activity induces changes to Foxo1 activity beyond its acute export from the nucleus.
- To determine the extent to which the endogenous Nrf2/ARE pathway can respond to intrinsic signals such as mild oxidative stress and the locus of its activation in our *in vitro* system of astrocyte containing primary cortical cultures.
- To investigate the mechanism(s) by which mild oxidative stress activates the endogenous Nrf2/ARE pathway.

As will be discussed in the concluding remarks of this thesis, our data provide a better insight into the regulation of intrinsic neuroprotective pathways, and therefore may point to ways in which they may be mimicked or enhanced for therapeutic effect.

Chapter 2
Materials and Methods

2.1. Neuronal cultures

Cortical neurons were cultured from embryonic day 17.5 (E17.5) CD1 mice or from E21 Sprague Dawley (SD) rat pups. The pregnant mothers and their pups were culled in accordance with schedule 1 of the home office guidelines on human killing of animals. Mouse pups were decapitated and rat pups were anaesthetized with an intraperitoneal injection of pentobarbital (Ceva Sante Animale, La Ballastiere, France) before decapitation. To dissect the cortices, the brains were removed and placed in plastic dishes containing dissociation medium (81.8 mM Na₂SO₄, 30mM K₂SO₄, 5.84mM MgCl₂, 0.252 mM CaCl₂, 1mM HEPES, 0.001% Phenol Red, 20 mM D-glucose,) (Sigma-Aldrich, Dorset, UK) supplemented with 1mM kyurenic acid and 10 mM MgCl₂ to prevent excitotoxic neuronal damage via NMDAR during the dissection and the enzymatic digestion stage. Micro-dissections were performed under a light microscope to isolate the cortices, which then were transferred to 15 ml round bottom plastic tubes and incubated with 1-2ml of dissociation media containing papain enzyme (10 enzymatic units/ml; Worthington Biochemical Corporation, Lakewood, NJ, USA) for 20 minutes at 37°C (waterbath), stirring every 10 minutes. After that, the papain solution is removed and replaced with a fresh one for a second round of 20 minutes incubation. Next the cortices were washed twice with pre-warmed dissociation medium after removing the papain solution to wash off any membranous or blood-containing tissues. Similarly, the cortices were washed twice but this time with pre-warmed growth medium consisting of Neurobasal-A medium (Invitrogen, Paisley, UK) supplemented with 1% rat serum (Harlan Laboratories), B-27 Supplement (1:50 of 50X stock, Invitrogen), 1mM glutamine, and 1:100 antibiotic/antimycotic (Gibco®, Life technologies, Paisley, UK). After the repeated washing steps, the cortices were homogenized by rapid trituration in 10 ml of pre-warmed growth medium using a 2 ml pipette, whereby repeated rounds of filling and emptying the barrel of the pipette facilitate the dissociation into single cells. The homogenized cells were then diluted with a Opti-MEM (Invitrogen) plating media supplemented with 20 mM D-glucose (Invitrogen) and 1:100 antibiotic/antimycotic. The cell suspension is then plated out at 0.5ml/well in 24-wells cell culture plates (Grenier Bio-One, Stonehouse, UK). These plates were pre-

coated with poly-D-lysine (1.33% w/v in H₂O; from BD Biosciences, Oxford, UK) and laminin (0.5% w/v) at 37°C for 2 h prior to the cells plating step. The cultures were then placed in a humidified 5% CO₂ atmosphere at 37°C for 2 h, after which the medium was replaced with 1ml growth medium. To obtain astrocytes containing cultures (AC), 1 ml of growth medium containing 9.6 μM cytosine β-D-arabino-furanoside hydrochloride (AraC) (Sigma-Aldrich, Dorset, UK) was added on (DIV04 Days *in vitro*) to prevent glial proliferation.

2.2. Nrf2 and Keap1 KO cultures

Nrf2 and Keap1 KO mice, originally developed by Prof. M. Yamamoto laboratory (University of Tohoku) (Itoh et al., 1997; Wakabayashi et al., 2003), were obtained from Prof. John. D. Hayes (University of Dundee). However, Nrf2 KO mice have been backcrossed over six generations onto C57BL/6 genetic background (Higgins et al., 2009b). An offspring of Nrf2 KO mice was generated through breeding of Nrf2 KO females and males. Matching C57BL/6 WT animals were used to generate parallel WT cultures. Keap1 heterozygote males and females were mated to produce WT and KO littermates. The offspring genotypes were verified through multiplex PCR analysis of cerebellum genomic DNA using DNA easy blood and tissue kit (Qiagen UK, Crawley, UK). The primers used are listed in table M1 and the PCR conditions were as follows: 5 minutes at 95°C followed by 34 cycles of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute, followed by an extension step at 72°C for 10 minutes.

The size of the bands produced by this reaction are distinctive based on the genotype of the sample DNA whereby Keap1 WT and KO samples generate bands of 235 and 420 base pairs (bp) respectively (Fig. 2.1).

Table 2.1. Keap1 genotyping PCR primers sequences.

PCR Primer	Sequence
D123	5'-CGGGATCCCCATGGAAAGGCTTATTGAGTTC-3'
2nd Ex 3'	5'-GAAGTGCATGTAGATATACTCCC-3'
Tv Neo	5'-TCAGAGCAGCCGATTGTGTGTTGTGCCAGTCA-3'

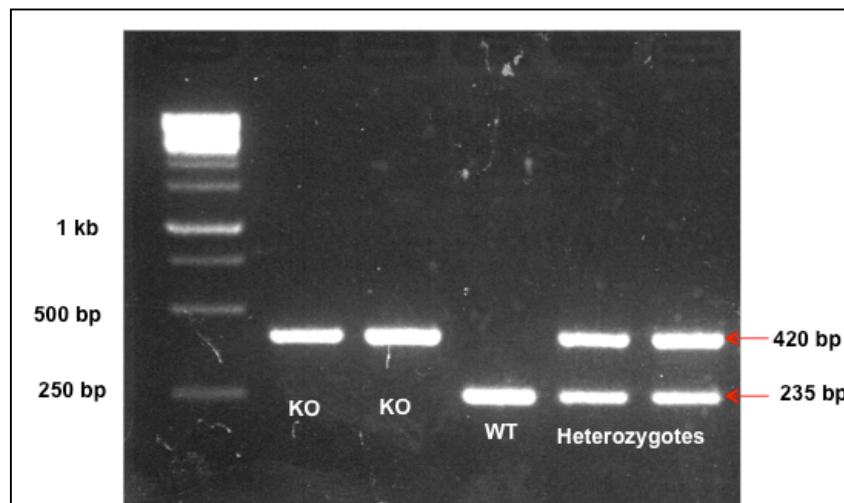


Figure 2.1. Keap1 genotyping PCR

Example of genotyping products obtained in Keap1 WT, KO, and heterozygotes mice.

2.3. Stimulations and reagents

Prior to performing the experiments, cells were placed into trophically deprived medium (TMO) containing 10% MEM (Invitrogen, Paisley, UK) and 90% salt-glucose-glycine (SGG) medium (114 mM NaCl, 0.219% NaHCO₃, 5.292 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES, 1 mM Glycine, 30 mM Glucose, 0.5 mM sodium pyruvate, 0.1% Phenol Red; osmolarity 325 mosm/l) (Bading et al., 1993) and allowed to equilibrate for (2-16h) before stimulation. Treatments of cultured cells were performed after 8-10 DIV. Reagents were either dissolved in H₂O or in dimethyl-sulfoxide (DMSO).

2.3.1. Induction of synaptic NMDAR activity in neurons

Stimulations of cultured neurons were done in most cases after a culturing period of 8–10 days, during which neurons develop a network of processes, express functional NMDA-type and AMPA/kainate-type glutamate receptors, and form synaptic contacts. Bursts of action potential firing were induced by treatment of neurons with 50 µM bicuculline (Tocris bioscience, Bristol, UK), a GABA_A receptor inhibitor, and 250 µM 4-amino pyridine, a K⁺ channel antagonist, to enhance burst frequency (Calbiochem, Merk Millipore, Germany) (Hardingham et al., 2001).

2.3.2. Other stimulations

Sub-toxic doses of H₂O₂ (Sigma-Aldrich) were utilized to model oxidative stress *in vitro*. tBHQ (Acros Organics, Fisher's scientific, Belgium) treatments were used to model pharmacological activation of the Nrf2 pathway. 50 µM LY 294002 (Calbiochem, Merk Millipore, Germany) was used to inhibit the PI3K pathway. Inhibition of GSK-3β, was performed using 2 µM CT-99021 (Cayman chemical, USA).

2.4. Assessment of neuronal cell death

For cell death quantification, neurons were fixed with 4% paraformaldehyde (PFA) and subjected to 4',6' diamidino-2-phenylindole (DAPI) (Vector Laboratories, California, USA) nuclear staining and cell death was quantified by counting the number of pyknotic nuclei as a percentage of the total. Approximately 1500 cells were scored across several random fields within 3-4 independent experiments.

2.5. Transfections

Neurons (on DIV08) or HEK-293 cells were transfected using Lipofectamine 2000 (Invitrogen). In a 24-wells culture plate, each well is transfected with a total up to 0.65µg of plasmid DNA (pDNA). The pDNA is then diluted in 33µl of TMO medium from the wells and in another tube 2.33µl of Lipofectamine was diluted in the same manner. The diluted preparations were then combined and incubated at room temperature for 20 minutes. Following the incubation period, 285µl of medium from the wells was added to the tube containing the pDNA and Lipofectamine mixture and mixed gently. Finally, the medium was removed from each well and replaced with 333µl transfection mixture, after which the cells were maintained at 37°C in a CO₂ for 2-4 h. Of note, transfections were scaled up as needed, in which reagents, DNA, cells and medium were used in proportion to the relative growth surface area. At the end of the transfection period, the medium on the cells was replaced with either fresh growth medium or TMO to prevent cytotoxicity. Transfection efficiency using this protocol is approximately 2-5%, of which >99% of the eGFP-expressing cells are NeuN-positive neurons and less than 1% GFAP positive astrocytes.

For astrocyte transfection, the same protocol was employed except that transfections are carried out on DIV02 instead of DIV08. The rationale behind the early transfection is the fact that quiescent (AraC-treated) glial cells are not amenable to transfection, whereas (non-AraC-treated) non-quiescent glial cells appear to be more amenable to transfection (Soriano et al., 2008). This observation was based on quantifying the eGFP-expressing astrocytes in AC cultures transfected with eGFP either on DIV02 or DIV8 and subjected to GFAP and GFP immunofluorescence.

The percentage of transfected astrocytes was calculated as the number of GFP⁺/GFAP⁺ cells over the total number of GFAP⁺ cells, and it was 6.7% and 1.6% for transfections carried out on DIV02 and DIV08 respectively.

2.6. Plasmids and reporter assays

2.6.1. Preparation and digestion of plasmids

Gift plasmids received on filter paper were soaked in 40 µl nuclease free water and placed at 4°C overnight. The following day, transformation was performed by mixing 10-15ng of pDNA with 50 µl of ice-cold JM109 competent cells (Promega, Southampton, UK) and incubated on ice for 30 minutes, then the mixture was subject to 45 seconds heat shock in 42°C water bath. After brief cooling of the mixture for few seconds on ice 1ml of Luria-Bertani (LB) broth media (Sigma-Aldrich) was added to each mixture tube and placed in a 37°C shaker for 45 minutes, following that the mixture was spread on LB agar plates containing 50 ng/ml of ampicillin and incubated overnight at 37°C. The following day, colonies were picked and seeded into 50ml of LB supplemented with ampicillin and were incubated at 37 °C for 12-16 hrs with aggressive shaking. The bacterial cells were then harvested by centrifugation at 6000Xg for 15 minutes at 4°C and plasmids were purified using the Qiagen Plasmid Midi kit (Qiagen).

Digestion procedure was carried out on ice by mixing 10-30 ng of pDNA with 5 units of the desired restriction enzyme and the appropriate buffer supplied with the enzyme, then the reaction volume was made up to 25 µl with PCR grade water. The mixture then was incubated at 37 °C for 2 hr or overnight. When the digestion was complete, few microliters of the resulting product was subjected to electrophoresis analysis on 0.8% agaros gel containing Syber Safe DNA gel stain (Invitrogen) and then visualized with a U:Genius UV illuminator (Syngene, Cambridge, UK). If the DNA fragments were required for subsequent procedures, the DNA bands were excised from the gel and DNA was extracted using QIAquick Gel Extraction kit (Qiagen).

2.6.2. Constructs generation

Foxo1-Luc construct was created by amplifying 1100bp segment of the rat *Foxo1* upstream promoter region from genomic DNA using PicoMaxx DNA Polymerase (Agilent Technologies, Cheshire, UK) with the following primers: forward, 5'- *atc tgc agt ctc taa aca ctc tcc tct gac c* -3'; reverse, 5'- *atg ata tca act taa ctt cgc tgg gtc ac* -3'. The resulting PCR product was digested with XhoI and EcoRV (sites within primers italicized) and cloned into the corresponding sites upstream of the firefly luciferase construct in PGL4.10.

For the construction of *Keap1*-Luc approximately 890bp of the rat *Keap1* upstream region was amplified from genomic DNA using PicoMaxx high fidelity PCR system (Agilent Technologies) with the following primers: forward 5'- *caa ctc gag gcg tga cag tcg ctc act ta*-3'; reverse, 5'- *cgt aga tct tcc acc act agc gat tag gg*-3'. The resulting PCR product was digested with XhoI and BglII (sites within primers italicized) and cloned into the corresponding sites upstream of the firefly luciferase construct in PGL4.10.

2.6.3. Luciferase reporter assay

For *Foxo1*-reporter assay, neurons were transfected on DIV08 with *Foxo1*-firefly luciferase (*Foxo1*-Luc) or the mutant variants, plus a pTK-renilla (Promega) normalization vector at a DNA ratio of 4:1 respectively. Whereas, in the experiments aiming to evaluate the responsiveness of *Foxo1* promoter to Foxo transcription factors, the cells were transfected with either *Foxo1*-Luc or the mutant variants and pTK-renilla along with a vector encoding either Foxo1, Foxo3, or beta-globin (control) at the following DNA ratio 2:1:4 for *Foxo1*-Luc: renilla: effector plasmid.

In the experiments aimed at evaluating the effect of synaptic activity on Nrf2-driven ARE-Luc activity, neurons were transfected on DIV08 with ARE-Luc along with pTK-renilla, and also pcDNA3.1/ V5HisBim Nrf2 or beta-globin (control), plus pcDNA3.1/V5HisCmKeap1 or beta-globin (control).

For the Gal4-based reporter assay, astrocytes containing neuronal cultures (AC-neurons) were transfected using an astrocyte specific transfection protocol (previously described in the Transfections section) where the astrocytes are transfected with the reporter plasmid Gal4-Luc and pTK-renilla and either of the effector plasmids; GBD-Nrf2, GBD-Neh(2-4), GBD-Neh5 or GBD only.

As for the Neh2-Luc reporter system, AC-neuron were transfected using the astrocyte transfection protocol, in which astrocytes were transfected with Neh2-Luc along with pTK-renilla normalization vector; or transfected with a plasmid encoding for the luciferase reporter gene not fused to Neh2 (for this purpose we utilized SV40 driven luciferase reporter) along with pTK-renilla. The DNA ratio of the plasmids transfected into the cells is 4:1 for Neh2-Luc or Luc only: renilla respectively.

Prior to performing the *Foxo1*-Luc, or ARE-Luc, or *Keap1*-Luc assays, neurons were stimulated with 50 μ M bicuculline and 250 μ M 4-aminopyridine for 24 h after 24 h of recovery from transfection. As for the Gal4 and Neh2 luciferases reporter systems, stimulations were performed 6 days post transfection whereby the cells were treated with either 10 μ M tBHQ or (25-75 μ M) H₂O₂ for 8 h prior to carrying out the luciferase assay.

Luciferase assays were performed using the Dual-Glo luciferase system kit (Promega) and relative light units were measured in a FLuostar OPTIMA luminometer (BMG Labtech, Aylesbury, UK). Firefly luciferase activity was

routinely normalized to *Renilla* luciferase activity and the results were obtained from at least 4 independent experiments, each carried out in duplicates.

Table 2.2. List of plasmids used.

Plasmid name	Description	Reference
<i>Foxo1</i> -Luc	1100bp segment of the rat <i>Foxo1</i> upstream promoter region fused to luciferase reporter gene (refer to construct generation)	This study (generated by Prof. Giles Hardingham)
PGL4.10	Luciferase reporter vector	Promega
pFoxo1myc	Myc epitope tagged mouse <i>Foxo1</i>	A gift from Dr. Domenico Accili (Nakae et al., 2000)
pGFP-Foxo3a	GFP epitope tagged mouse <i>Foxo3</i>	A gift from Dr. Domenico Accili
pTK-RL	Thymidine kinase promoter driven <i>Renilla</i> luciferase reporter	Promega
<i>Keap1</i> -Luc	890bp of the rat <i>Keap1</i> upstream region fused to luciferase reporter gene (refer to construct generation)	This study (generated by myself)
ARE-Luc	Human <i>Hmox1</i> ARE fused to luciferase reporter	A gift from Dr. Satoshi Numazawa (Numazawa et al., 2003)
pcDNA3.1V5HisBim Nrf2	Murine <i>Nrf2</i> coding sequence	A gift from Dr. Michael McMahon (McMahon et al., 2003)
pcDNA3.1/V5HisCmKeap1	Murine <i>Keap1</i> coding sequence	
Gal4-Luc	Luciferase reporter bearing 4 copies of Gal4-binding site	Promega
GBD-Nrf2	Gal4 DNA binding domain fused to the full length <i>Nrf2</i>	A gift from Prof. Masayuki Yamamoto (Kato et al., 2001)
GBD-Neh5	Gal4 DNA binding domain fused to <i>Neh5</i> domain (153-227 amino acids)	
GBD-Neh(2-4)	Gal4 DNA binding domain fused to both <i>Neh2</i> and <i>Neh4</i> domains (1-156 amino acids)	
GBD	Gal4 DNA binding domain only	
<i>Neh2</i> -Luc	<i>Neh2</i> domain fused to luciferase gene (<i>Neh2</i> -Luc)	a gift from Dr. Irina Gazaryan (Smirnova et al., 2011)

2.6.4. Site directed mutagenesis

Foxo1 promoter mutants were generated by Prof. Giles Hardingham using the QuikChange II XL site-directed mutagenesis kit (Stratagene), following the manufacturer's instructions. The proximal Foxo site on *Foxo1* was mutated to **GTCGACAA** (bold indicates nucleotides changed, underlined sequence indicates creation of Sall diagnostic site). The distal IRS-like site on *Foxo1* was mutated to **TCTAGACAAA** (bold indicates nucleotides changed, underlined sequence indicates creation of XbaI diagnostic site).

For the GBD-Neh5 mutation studies, the reactive cysteine residue 191 (Cys-191) was substituted with alanine. To obtain this, QuikChange II XL site-directed mutagenesis kit (Stratagene) was used along with the mutagenic primer and its reverse complementary sequence containing the mismatches 5'**CATTCCCGAATTA** **CAGGCCCT** TAATACCGAAAACAAG-3' (bold indicates nucleotides changed, underlined sequence indicates creation of Eco01091 diagnostic site). All mutants were verified by performing diagnostic cuts with the indicated restriction enzymes and were further confirmed by sequencing (SBS sequencing service, University of Edinburgh).

2.7. Immunocytochemistry

Cells grown in 24-well plates were fixed with PFA for 20 minutes at room temperature, washed once with 1X phosphate buffer saline (PBS) (Invitrogen) and permeabilized with NP40 (Invitrogen) for 5 minutes, and then rinsed twice with 1X PBS. Fixed cells were incubated with 0.5 ml of the primary antibody diluted in PBS overnight at 4°C with gentle rocking. The next day, the primary antibody is retained and the cells were incubated with either fluorophore-conjugated or biotin-conjugated secondary antibody/antibodies, away from light for 1 h at room temperature after rinsing the cells twice with 1X PBS. Following secondary antibody incubation, cells were washed rinsed twice with 1X PBS. In the case of biotin-conjugated secondary antibody, an additional incubation with Cy3-streptavidin-conjugated antibody (Jackson ImmunoResearch Laboratories, Inc., Pennsylvania, USA) diluted at 1:500 in PBS was carried out for 1 h in the same conditions. Finally, the cells were rinsed twice with 1X PBS and then one drop of DAPI was added after aspirating off the PBS and each well was covered with a glass cover slip. Primary antibodies used in immunocytochemistry were as follows: rabbit anti-HO1 used at (1:1000) (Stressgen Biotechnologies Corporation, British Columbia, Canada), mouse anti-GFAP used at (1:400) (Sigma-Aldrich), rabbit anti-GFP used at (1:750) (Invitrogen). Secondary antibodies were all used at (1:200) dilution in 1X PBS and were as follows; biotin-SP conjugated anti-mouse and Dylight 488-conjugated anti-rabbit (Jackson ImmunoResearch Laboratories). Images were obtained using Leica AF6000 LX imaging system, with a DFC350 FX monochrome digital camera. Using the appropriate filter set, images were initially taken in black and white, and colour was applied after image acquisition.

For measuring Hmox1 immunostaining intensity, non-saturating pictures were taken on a Leica AF6000 LX imaging system, with a DFC350 FX digital camera at a magnification of 20X and fluorescence intensity quantified on Image J. Gray scale images were obtained at the following settings; exposure time (751-935ms), gain (5) and intensity (4). To ensure consistency, all settings were kept the same for pictures taken of other conditions within each experiment. Fluorescence intensity was quantified for each cell across 4-5 fields, each field containing 10-18 cells. Background intensity was subtracted from each cell's intensity and then the mean of

cells intensities was calculated for each condition and normalized to the control condition.

2.8. Western blotting and antibodies

Cells were cultured in 35 mm dishes, and lysed in 110 μ l of 1.5 X sample buffer consisting of (1.5 M Tris pH 6.8; Glycerol 15%; SDS 3%; β -mercaptoethanol 7.5%; bromophenol blue 0.0375%). Total cell lysate was then boiled at 100°C for 5 minutes and the samples were resolved in precast gradient (4–12%) NuPAGE-Novex- Bis-Tris gels (Invitrogen). Electrophoresis was performed using Xcell Surelock system (Invitrogen). 20 μ l of each protein sample was loaded per well and electrophoresis was carried out using 1 X NuPAGE® MOPS SDS running buffer (Invitrogen) at 160V for ~ 1.5 h. When the protein migration was satisfactory, the protein samples were then electrophoretically transferred from the gel onto a polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Germany) in a buffer consisting of (96mM glycine, 12mM Tris and 20% Methanol) at 45 V for ~ 2 h. Once the transfer is complete, the membrane was then blocked for 1 h at room temperature in TBS solution (20mM Tris, 137 mM NaCl and 0.1% Tween 20%) supplemented with 5% skimmed dried milk. The membrane was then incubated overnight at 4°C in TBS+5% milk solution containing the appropriate dilution of the primary antibody. The next day, the membrane was rinsed three times for 5 minutes in TBS, and placed in a solution consisting of the appropriate horseradish peroxidase-conjugated antibody prepared at the desired dilution in TBS+5% milk. The membrane was left to incubate in this solution at room temperature for 1 h, after which the secondary antibody was removed by rinsing the membrane three times for 5 minutes in TBS. Proteins were detected by enhanced chemiluminescence, whereby the membrane is incubated for 1 minute in LumiGlo reagent and peroxide (Cell signaling technology, Massachusetts, USA) and then a Kodak X-Omat film was exposed to the membrane. Western blots were analysed by digitally scanning the blots, followed by densitometric analysis using (imageJ) software. The densities of the bands were normalized to an appropriate loading control, which was obtained by

stripping the membrane of the antibodies using Reblot Plus Strong Stripping Solution (Merck Millipore, Germany). The procedure involves incubating the membrane for 15 minutes in the stripping solution, followed by three washes with TBS, and then re-probing with the desired anti-body following the primary anti-body incubation protocol described earlier.

The primary antibodies used were: rabbit anti-FOXO1 (1:1000, Cell signaling technology), mouse anti-Akt (1:500, Cell signaling technology), goat anti- Keap1 (1:500, Santa Cruz Biotechnology, California, USA), mouse anti- β -tubulin isotype III (1 : 125 000, Sigma-Aldrich), mouse anti-GAL4 (DBD) (1:500, Santa Cruz Biotechnology), and rabbit anti-beta actin (1:2000, abcam, Cambridge, UK). FOXO1 bands were normalized to Akt, Keap1 bands were normalized to β -tubulin and GAL4 bands were normalized to beta-actin. Horse-radish peroxidase-conjugated secondary antibodies used in this analysis are anti-mouse, anti-goat (1:1000, Dako, Cambridgeshire, UK), and anti-rabbit (1:2000, Santa Cruz Biotechnology).

2.9. RNA isolation, RT-PCR and Quantitative-PCR

Total RNA was isolated using High Pure RNA Isolation Kit (Roche, Welwyn Garden City,UK) including a DNase-treatment step to degrade genomic DNA and samples were eluted in 35-45 μ l of the elution buffer provided with the kit. cDNA was synthesized from 1-5 μ g RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche). Briefly, the reverse transcription reaction was carried out on ice by mixing 7 μ l of RNA with 13 μ l RT mix containing Anchored-oligo(dT) primer: random hexamer primer 1:2 (total 3 μ l), 4 μ l Transcriptor Reverse Transcriptase Reaction Buffer (5X), 0.5 μ l Protector RNase Inhibitor (40 U/ μ l), 2 μ l Deoxynucleotide Mix (1mM each:dATP,dGTP,dTTP, and dCTP), 0.5 μ l Transcriptor Reverse Transcriptase (20 U/ μ l) and 3 μ l nuclease free water. In parallel, at least one NoRT control was generated per isolation and all samples were vortexed and spun down before placing them in a thermal block cycler with a heated lid. cDNA synthesis reactions were all carried out at 25°C for 10 minutes for primer

annealing, 30 minutes at 55°C for RT reaction, 5 minutes at 85°C for enzyme inactivation and then cooled down to 4°C. The resulting cDNA was then diluted to 6 ng and was used to perform real-time quantitative PCR using FS Universal SYBR Green MasterRox (Roche) and the reaction was carried out in an Mx3000P qPCR system (Agilent Technologies, Cheshire, UK). In short, 15 µl of the qPCR reaction mix was pipetted into each well, containing 1 µl template cDNA, 7.5 µl SYBR Green master mix (containing optimized amount of DNA polymerase, dNTP, reaction buffer and Rox dye), 0.6 µl forward and reverse primers at a final concentration of 200 nM and 5.3 µl nuclease free water. In each experiment, technical duplicates were used for every sample including NoRT and No-template controls. The cycling parameters were as follows: 10 minutes of initial denaturation at 95°C; 40 cycles of 30 seconds at 95°C, 40 seconds of annealing at 60°C with detection of fluorescence and 30 seconds of extension at 72°C; followed by one cycle of 1 minute at 95°C, the temperature was ramped from 55°C to 95°C over 30 seconds at 1°C per step with continuous fluorescence detection (for dissociation curve analysis to confirm the amplification of a single product). The mRNA level of the gene of interest was normalized to Gapdh levels and expressed compared to levels in control samples using the $2^{(-\Delta\Delta Ct)}$ efficiency corrected method (Livak and Schmittgen, 2001). For each set of primers, the amplification efficiencies were measured using a 5-fold dilution standard curve and were limited between 1.74 and 2.112. Primers sequences, and efficiencies are listed below.

Table 2.3. List of qPCR primers used.

Gene	Efficiency (%)	Forward primer (5'-3')	Reverse primer (5'-3')
<i>Gapdh</i> (Mouse)	104	GGGTGTGAACCACGAGAAT	CCTTCCACAATGCCAAAGTT
<i>Gapdh</i> (Rat)	71.8	AGAAGGCTGGGGCTCACC	AGTTGGTGGTGCAGGATGC
<i>xCT</i> (Mouse)	74	ATACTCCAGAACACGGGCAG	AGTTCACCCAGACTCGAAC
<i>Srxn1</i> (Mouse)	102	GACGTCCTCTGGATCAAAG	GCAGGAATGGTCTCTCTCTG
<i>Hmox1</i> (Mouse)	112	AGCACAGGGTGACAGAAGAG	GGAGCGGTGTCTGGGATG
<i>Foxo1</i> (Rat)	85	CCGACCTCATCACCAAGG	TCTCCAGGACCCTCTTGC
<i>Keap1</i> (Rat and mouse)	96	GACTTCCTCGTGCAGCAG	GAACACCTCGGACTCGC

2.10. Statistical analysis

All results are presented as mean \pm standard error of the mean. Statistical testing involved a 2-tailed paired student *t* test (Excel Microsoft). For studies using multiple testing a one-way ANOVA was used followed by Fisher's LSD *post hoc* test.

Chapter 3
Synaptic NMDAR activity control of Foxo1 and Nrf2
transcription factors

3.1. Summary

The thioredoxin-peroxiredoxin system provides protection against oxidants by neutralizing ROS and maintaining normal redox homeostasis. (Chae et al., 1994; Schulze et al., 2004). Thioredoxin anti-oxidative function is suppressed by an endogenous inhibitor known as thioredoxin interacting protein (Txnip). (Schulze et al., 2004; Yoshida et al., 2005). Our group has previously shown that synaptic activity enhances thioredoxin activity by causing *Txnip* down regulation, whereby synaptic activity promotes PI3K-directed nuclear exclusion of Foxo (Papadia et al., 2008). However, the Foxo-inhibiting capacity of PI3K/Akt pathway is thought to be short-lived. This is attributed to a key aspect of Foxo regulation, which is once PI3K/Akt activity has returned to baseline, Foxos localize to the nucleus to resume the activation of their downstream genes.

In this study, I demonstrate that synaptic NMDAR activity not only triggers Foxo export, but also suppresses the expression of Foxo1. I also found that blockade of PI3K activity prevented both Foxo3 nuclear export and suppression of Foxo1 expression, raising the possibility that Foxo1 is itself a Foxo target gene. Moreover, the data presented here revealed that Foxo3, and to a lesser extent Foxo1 transactivates the *Foxo1* promoter via a consensus Foxo binding site (GTA AAC AA), and also an upstream sequence resembling a classical Foxo-binding insulin response sequence (CAA AAC AA). The proximal (GTA AAC AA) sequence mediated both the activity-dependent and the IGF-1-dependent suppression of Foxo1 promoter. Thus, through a feed-forward inhibition mechanism, synaptic activity triggers Foxo export resulting in suppression of Foxo1 expression. These published results suggest that Foxo-inactivating signals are likely to result in longer-term inhibition of Foxo target gene expression than previously thought (Al-Mubarak et al., 2009).

In addition to its suppressing effect on Foxo, I found that synaptic activity downregulates the Nrf2/ARE antioxidant pathway inhibitor, Keap1. I have shown that synaptic activity enhances Nrf2 capacity to drive ARE-reporter and reduces Keap1 expression at both mRNA and protein levels in an NMDAR-dependent fashion. I also demonstrated that the activity-dependent regulation of Keap1 occurs, at least in part, at a transcriptional level.

3.2. Results

3.2.1. Foxo1 expression is suppressed by synaptic activity

Synaptic activity had been observed to rapidly inhibit Foxo pro-oxidative target gene *Txnip*, through promoting Foxo1 cytoplasmic retention and its dissociation from the *Txnip* promoter (Papadia et al., 2008). In line with this reported finding, I sought to determine whether synaptic activity induced longer-lasting changes to Foxo1 activity other than its acute export from the nucleus. Rat cortical neurons were placed in trophically deprived medium and synaptic activity was stimulated using the established method of network disinhibition, in which the GABA_A receptor antagonist bicucullin (BiC) was co-applied with the K⁺ channel antagonist 4-aminopyridine (4-AP) (which enhances burst frequency, referred to hereafter as BiC) (Hardingham et al., 2001; Papadia et al., 2005). In time course experiments, the expression of Foxo1 was strongly suppressed 4h after enhancing synaptic activity with BiC and this was maintained at 24 h post-stimulation (Fig.3.1A). However, MK-801 (an established NMDAR antagonist) co-treatments attenuated *Foxo1* transcriptional suppression indicating that the inhibitory effect of synaptic activity is NMDAR-dependent. Next, the level of Foxo1 protein was assayed by western blot 24 h post-stimulation. The results confirmed the substantial down regulation of Foxo1 compared to control (Fig. 3.1B).

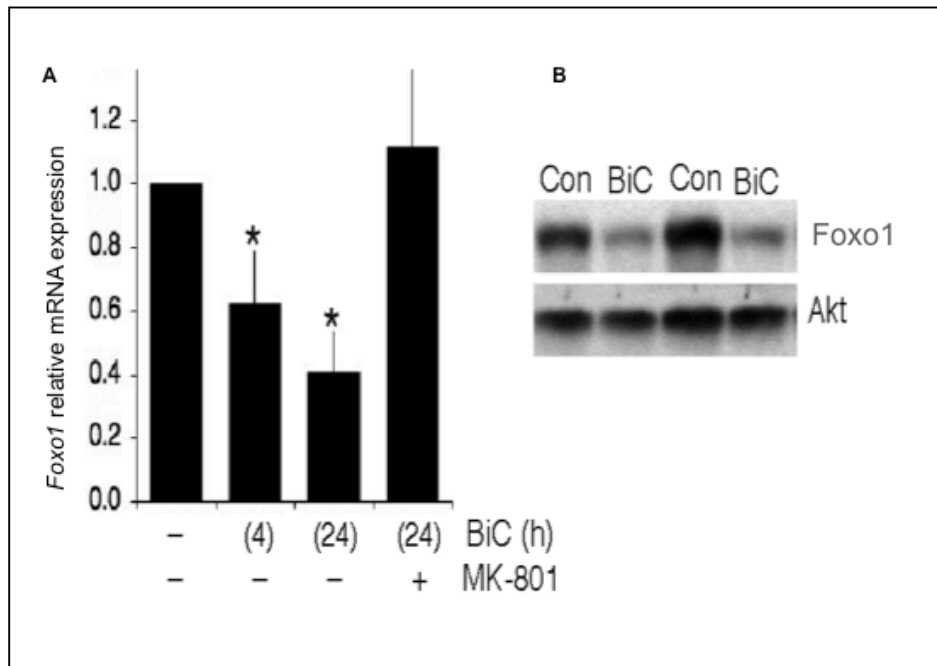


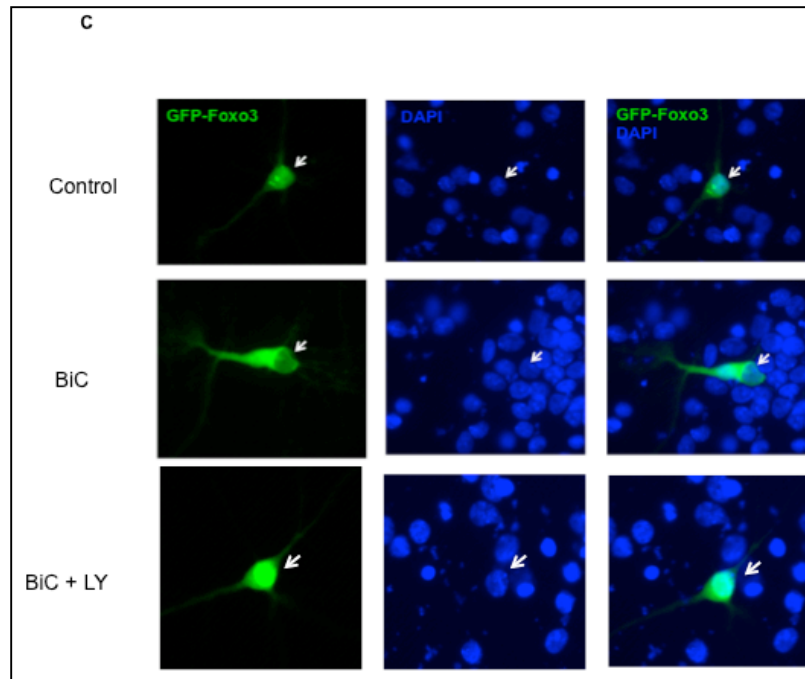
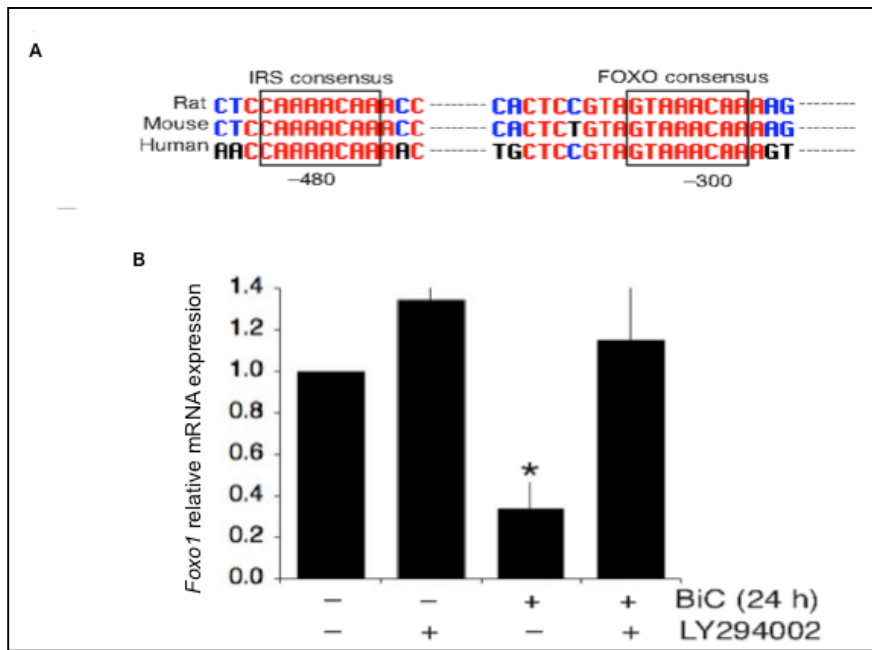
Figure 3.1. Synaptic NMDAR activity suppresses expression of Foxo1.

A) QRT-PCR analysis of *Foxo1* mRNA levels in neurons placed in trophically deprived medium and stimulated as indicated. Levels of *Foxo1* mRNA are normalized to those of GAPDH. $n=3$, $*p<0.05$. **B)** Example western blot demonstrating that activity-dependent suppression of *Foxo1* mRNA levels results in lower protein expression. Example western of total protein lysates are shown to illustrate the effect of BiC stimulation on Foxo1 protein levels after 24 h. For comparison, Akt levels are shown to be unaltered. The experiment was repeated three times across three independent cultures

3.2.2. Synaptic activity regulation of Foxo1 is PI3K-dependent

Having shown that synaptic activity down regulates Foxo1 expression, we sought to decipher the mechanism by which Foxo1 expression is suppressed by synaptic activity. Since Foxo itself, is subject to activity dependent nuclear export via the PI3K-Akt pathway (Papadia et al., 2008; Martel et al., 2009), we first sought to analyse the *Foxo1* promoter. Interestingly, two potential Foxo binding sites were detected. A proximal site at -306 nt. near the transcription start site (GTA AAC AA) matching the exact Foxo-binding consensus (Furuyama et al., 2000) and a consensus insulin-response sequence (IRS, CAA AAC AA) at -483 nt (Guo et al., 1999). The sequence of these elements is evolutionarily conserved in rodents and humans genomes, indicative of functional importance (Fig. 3.2A). Of note, the *in silico* studies as well as the promoter constructs generation was all performed by Prof. Giles Hardingham. Before evaluating the significance of these consensus sites directly, we sought to identify which signal transduction pathway was required in the activity dependent down regulation. Given the observed blockade of the activity-dependent nuclear export of the Foxo1 (Papadia et al., 2008; Martel et al., 2009), I sought to determine whether synaptic activity promotes the nuclear exclusion of Foxo3 in the same manner. To that end, neurons were transfected with GFP-tagged Foxo3 and 24 h post transfection the cells were stimulated with BiC for 1 h in the presence or absence of LY294002 and GFP-Foxo3 subcellular localization was assessed by immunofluorescence using anti-GFP antibody. Subcellular distribution of GFP-Foxo3 was scored as either nuclear (higher levels of GFP immunostaining in nucleus than cytoplasm or when a defined nuclear border is visible), nuclear + cytoplasmic (even distribution in nucleus and cytoplasm), or cytoplasmic (higher levels of GFP immunostaining in cytoplasm than nucleus) and the percentage of each category was calculated for each condition. The analysis revealed that synaptic activity triggers Foxo3 export in a PI3K-sensitive manner as pre-treatments with LY294002 attenuated the activity-dependent nuclear export (Fig. 3.2 C and D) Next, I wanted to test if synaptic activity exploits the same signalling transduction pathway to elicit Foxo1

suppression. To that end, neurons were pre-incubated with LY294002 then treated with BiC for 24 h. The BiC derived suppression of Foxo1 expression was completely reversed by LY294002 confirming that synaptic activity suppresses Foxo1 in a PI3K-dependent manner (Fig. 3.2B). Similarly, LY294002 was shown to block the activity-dependent down regulation of *Txnip*, which was found to be a Foxo target gene (Papadia et al., 2008).



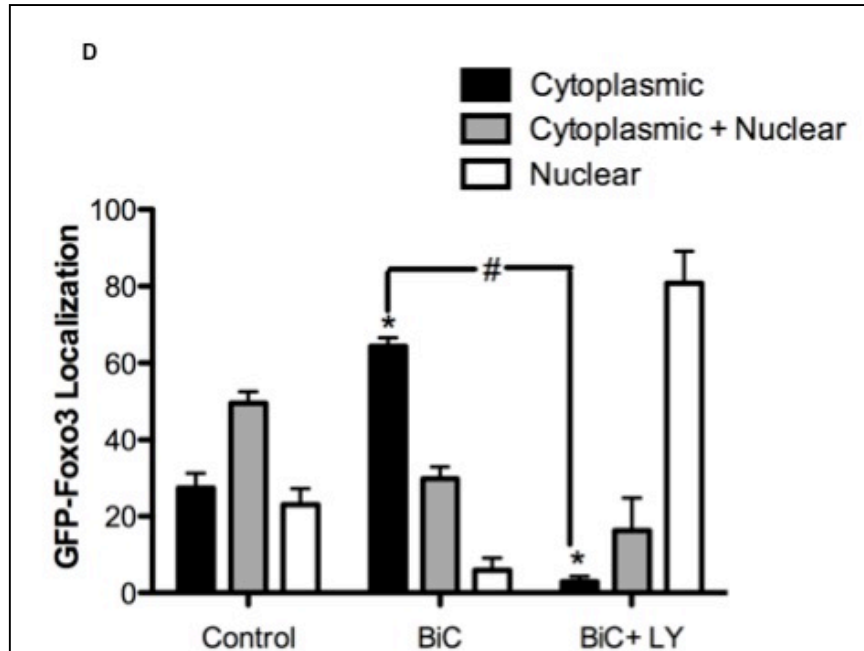


Figure 3.2. Synaptic activity suppresses Foxo1 and promotes Foxo3 nuclear export in a PI3K-dependent manner.

A) Schematic illustrating the position and conservation of the putative FOXO-response elements within the Foxo1 promoter. Nucleotide positions refer to the rat gene. **B)** QRT-PCR analysis of Foxo1 mRNA levels in neurons placed in trophically deprived medium and stimulated with BiC in the presence or absence of LY294002 (50 μ M), $n=3$, $*p < 0.05$. **C, D)** Neuronal activity promotes the nuclear export of Foxo3 via PI3K. Neurons were transfected with GFP-tagged Foxo3 and 24 h later were stimulated with BiC for 1 h in the presence or absence of PI3K inhibitor LY294002 (50 μ M). The cells were then fixed and processed for immunofluorescence with an anti-GFP antibody as described in section 2.7. **C)** Example pictures are shown from the experiments performed. **D)** Quantification of the subcellular distribution of GFP-Foxo3 using the scoring system described earlier in section 3.2.2. 15-35 cells were analysed for each condition, $n=4$, $*p < 0.05$ assessment of the effect of the indicated treatment on GFP-Foxo3 localization compared to control, $\#p < 0.05$ assessment of the effect of blocking the PI3K pathway on the activity-derived Foxo3 nuclear exclusion.

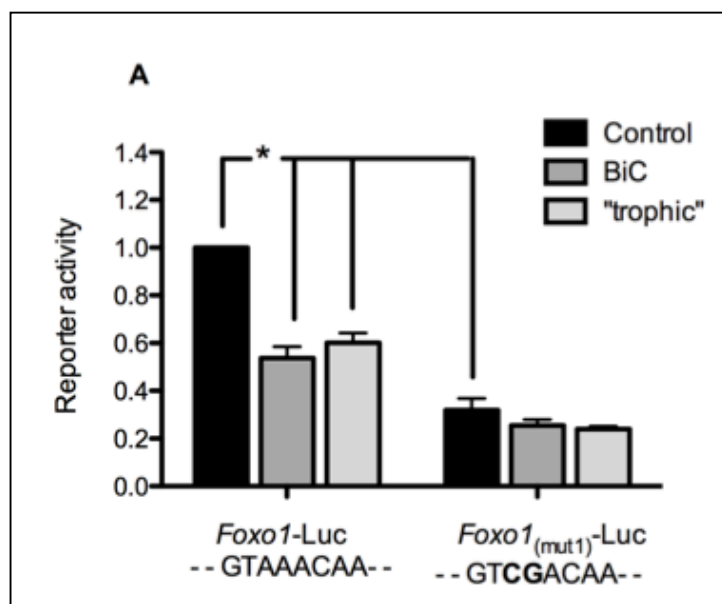
3.2.3. *Foxo1* is a Foxo target gene

To test the hypothesis that *Foxo1* is a Foxo target gene, we isolated a 1 kb fragment of the *Foxo1* promoter and cloned it in front of a luciferase reporter gene. We next created a mutant version of it with the proximal putative Foxo consensus site mutated to **GTCGACAA** (*Foxo1*(mut1)-Luc, bold underlined indicates the altered nucleotides). Of note, the *Foxo1*-Luc construct and the mutant versions were generated by Prof. Giles Hardingham, while *Foxo1*-Luc reporter activity experiments were carried out by myself and Prof. Giles Hardingham. Transfections were performed on neurons with *Foxo1*-Luc and 24 h later cells were stimulated for 24 h with BiC or left untreated. We observed that *Foxo1*-Luc activity was strongly repressed by BiC-induced neuronal activity. (Fig.3.3A). Mutation of the proximal Foxo consensus site produced a significant reduction in the basal *Foxo1* promoter activity. Altogether, our results suggest that the proximal (GTA AAC AA) Foxo consensus site is involved in the activity-dependent *Foxo1* promoter activity suppression.

In accordance to published work on the trophic factors-triggered *Foxo1* nuclear export (Soriano et al., 2006), we performed the same assay placing the neurons in trophic rich medium containing insulin, which activates neuronal IGF-1 receptors and obtained a similar effect as inducing synaptic activity-strong suppression of *Foxo1* promoter activity that is blocked by mutation of the Foxo consensus site (Fig. 3.3A).

In order to investigate whether the identified proximal site is a Foxo- responsive element, neurons were co-transfected with either *Foxo1*-Luc or (*Foxo1*(mut1)-Luc, along with vectors driving the constitutive expression of either the major neuronal Foxos, *Foxo1* and *Foxo3*, or a control (β -globin). Our data shows that expression of *Foxo3* and, to a lesser extent *Foxo1* both strongly enhanced the wild type *Foxo1* promoter activity. In contrast, expression of *Foxo3* and *Foxo1* produced a

modest increase in *Foxo1*(mut1)-Luc activity (Fig. 3.3B). These results indicate that the proximal Foxo consensus site on the *Foxo1* promoter is indeed Foxo-responsive. However, the small increase in *Foxo1*(mut1)-Luc activity observed following Foxo expression, is suggestive of the presence of an additional Foxo-responsive element within the *Foxo1* promoter (Fig. 3.3B). One obvious candidate was the upstream IRS consensus sequence (CAA AAC AA). We therefore tested the effect of mutating this site on the responsiveness of the *Foxo1* promoter to Foxo3 (since Foxo3 has the largest transactivating effect on *Foxo1* promoter). Mutation of the IRS-like element to CTAGACAA also reduced Foxo-responsiveness of the Foxo1 promoter both when introduced independently *Foxo1*(mut2)-Luc and also when combined with the first mutation *Foxo1*(mut1+2)-Luc (Fig. 3.3C). Thus our data underscore the need for both elements for achieving full Foxo responsiveness. To our surprise, the doubly mutated *Foxo1*(mut1+2)-Luc reporter displayed a higher basal activity compared with the *Foxo1*(mut1)-Luc (Fig. 3.3 C). The reason behind this is not clear, since mutation of the IRS site alone does not raise basal promoter activity (compare WT *Foxo1*-Luc with *Foxo1*(mut2)-Luc, Fig. 3.3C). While these data indicate that both sites are required for full activation of the *Foxo1* promoter by Foxos, they don't however, assess whether Foxo1 or Foxo3 bind directly to *Foxo1* promoter. On the same note, Essaghir *et al.* reported a direct association of FOXO1 and FOXO3 with *FOXO1* promoter in HEK293T cells as seen by chromatin immunoprecipitation (CHIP) assay (Essaghir *et al.*, 2009). However, whether this direct interaction exists in neurons could be addressed in future studies by performing CHIP assay with anti-Foxo antibodies.



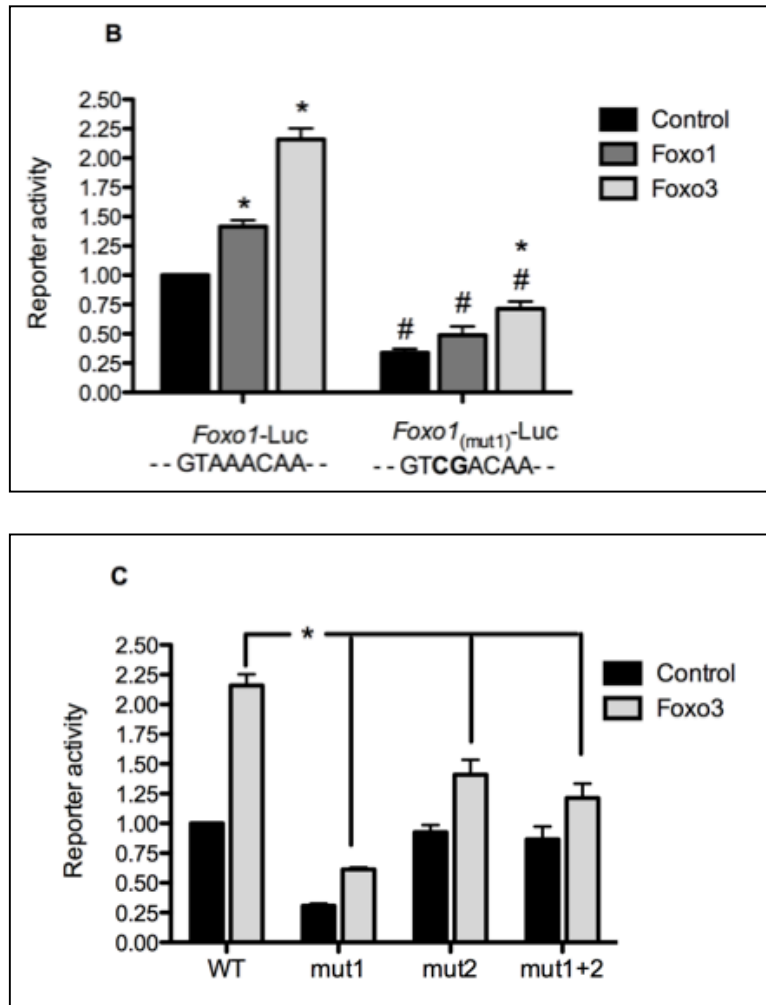


Figure 3.3. Foxo consensus site mediates the activity-dependent suppression and Foxo-mediated transactivation of Foxo1 promoter.

A) Neurons were transfected with either *Foxo1*-Luc or *Foxo1*(mut1)-Luc, plus a pTK-renilla normalization vector. Neurons were stimulated where indicated with BiC or placed in trophically deprived medium containing insulin for 24 h after which reporter activity levels were measured (normalized to renilla levels). n=6, **B**) Neurons were transfected with either *Foxo1*-Luc or *Foxo1*(mut1)-Luc, plus a pTK-renilla normalization vector. In addition, they were co-transfected with a vector encoding Foxo1, Foxo3, or beta-globin (control). Reporter activity levels were measured (normalized to renilla levels) at 48h post-transfection. * $p < 0.05$ assessment of the effect of Foxo expression relative to appropriate control, # $p < 0.05$ assessment of the effect of the mutation on basal, and Foxo-induced activity of the *Foxo1* promoter (n =7). **C**) Neurons were transfected with either *Foxo1*-Luc, or mutated variants, plus a pTK-renilla normalization vector. In addition, they were cotransfected with a vector encoding either Foxo3, or beta-globin (control). Reporter activity levels were measured (normalized to renilla levels) at 48 h post-transfection. * $p < 0.05$ (n = 5).

3.2.4. Synaptic activity induces Nrf2-driven ARE reporter activity

Having established that synaptic activity may promote neuronal-survival signalling through inhibiting Foxo1, I wanted to determine whether synaptic activity could boost antioxidant defences particularly those regulated by the ARE/ Nrf2/ Keap1 pathway. Our group has previously reported that Keap1 forced expression reduces the activity-dependent induction of *Srxn1* reporter (Soriano et al., 2008). Based on this observation, I wanted to investigate whether if synaptic activity can elicit a similar effect on the Nrf2-driven ARE activity and whether it was Keap1 sensitive. Therefore, I studied the effect of Keap1 overexpression on the Nrf2-driven ARE-Luc activity in control and BiC-stimulated rat cortical neurons. The analysis revealed that synaptic activity dramatically increases Nrf2 capacity to induce ARE-Luc activity (Fig. 3.4), however this induction was blocked upon Keap1 co-expression suggesting that the activity dependent induction of Nrf2 driven ARE-Luc activity is exerted via Keap1 suppression.

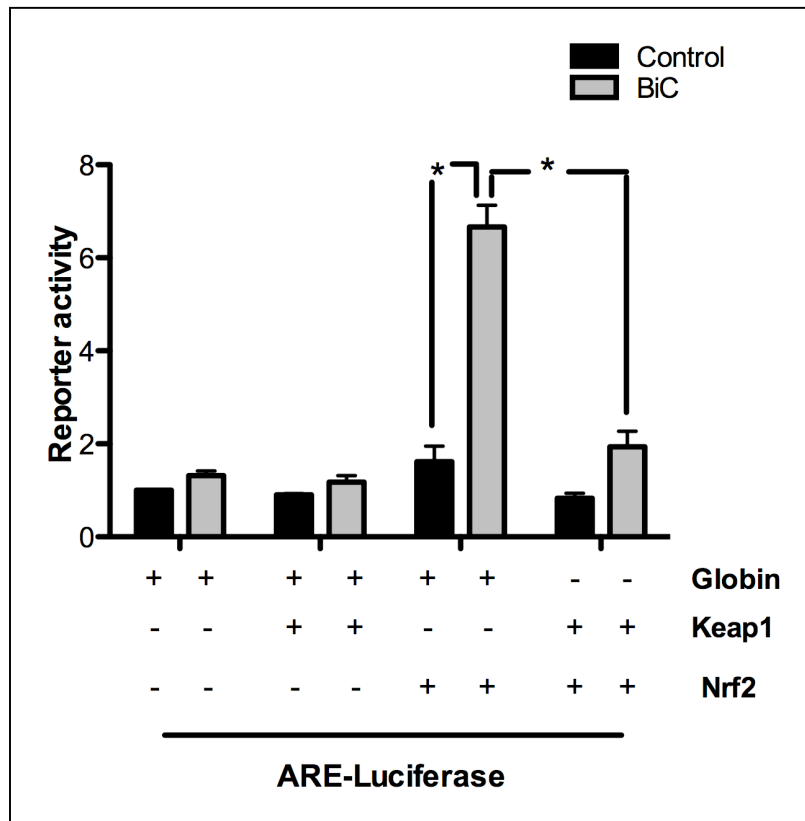
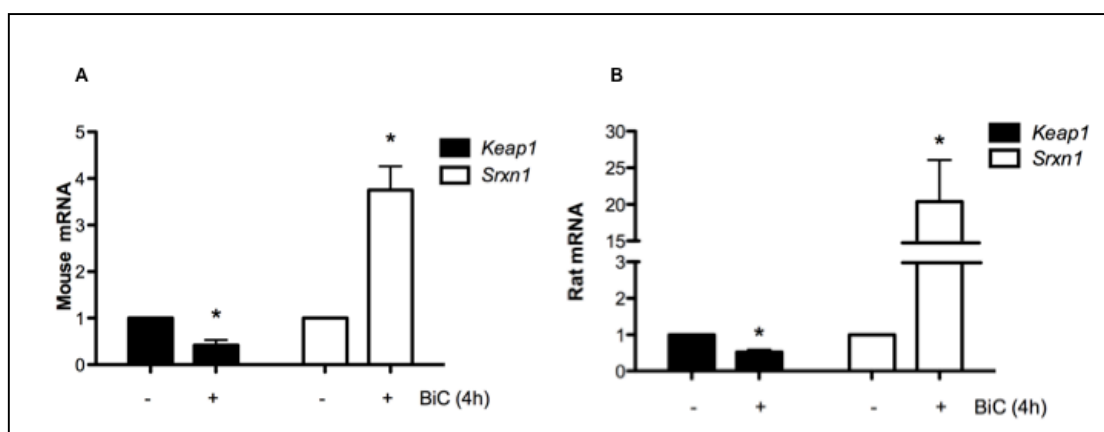


Figure 3.4. Synaptic NMDAR activity induces Nrf2-driven-ARE-promoter activity in a Keap1 sensitive manner.

Neurons were transfected with ARE-Luc plus pcDNA3.1-Nrf2 (or pGlobin), plus pcDNA3.1-Keap1 (or pGlobin). 24h post- transfection, neurons were stimulated where indicated with BiC for 24 h, after which reporter activity levels were measured (normalized to renilla levels). * $p < 0.05$, (n = 6).

3.2.5. Characterization of Keap1 suppression by synaptic activity

A previous mouse microarray expression data showed marked down regulation of mouse *Keap1* (Nrf2 negative regulator) mRNA by BiC treatment (in MK-801 sensitive manner) at 4 h time point. To confirm this finding, mouse and rat cortical cultures were treated with BiC for 4h, before harvesting RNA and the subsequent evaluation of *Keap1* and *Srxn1* (as a an example of an activity-regulated gene) mRNA expression by qRT-PCR (Fig. 3.5 A and B). The results revealed a large reduction in *Keap1* mRNA levels following BiC treatment in both mouse and rat primary neuronal cultures (Fig. 3.5 A and B) supporting the initial microarray results. In contrast, synaptic activity strongly induced *Srxn1* mRNA expression consistent with previous studies (Papadia et al., 2008). The suppressing effect of synaptic activity on *Keap1* mRNA was reduced by co-application of MK-801, which restored *Keap1* mRNA expression to a level slightly lower than that of the control but not significantly different, indicating that synaptic NMDAR activity is important for the suppressing function of synaptic activity (Fig. 3.5 C). The repression of *Keap1* expression by synaptic activity was also reflected at the protein level as evaluated by western blot, whereby 4 h of BiC treatment resulted in a significant down-regulation of Keap1 protein (Fig. 3.5 D and E).



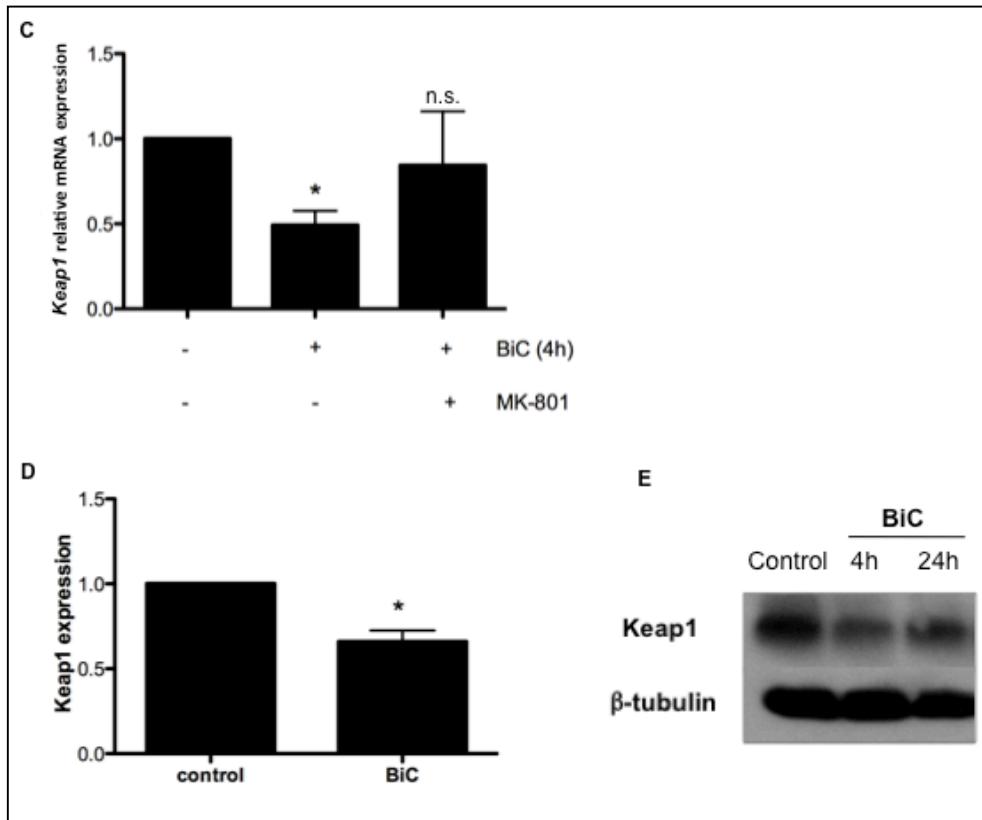


Figure 3.5. Synaptic NMDAR activity suppresses Keap1 expression.

QRT-PCR analysis of rat *Keap1* mRNA levels in neurons placed in trophically deprived medium and stimulated with BiC. Levels of Keap1 mRNA are normalized to GAPDH. **A**, **B**) Synaptic activity down regulates *Keap1* mRNA expression and up-regulates *Srxn1* mRNA expression in **A**) mouse and **B**) rat neurons at 4 h time point. * $p < 0.05$, (n=3-4). **C**) Neurons were treated with BiC in the presence or absence of the NMDAR antagonist MK-801 (10 μ M). * $p < 0.05$, not significant (n.s.), (n=6-7). **D**) Quantification of Keap1 protein expression in response to 4h treatment with BiC normalized to β -tubulin. * $p < 0.05$ compared with control (n=5). **E**) Example western blot picture of Keap1 expression and β -tubulin loading control.

3.2.6. Synaptic activity regulates Keap1 at a transcriptional level

I next wanted to investigate the mechanism by which synaptic activity down-regulates Keap1. The most likely mechanism of activity-dependent suppression of Keap1 expression is transcriptional, although other potential mechanisms may exist, such as regulation of mRNA stability. To address this, I created a *Keap1*-luciferase reporter construct by cloning approximately 890 bp of the rat *Keap1* promoter and 5' UTR upstream of a luciferase reporter gene. Neurons were transfected with *Keap1*-Luc and 24 h later were treated for another 24 h with BiC or left untreated before carrying out the luciferase assay. I found that BiC stimulations reduce *Keap1*-Luc activity by around 40% compared to the unstimulated neurons and this suppressive effect was reversed by co-application of MK-801 (Fig. 3.6A). These results suggest that synaptic activity elicits its suppressing effect on Keap1 by directly downregulating the transcriptional activity of *Keap1* promoter. Given this, I wondered if Keap1 could be a downstream target of Foxo, since Foxos are subject to synaptic activity regulation. To test this, I looked at the effect of LY294002 treatment on synaptic activity-induced downregulation of Keap1 mRNA levels. The rationale behind this, is if Keap1 is a Foxo target gene and given the pre-established involvement of PI3K pathway in the activity-dependent regulation of Foxo, then LY294002 pre-treatments shall attenuate the suppressive effect of BiC on Keap1 mRNA expression. However, data obtained from qPCR analysis showed no change either in Keap1 basal expression or in the activity-induced repression of Keap1 mRNA, suggesting that Keap1 is unlikely to be a Foxo target gene (Fig. 3.6B).

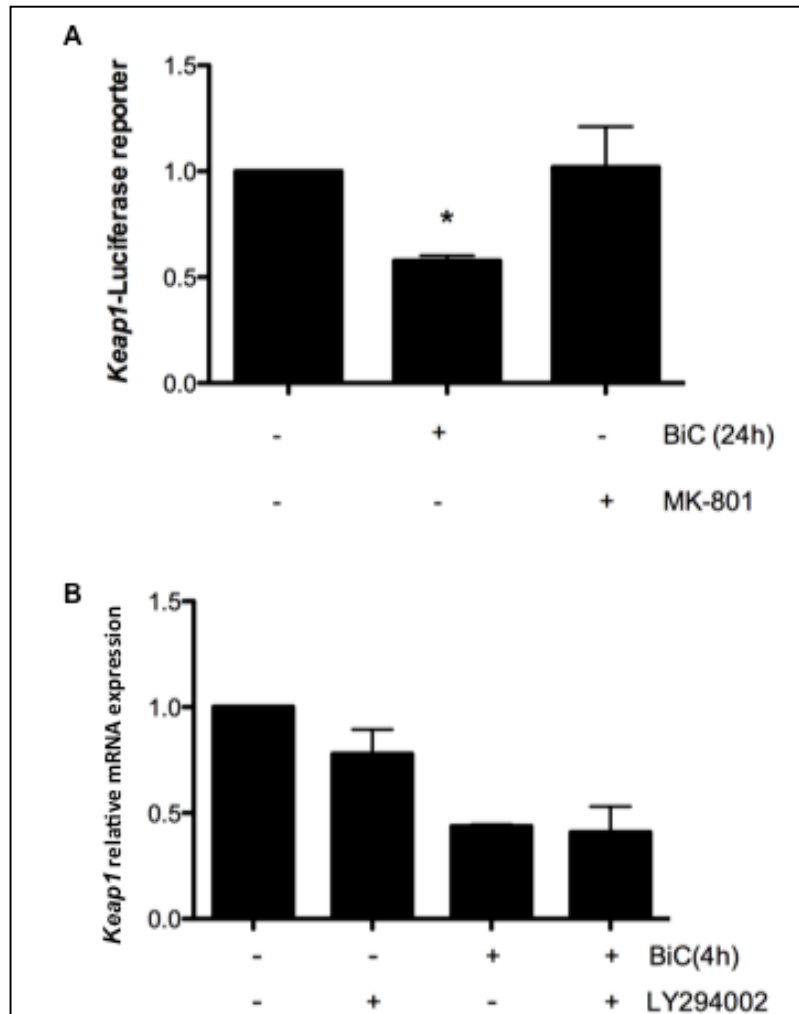


Figure 3.6. Synaptic NMDAR activity downregulates the transcriptional activity of *Keap1* promoter.

A) Neurons were transfected with Keap1-luciferase reporter, plus a TK-Renilla control vector. At 24 h after transfection, neurons were stimulated with the indicated drugs for 24 h, and firefly luciferase reporter activity was measured, normalized to the Renilla control. * $p < 0.05$, ($n=4$). **B)** QRT-PCR analysis of Keap1 mRNA levels in neurons placed in trophically deprived medium and stimulated as indicated in the presence or absence of the PI3K inhibitor 50 μ M LY294002, ($n=2$).

3.3. Discussion

3.3.1. Synaptic NMDAR activity suppresses Foxo1 expression via a cis-acting FOXO binding site

In this study we have demonstrated that *Foxo1* is a Foxo target gene and as a result, its expression is subject to feed-forward inhibition by synaptic NMDAR activity, which promotes Foxo nuclear exclusion. Thus, the suppressive effects of Foxo export on the expression of Foxo target genes may last considerably longer than previously thought due to the long-lasting effects of transcriptional suppression.

Synaptic activity exerts its inhibitory effect on Foxo1 activity not only through inducing post-translational changes (Foxo1 nuclear export, Papadia et al., 2008; Martel et al., 2009) but also through altering *Foxo1* expression. I found that Foxo1 expression was strongly suppressed following synaptic activity stimulation (Fig. 3.1). The activity-induced suppression of *Foxo1* and export of Foxo3 was PI3K- dependent, as treatments with LY294002 completely blocked the effect of synaptic activity (Fig. 3.2B and C). This observation led us to analyse the promoter region of *Foxo1*, which revealed two potential Foxo binding sites (Fig. 3.2A). Mutating the proximal Foxo consensus site reduced *Foxo1* promoter activity and abolished both the effect of synaptic activity and the effect of IGF-I on *Foxo1* promoter, emphasizing the importance of this consensus site in mediating Foxo-inactivating signals (Fig.3.3A). We also found this consensus site to be strongly activated by Foxo3 and, to a lesser extent Foxo1 (Fig 3.3B). Moreover, the proximal consensus site was not the only Foxo-responsive element within the *Foxo1* promoter, as a small but significant increase was observed in the activity of *Foxo1*(mut1)-Luc following the expression of Foxo3. This observation led us to identify the upstream IRS consensus sequence as a Foxo responsive element (Fig. 3.3C).

In neurons, Foxos are mostly involved in promoting cell death in response to various stress stimuli such as oxidative stress (Lehtinen et al., 2006), loss of trophic factor (Gilley et al., 2003) and ischemic injury (Fukunaga and Shioda, 2009). However, activation of synaptic NMDAR has been shown to render cultured neurons more resistant to oxidative stress, this effect was in part attributed to the suppression of Foxo1 target gene *Txnip* through promoting the PI3K-dependent Foxo1 nuclear exclusion. In contrast, elimination of NMDAR activity upregulates *Txnip* both *in vivo* and *in vitro* and promotes vulnerability to oxidative stress (Papadia et al., 2008). On the other hand, activation of extrasynaptic NMDAR promotes Foxo3 nuclear accumulation, which subsequently contributes to NMDAR-mediated neuronal death in hippocampal neurons. However, neurons depleted of endogenous Foxo3 or undergoing prolonged periods of synaptic activity are more resistant to excitotoxic insults (Dick and Bading, 2010). Furthermore, exposure of neurons to the phenolic antioxidant tBHQ protects against NMDA-evoked excitotoxicity, at least in part, by promoting Foxo3 cytoplasmic retention (Bahia et al., 2012). Neurotrophic factors are thought to promote neuronal survival at least in part through triggering the nuclear export of Foxo and the subsequent suppression of Foxo-directed gene expression, and the same may be true of synaptic NMDAR activity (Gan et al., 2005; Hardingham, 2006).

Although a large body of evidence shows that cell death is the most likely outcome of Foxos activation in neurons, work from the Kalb group has shown that genetic or pharmacological manoeuvres that promote nuclear accumulation of Foxo3 confers protection against known causes of motor neuron diseases in mouse spinal cord cultures (Mojsilovic-Petrovic et al., 2009). Their findings are in direct contrast to previous studies where overexpression of Foxo3 led to the death of motor neurons and cerebellar granule neurons (Brunet et al., 1999; Barthelemy et al., 2004). The disparities between these two observations may relate to the levels of transgene expression, the nature of stimulus used or differences in the neuronal cell types used

(Nemoto and Finkel, 2002). However, it is plausible that Foxo may need to be activated in the proper ‘window’ of time/magnitude in order to confer neuroprotection, otherwise it is detrimental.

The regulation of *Foxo1* promoter by Foxos may serve as an important feed-forward mechanism aimed to reinforce the effect of Foxo-inactivating or -activating signals such as synaptic activity or oxidative stress respectively, on FOXO1-target genes expression. This aspect of Foxo1 regulation could be unfavourable particularly in tumours, where abnormal activation of the PI3K-Akt pathway is an important step in their initiation and maintenance, Akt-mediated Foxo export may then lead to suppression of Foxo1 expression, exacerbating the situation further.

Shortly before the publication of this study, the Demoulin group performed a study on FOXO regulation in human fibroblast and arrived at the same conclusion that *FOXO1* is a FOXO target gene (Essagher et al., 2009). In agreement with our observations, they reported that FOXO3 displayed a stronger transactivating effect on *FOXO1* promoter in comparison to FOXO1 (Fig 3.3B). They also identified the same proximal Foxo consensus site (GTA AAC AA) as mediating a large portion of the observed Foxo responsiveness, which was responsible for growth factor-induced transcriptional suppression. Interestingly, they found that mutation of this proximal site reduced the activation of the promoter by FOXO3 overexpression, but did not abolish it completely. This raises the possibility that the IRS-like sequence identified in our study may be mediating the remaining induction. It is therefore likely that Foxo-inactivating signals are capable of suppressing Foxo1 expression in a variety, if not all, cell types.

3.3.2. Synaptic NMDAR activity suppresses Keap1 expression

Keap1 was initially described as a cytoplasmic inhibitor of Nrf2 that binds to actin cytoskeleton and Nrf2 to retain Nrf2 in the cytoplasm and target it for subsequent ubiquitination and degradation. In this study, the results suggest that synaptic NMDAR activity could induce the Nrf2/ARE signalling pathway by suppressing Keap1. Synaptic activity enhanced exogenous Nrf2 capacity to induce ARE-Luc activity in a Keap1 sensitive manner (Fig. 3.4) In addition, I showed that induction of synaptic activity results in the downregulation of Keap1 mRNA and protein expression in an NMDAR-dependent manner (Fig 3.5). The activity-dependent regulation of Keap1 appears to occur, at least in part, at a transcriptional level as BiC stimulations results in a significant suppression of *Keap1* promoter activity (Fig. 3.6A). Although Keap1 suppression could be a plausible mechanism by which synaptic activity could boost the Nrf2-controlled antioxidant defences, I didn't pursue this study further, as I find later in section 4.2.3 that the endogenous Nrf2 pathway is inactive in neurons and that it is predominantly astrocytic. Nevertheless, it is conceivable that synaptic activity could promote cell survival through repressing Keap1-mediated pro-death signalling.

Besides binding to its well-characterized substrate, Nrf2, Keap1 has been reported to bind other proteins directly or indirectly through its DGR domain. Recent studies have suggested a role for Keap1 in regulating cellular apoptosis through altering the activity of pro-apoptotic and anti-apoptotic proteins such as ASK1 and Bcl-2/Bcl-xL respectively (Stepkowski and Kruszewski, 2011). Phosphoglycerate mutase 5 (PGAM5), a recently characterized atypical member of the phosphoglycerate mutase family and a direct binding partner of the Bcl-xL anti-apoptotic protein, have been reported to interact with the DGR domain of Keap1 (Niture and Jaiswal, 2011a). PGAM5 acts as a bridge mediating Keap1 interaction with Bcl-xL and controlling Keap1-mediated degradation of Bcl-xL.

Further studies revealed that overexpression of Keap1 leads to increased degradation of both PGAM5 and Bcl-xL, which increases/activates pro-apoptotic factors and eventually resulting in increased cell apoptosis and decreased cell survival. On the other hand, siRNA inhibition of Keap1 or treatments with antioxidants such as tBHQ leads to the stabilization of Bcl-xL and increases cell survival (Niture and Jaiswal, 2011a).

Bcl-2 is another recently reported substrate for Keap1. Unlike Bcl-xL, Keap1 DGR domain directly interacts with the BH2 domain of Bcl-2 and facilitates the Keap1-Cul3 mediated ubiquitination of Bcl-2 lysine17 residue and degradation of Bcl-2 (Niture and Jaiswal, 2011b). Overexpression of Keap1 can promote apoptosis by degrading Bcl-2 and decreasing Bcl-2: Bax heterodimers. For instance, Keap1 overexpression enhanced etoposide-mediated apoptosis in cancer cell. However, antioxidants antagonize Keap1: Bcl-2 interaction, leading to the release and stabilization of Bcl-2, increased Bax:Bcl-2 heterodimer and cell survival. The stabilization of the anti-apoptotic proteins Bcl-2 and Bcl-xL and prevention of apoptosis are presumably important mechanisms by which the cells protect themselves from dying in acute stress (Niture and Jaiswal, 2011b).

ASK-1 was identified as the main MAPK kinase kinase and a pivotal component in the mechanism of cytokine- and stress-induced apoptosis (Saitoh et al., 1998). Dephosphorylation of ASK-1 by PGAM5 leads to its activation, which contributes to apoptosis in various stress conditions such as toxification, neurodegenerative diseases, immune responses (Stepkowski and Kruszewski, 2011) and oxidative stress (Niso-Santano et al., 2010). Whereas thioredoxin, an Nrf2-target gene, binds and inhibits ASK-1. The link between ASK-1 and Nrf2/Keap1 pathway was demonstrated in the study of paraquat-induced cell death, in which Keap1-deficient mouse embryonic fibroblasts (MEFs) were insensitive to paraquat-mediated cell death and this effect was attributed to the inability to activate ASK-1, whilst ASK-1 was constitutively activated in Nrf2-deficient MEFs displaying hypersensitivity to the same insult (Niso-Santano et al., 2010). However, whether Keap1/PGAM5 binding affects ASK-1

dephosphorylation is unclear.

The activation of NF- κ B family of transcription factors has been associated with the development and/or progression of cancer by regulating the expression of genes involved in cell growth and proliferation. Recently, I κ B kinase (IKK β), a positive regulator of NF- κ B, was found to be destabilized by Keap1, which resulted in the inhibition of NF- κ B-induced tumourgenesis (Lee et al., 2009; Kim et al., 2010). Moreover, genomic alterations of Keap1 fail to facilitate IKK β degradation, which then leads to the activation of NF- κ B pathway in human cancers (Lee et al., 2009). Thus, Keap1 acts as a tumour suppressor through negative regulation of three substrates Nrf2, IKK β and Bcl-2/Bcl-xL.

The notion that the activity-dependent inhibition of Keap1 may promote cell survival requires further investigation. Future studies will be needed to address the effect of synaptic activity on the stability of the anti-apoptotic proteins Bcl-2 and Bcl-xL and/or the activity of ASK-1 and NF- κ B, and to determine whether this effect is conferred through Keap1 suppression. However, this is beyond the scope of this thesis as we are primarily interested in the regulation of the endogenous Nrf2/ARE pathway in response to intrinsic stimuli rather than the regulation of Keap1-mediated pro-death signalling.

Chapter 4

Mild oxidative stress activates the Nrf2 pathway in astrocytes but not neurons

4.1. Summary

Since Nrf2 plays an important role in sensing and combating oxidative stress (Kensler et al., 2007; Cullinan et al., 2004), I postulated that sub-toxic levels of oxidative stress might be sufficient to turn on the Nrf2-dependent cytoprotective genes expression. To recreate oxidative stress *in vitro*, I utilized sub-toxic doses of H₂O₂. I chose three previously established markers of Nrf2 activation to evaluate: *Srxn1* (Soriano et al., 2008) *Hmox1* and *xCT* (Alam et al., 2000; Ishi et al., 2000; Shih et al., 2003). I found that all three markers were transcriptionally induced in astrocytes containing neuronal cultures (AC-neurons) following sub-toxic H₂O₂ application. However, the inductive effect of H₂O₂ was abolished in cultures prepared from *Nrf2*^{-/-} mice, confirming Nrf2-dependent mechanism of action. I also found that application of H₂O₂ or tBHQ to AC-neurons induces Hmox1 immunostaining in astrocytes but not neurons. Moreover, neither mild oxidative stress, tBHQ nor Keap1 genetic ablation could induce Nrf2 target gene expression in neuronal cultures devoid of astrocytes. However, Nrf2 forced expression in neurons was capable of inducing endogenous Hmox1 expression. These results therefore, demonstrate that the endogenous Nrf2 pathway is responsive to mild oxidative stress and that astrocytes are the sole locus of Nrf2 activation in our model. Of note part of the work presented in this chapter has been published (Bell et al., 2011a).

4.2. Results

4.2.1. Establishment of culture systems and H₂O₂ stimulations

Cortical mouse neurons from E17.5 CD1 mice were cultured as described in section 2.1. In this study I utilized three types of cortical cultures: astrocytes containing neuronal cultures, astrocyte-free neuronal cultures designated as (AC-neurons) and (AF-neurons) respectively and astrocytes enriched cultures (astrocytes). To control for non-neuronal cell proliferation, cultures were grown in the presence or absence of the anti-mitotic AraC. To obtain (AC-neurons) comprising (more than 90% NeuN⁺ and less than 10% GFAP⁺ cells), cultures were treated with AraC on DIV04 (Fig. 4.1 A). For (AF) cell population, comprising more than 98% Neu N⁺ and less than 0.2% GFAP⁺ cells, AraC was added to cultures on DIV0 (Fig. 4.1 B). Astrocyte cultures were prepared as described in section 2.1 and grown in the absence of AraC, which results in a cell population comprising of more than 96% GFAP⁺ and less than 4% Neu N⁺ cells (Fig. 4.1 C) (Bell et al., 2011a).

Various experimental approaches have been developed to model oxidative stress in primary neural cell cultures, which include exposure to pro-oxidative conditions such OGD (Furuichi et al., 2005), GSH-deprivation (Ratan et al., 1994) and addition of external chemical compounds with the capacity to undergo redox cycling such as Paraquat (PQT) (Comporti, 1989; Escartin et al., 2011) or addition of H₂O₂ (Rojo et al., 2008) Papdia et al., 2008; Soriano et al., 2008). In addition, some studies utilize tBHQ to induce oxidative stress-mediated Nrf2 activation, however, reports differ on the involvement of oxidative stress in the tBHQ-mediated Nrf2 activation. While tBHQ was shown to lead to OH[•] radical formation, which was inhibited upon the addition of catalase in human hepatoma cells (Pinkus et al., 1996), activation of the ARE by tBHQ in human neuroblastoma cells (Lee et al., 2001) or in primary cortical cultures derived from ARE-hPAP reporter mice (Johnson et al., 2002) was independent of oxidative stress as pre-treatment with antioxidants or antioxidant enzymes didn't inhibit tBHQ-induced ARE activation.

In the present study, I have utilized H₂O₂ to model oxidative stress *in vitro* because it's endogenously produced and can easily diffuse through cell membranes (Halliwell

and Gutteridge, 1989; Hogg et al., 1992). Cells were pre-incubated in TMO for ~ 16 h prior to 6 h stimulation with sub-toxic doses of H₂O₂ (25μM-100μM) (Fig. 4.1D).

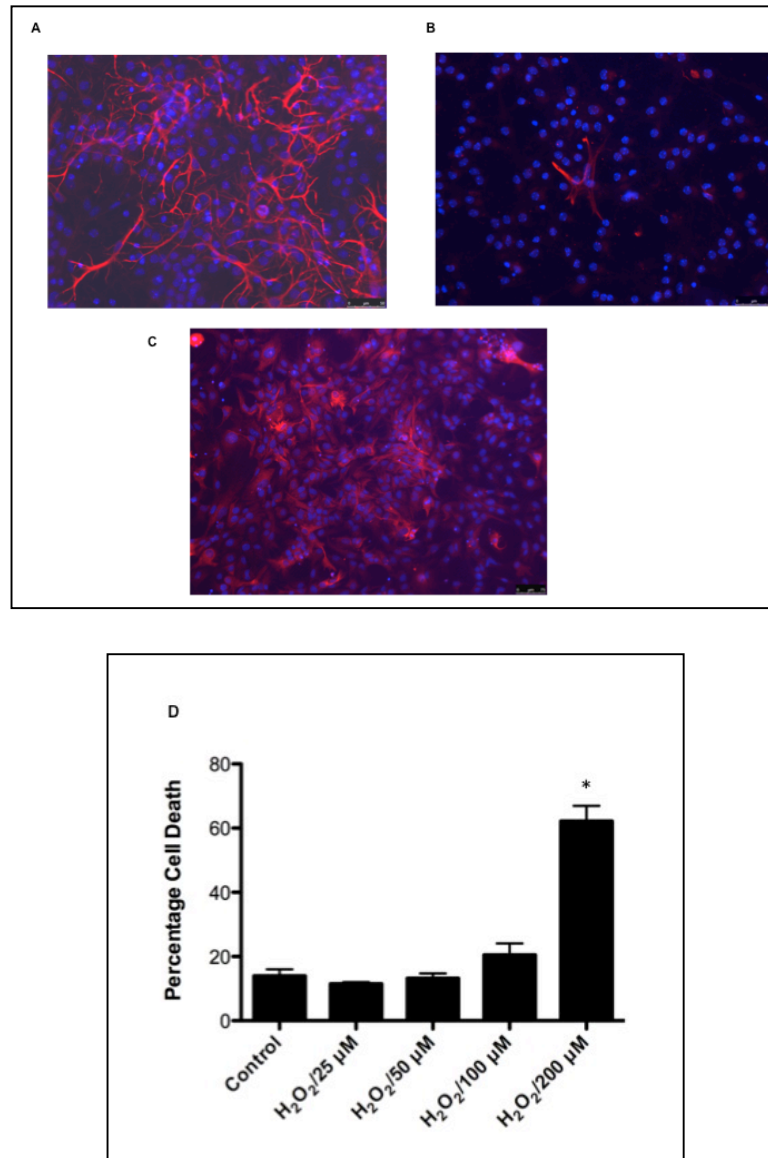


Figure 4.1. Establishment of cell culture systems and oxidative stress model.

Example pictures showing GFAP immunostaining (red) with nuclear DAPI stain (blue). **A)** AC-neurons. **B)** AF-neurons **C)** Astrocytes. **D)** To induce oxidative stress a range of H₂O₂ doses (25μM-100μM) was utilized and cell viability was evaluated in AC- neurons by assessing nuclear integrity as described in section 2.4. (n=3), **p*<0.05.

4.2.2. Mild oxidative stress upregulates Nrf2-dependent gene expression

In an effort to determine whether mild oxidative insult could activate the Nrf2 pathway, AC-neurons were exposed to either 10 μ M tBHQ, as a positive control for Nrf2-dependent gene expression induction (Lee et al., 2003), or to a range of H₂O₂ doses (25-100 μ M) for 6 h after which RNA was harvested and converted to cDNA for subsequent qPCR analysis. Treatments with H₂O₂ showed marked upregulation of *Srxn1* and *Hmox1* gene expression (Fig. 4.2A, B). As for *xCT* mRNA levels, a modest yet significant induction was observed (Fig. 4.2 C). To look at dependence on Nrf2, cultures derived from *Nrf2*^{-/-} mice were subjected to the same protocol. In these cultures, H₂O₂ was unable to induce Nrf2-target genes expression, highlighting the central role of Nrf2 in mediating the H₂O₂-induced gene upregulation (Fig. 4.2). Loss of Nrf2 not only abolished the inductive effect of H₂O₂, but also dramatically reduced *Hmox1* basal expression. Similar observation was reported in human endothelial cells, whereby transfection with Nrf2 siRNA produced a significant reduction in HMOX-1 mRNA expression (Jyrkkanen et al., 2008). Moreover, *xCT* basal expression was reduced but to a lesser extent in *Nrf2*^{-/-} cultures, while *Srxn1* basal levels remained unchanged.

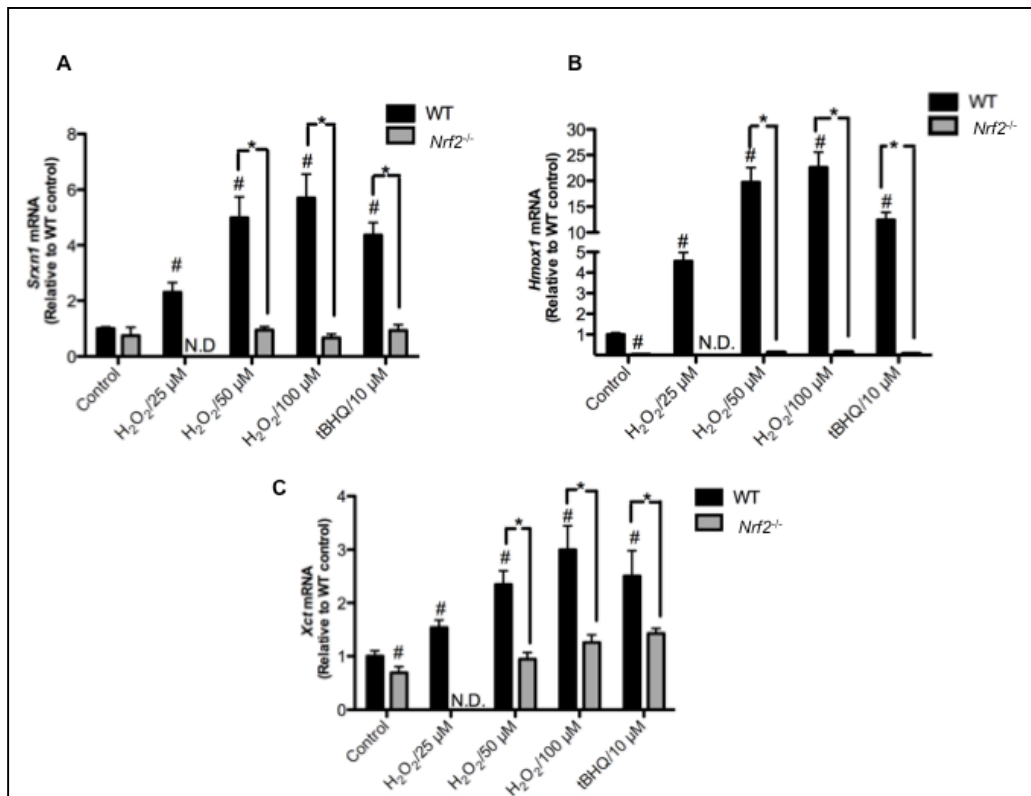


Figure 4.2. Mild oxidative stress-mediated upregulation of phase II genes expression is Nrf2 dependent.

AC-neurons were treated for 6 h in trophically deprived medium with H₂O₂ or tBHQ at the indicated doses, followed by RNA isolation and qPCR analysis of gene expression (normalized to Gapdh) and compared to WT-control. (n=5-6) #*p*<0.05 significantly different from WT-control ; **p*<0.05 significantly different from corresponding treatment in *Nrf2*^{-/-}, not done (N.D). **A)** *Srxn1* mRNA levels. **B)** *Hmox1* mRNA levels. **C)** *xCT* mRNA levels.

4.2.3. Mild oxidative stress-mediated Nrf2 activation is restricted to astrocytes

The site of Nrf2 activation has been a matter of controversy. While most reports suggest that Nrf2 activation is predominantly astrocytic (Shih et al., 2003; Kraft et al., 2004; Vargas and Johnson, 2009), several lines of evidence show that Nrf2 could be induced in neurons *in vitro* (Johnson et al., 2002) and *in vivo* (Kraft et al., 2007). I therefore resolved to determine the locus of Nrf2 activation, in our *in vitro* model of oxidative stress by measuring the mRNA levels of Nrf2 downstream genes in AC-neurons following 6 h exposure to H₂O₂ or tBHQ. The same experiment was performed with AF-neurons and astrocytes. QPCR analysis revealed a large induction of *Srxn1*, *Hmox1*, and *xCT* gene expression in both AC-neurons and astrocyte post H₂O₂ application (Fig. 4.3). By contrast, the absence of astrocytes in AF-neurons completely abolishes the H₂O₂-mediated gene induction (Fig. 4.3). However, in astrocytes, H₂O₂ triggers a stronger Nrf2-dependent gene expression (Fig 4.4) compared to the other culture types. These results strongly indicate that astrocytes are the primary locus for Nrf2 activation by mild oxidative stress.

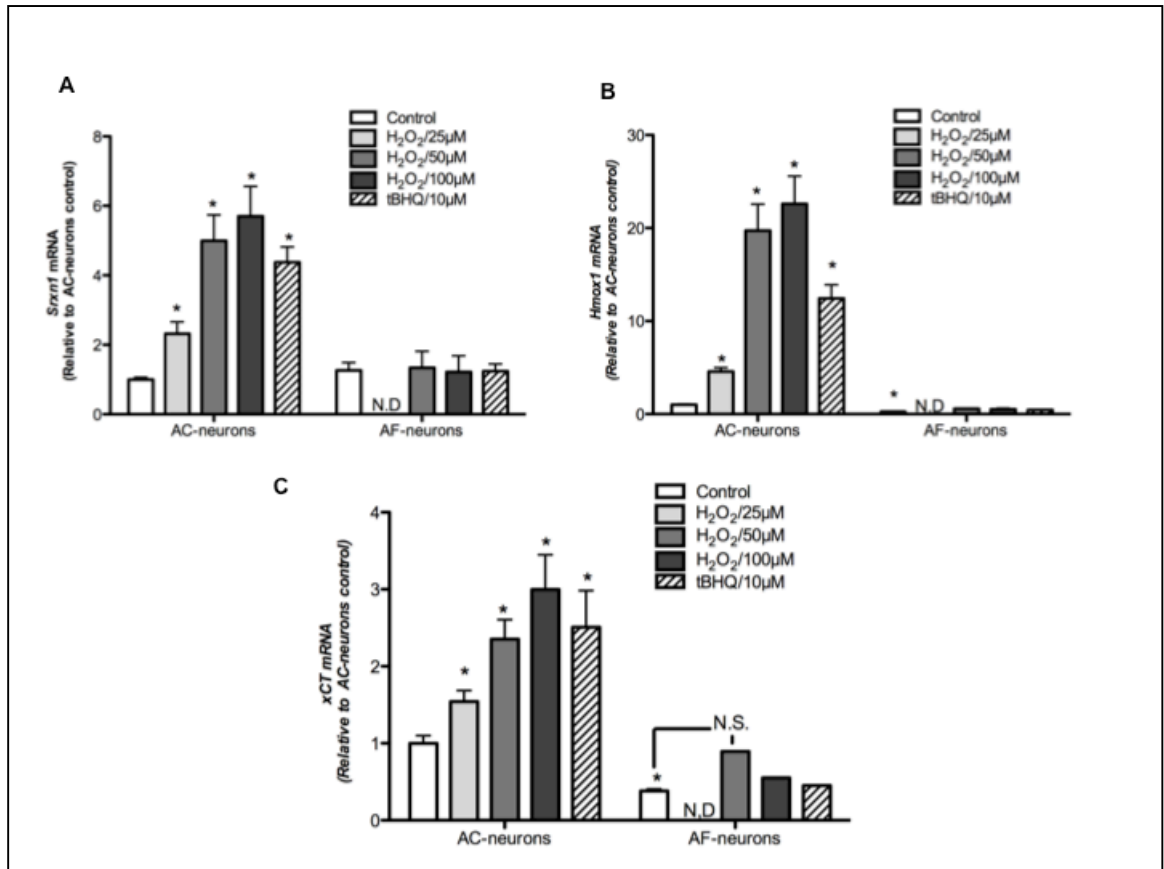


Figure 4.3. Nrf2 activation by mild oxidative stress is astrocytes dependent.

AC and AF-neurons were treated in parallel with H₂O₂ or tBHQ at the indicated doses for 6 h, before harvesting RNA for qPCR analysis of gene expression (normalized to Gapdh). (n=5-9), **p*<0.05 significantly different from AC-neurons control. **A)** *Srxn1* mRNA levels. **B)** *Hmox1* mRNA levels. **C)** *xCT* mRNA levels.

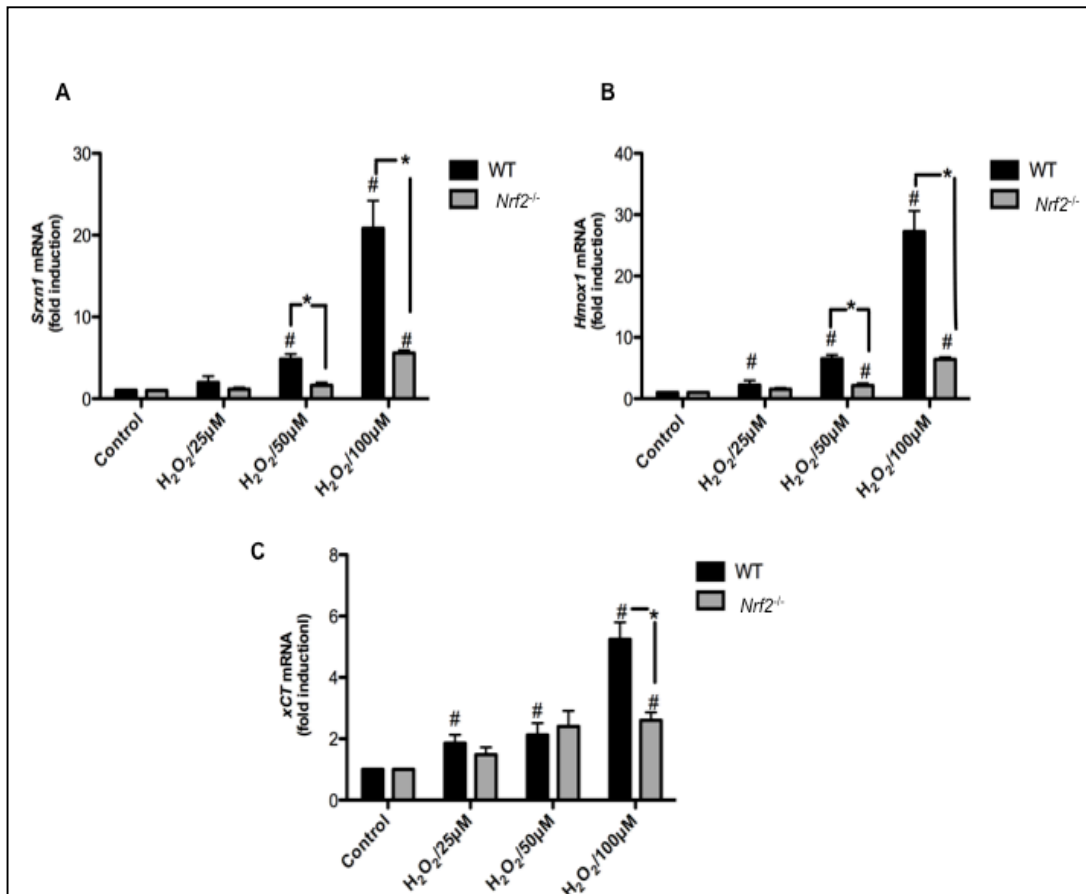


Figure 4.4. H₂O₂ treatments trigger robust Nrf2-dependent induction in astrocytes.

Astrocytes were treated with the indicated doses of H₂O₂ for 6 h, before harvesting RNA for qPCR analysis of gene expression (normalized to Gapdh). (n=5-9), #*p*<0.05 significantly different from control ; **p*<0.05 significantly different from corresponding treatment in *Nrf2*^{-/-}. **A)** *Srxn1* mRNA levels. **B)** *Hmox1* mRNA levels. **C)** *xCT* mRNA levels.

4.2.4. The Nrf2 pathway can't be activated in neurons even when surrounded by astrocytes

The induction of Nrf2-dependent gene expression observed in AC-neurons is most likely attributable to Nrf2 activation in astrocytes, since AF-neurons devoid of astrocytes fail to respond to Nrf2 inducers (Fig. 4.3). However, I sought to rule out the possibility that Nrf2 could be activated in neurons when surrounded by astrocytes. One way to examine this notion is by performing immunofluorescence in AC-neurons using antibody to Hmox1 protein in conjunction with an antibody to the astrocytic marker GFAP. The analysis demonstrated an increase in Hmox1 expression co-localized to GFAP positive cells following treatment with either tBHQ or H₂O₂ (Fig. 4.5A), whereas cells displaying neuronal morphology not only failed to respond to the treatments but also exhibited significantly lower basal expression compared to astrocytes (Fig. 4.5B). These results suggest that the Nrf2 pathway is inactive in neurons even when in contact with astrocytes and further confirms that astrocytes are the sole locus for Nrf2 activation.

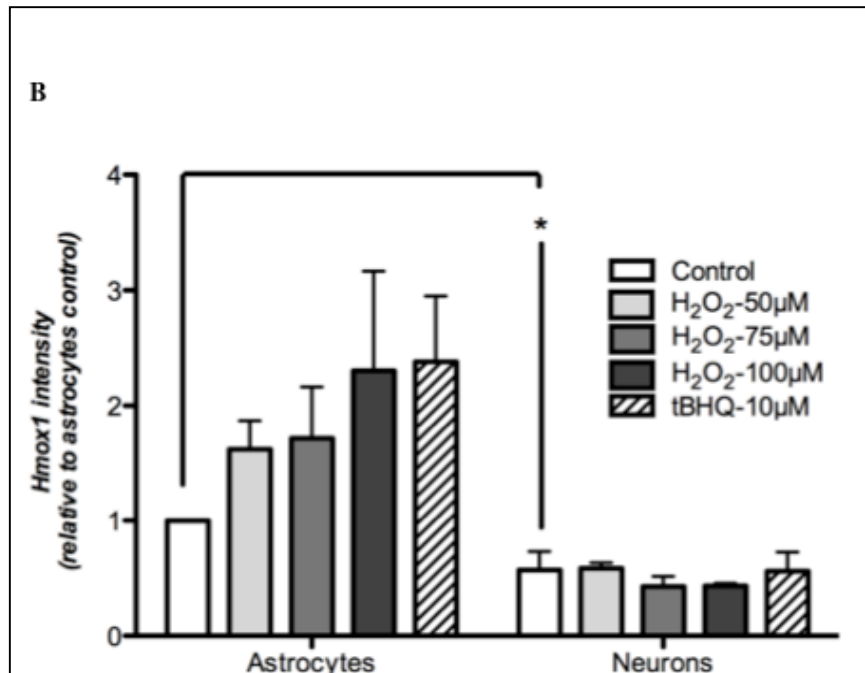
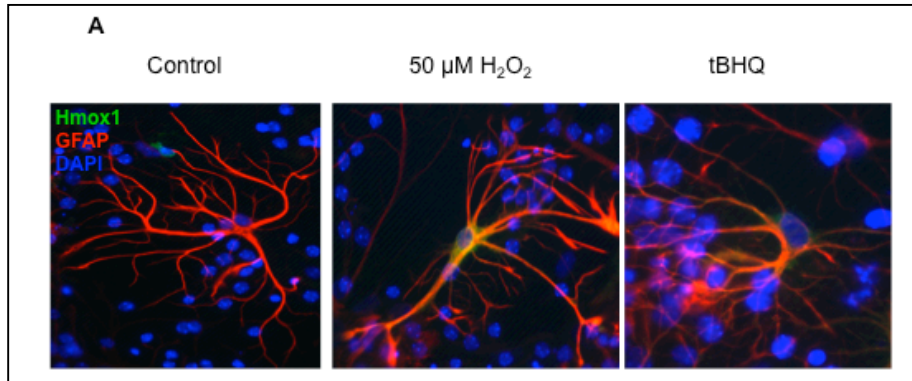


Figure 4.5. Activation of the Nrf2 pathway is confined to astrocytes.

AC-neurons were treated with either H₂O₂ or tBHQ at the indicated doses for 24 h and then were stained with anti-Hmox1 (green) and anti-GFAP (red) and with DAPI (blue). **A**) Representative images of Hmox1 immunofluorescence staining showing that Hmox1 induction in response to either H₂O₂ or tBHQ is specific to GFAP-positive astrocytes. **B**) Quantification of Hmox1 staining. (n=3), **p*<0.05, versus astrocytes control.

4.2.5. Alleviating the Keap1-mediated Nrf2 suppression fails to activate the endogenous Nrf2 pathway, while forced Nrf2 expression rescues the pathway in neurons

The failure to activate the Nrf2 pathway following the application of H₂O₂ or tBHQ in AF-neurons (Fig. 4.3) or even in neurons when surrounded by astrocytes in the AC-neurons (Fig. 4.5) led me to hypothesize that this was due to increased Keap1 inhibition of Nrf2 in neurons. To test this, I evaluated the mRNA expression of Nrf2-downstream genes in cultures derived from *Keap1*^{-/-} mice. Constitutively active Nrf2 has been reported in Keap1 null MEF (Wakabayashi et al., 2003) and in Keap1 knockdown cortical astrocytes (Gan et al., 2010) leading to the induction of Nqo1. Similarly, cortical mixed neuron/astrocyte cultures derived from *Keap1*^{-/-} mice displayed an upregulation in phase II enzymes resulting in increased resistance of neurons against oxidative stress (Sato et al., 2009). In agreement with the previous reports, I have observed an elevation in the basal expression of Nrf2-target genes in AC-neurons and astrocytes prepared from *Keap1*^{-/-} mice, whereas Keap1 deletion had no effect in AF-neurons (Fig. 4.6). The possibility that the lack of Nrf2 activity in neurons is due to low Nrf2 (*Nfe2l2*) gene expression was tested in our laboratory. A qPCR analysis of *Nfe2l2* and *Keap1* basal mRNA levels in the three culture types performed by Dr. Bell showed no significant difference in *Keap1* expression between neurons and astrocytes, while interestingly *Nfe2l2* mRNA levels were substantially lower in neurons (0.079 ± 0.027 and 4.9 ± 1.53 in AF- and astrocyte respectively, compared to AC-neurons). The diminished Nrf2 expression could account in part for the lack of Nrf2 activity in neurons. However, limited Nrf2 access to its target genes promoters is another plausible explanation for the lack of Nrf2 activity in neurons. To this end, I resolved to test if ectopic expression of Nrf2 in neurons could revive the pathway. Using a neuron-specific transfection protocol, neurons were co-transfected with Nrf2 driven by the elongation factor-1 α promoter (EF-Nrf2) and a CMV promoter driven eGFP (used as marker for transfected cells). GFP-positive neurons overexpressing Nrf2 displayed an increased Hmox1 staining as seen by immunofluorescence analysis (Fig. 4.7). This preliminary result suggests that ectopic Nrf2 is capable of transactivating its target genes and could be activated in neurons when available at sufficient levels, however, the same experiment should be repeated

to include appropriate controls in which neurons are transfected with a control plasmid such as Globin along with the eGFP and the intensity of Hmox1 expression should be evaluated in Nrf2 and Globin overexpressing neurons. All together these results suggest that the inactivity of endogenous Nrf2 in neurons and the failure to boost it through lifting the Keap1-mediated suppression is a result of Nrf2 low expression levels in neurons.

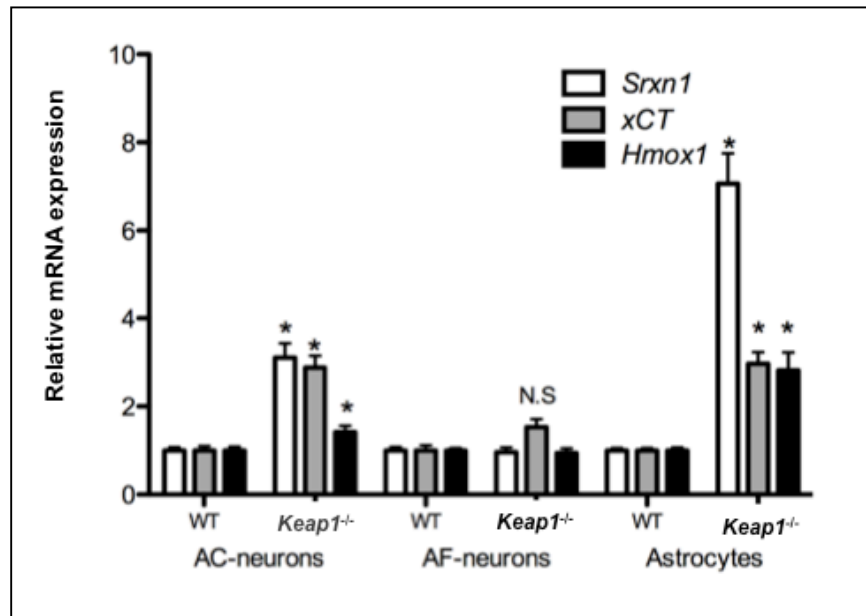


Figure 4.6. Keap1 genetic ablation is not sufficient to activate Nrf2 transcriptional pathway in neurons.

AC-neurons, AF-neurons and astrocytes were prepared from WT or *Keap1*^{-/-} mice littermates. Cells were lysed and RNA was harvested for qPCR analysis of *Srxn1*, *xCT* and *Hmox1* constitutive mRNA levels (normalized to Gapdh). (n=6-15), **p* <0.05 significantly different compared to WT.

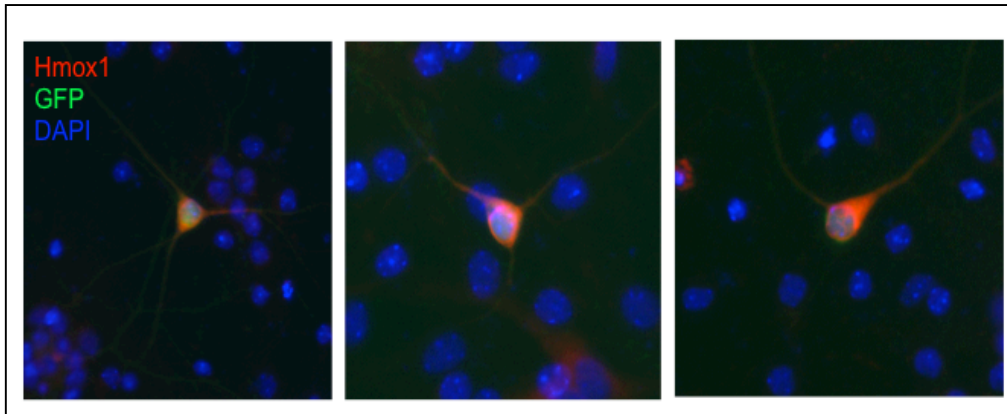


Figure 4.7. Nrf2 ectopic expression induces endogenous Hmox1 expression in neurons.

Example pictures from preliminary immunocytochemistry experiments showing that Nrf2 forced expression could induce endogenous Hmox1 expression, however, inclusion of appropriate controls is required for more conclusive results. AC-neurons were co-transfected with pEF-Nrf2 and eGFP as a transfection marker (green) and after 48h subjected to immunocytochemical analysis of Hmox1 expression (red).

4.3. Discussion

Data presented herein are the first demonstrating that intrinsic cellular signals such as mild oxidative stress are capable of activating the endogenous Nrf2 pathway and mediating Nrf2-dependent phase II gene induction in an astrocyte specific manner. I also report the lack of neuronal response to Nrf2 activation by H₂O₂, Nrf2 specific inducer tBHQ or Keap1 genetic ablation. In addition, restricted induction of Hmox1 to astrocytes in AC-neurons, excludes the possibility of neuronal contribution to the Nrf2-dependent gene upregulation observed post H₂O₂ or tBHQ treatments as a results of a specific neuron-astrocyte signalling.

4.3.1. Nrf2-dependent gene expression is strongly induced in response to mild oxidative stress

In this current study I have shown that endogenous Nrf2-dependent antioxidant response could be strongly triggered by mild oxidative stress (Fig. 4.2). Robust induction was observed in *Hmox1* gene expression levels following treatment with H₂O₂ (Fig. 4.2B), this elevation was also reflected at the protein level as seen by increased Hmox1 immunofluorescence staining (Fig. 4.5A). Similarly, sub-toxic H₂O₂ doses increased *Srxn1* and *xCT* mRNA levels (Fig. 4.2 A, C). I confirmed the Nrf2 specific requirement in the H₂O₂-mediated upregulation of phase II genes expression, as loss of Nrf2 completely ablated the inductive effect of H₂O₂. The impact of Nrf2 loss was not limited to the inducible levels of these genes, it also, as anticipated altered the basal mRNA expression of *Hmox1* and *xCT*. *Hmox1* basal levels dropped markedly in *Nrf2*^{-/-} AC-neurons to about one tenth of that in WT, whereas *xCT* constitutive expression was reduced by around 30% in *Nrf2*^{-/-} cells (Fig. 4.2B, C). Moreover, Keap1 deficient cultures displayed constitutive upregulation of *Hmox1* and *xCT* gene expression, further confirming the role of Nrf2 in controlling the basal expression (Fig. 4.6). This is in agreement with previous reports, documenting a significant upregulation of phase II enzymes gene expression or protein expression in the absence of Keap1 (Wakabayashi et al., 2003; Satoh et al.,

2009; Gan et al., 2010). Interestingly, while loss of Keap1 induced *Srxn1* basal mRNA levels, no change was observed in *Nrf2*^{-/-} cultures. Given that *Srxn1* harbours composite ARE/AP-1 sites within its promoter (Soriano et al., 2008), its plausible that compensatory genetic mechanism controlled through the AP-1 site accounts for the lack of change in *Srxn1* basal mRNA levels in Nrf2 deficient cells.

H₂O₂ has been implicated in the pathway leading to the Ischemic-preconditioning phenomenon (IPC), in which sub-lethal ischemic episode renders the cells more resistant to a subsequent and normally lethal ischemic insult (Furuichi et al., 2005). In regard to this, our group has recently shown that Nrf2 activation contributes to the neuroprotective effect of IPC in both *in vitro* and *in vivo* models of ischemia. Exposure to oxygen glucose deprivation (OGD) preconditioning stimulus resulted in a significant induction of *Srxn1* and *Hmox1* gene expression. This effect was also extended to include upregulation of the glutathione pathway genes expression *in vitro* (Bell et al., 2011b). Similar results were obtained when adult mice were subjected to transient occlusion of the middle cerebral artery (MCAO). Moreover, mild oxidative stress-induced *Srxn1* and *Hmox1* gene up-regulation in human embryonic stem cell (HESC)-derived astrocytes (Bell et al., 2011a).

A recent study identified an Nrf2-independent mechanism for H₂O₂-induced astrocyte-dependent neuroprotection (Haskew-Layton et al., 2010). A unique enzymatic system was employed in this study to allow for the control of duration and level of intracellular H₂O₂ production in astrocytes. To that end, H₂O₂-producing enzyme (D-amino acid oxidase) was heterologously expressed in astrocytes that metabolizes an exogenous substrate (D-alanine) enabling regulated generation of H₂O₂. The controlled continuous sub-toxic H₂O₂ production in astrocytes rendered adjacent neurons resistant to an oxidative insult. However, they reported that the sub-toxic doses of H₂O₂ fail to activate the Nrf2 pathway in astrocytes and trigger neuroprotection via a mechanism independent of Nrf2 and possibly involving the inhibition of protein tyrosine phosphatase (Haskew-Layton et al., 2010). One potential explanation for the discrepant findings between the two studies is that H₂O₂ doses greater than 30 μM were not explored in their study, because 100 μM H₂O₂ was reported to be toxic to astrocytes as determined by 3-(4,5-Dimethylthiazol-2-yl)-

2,5-diphenyltetrazolium (MTT) assay or by ethidium homodimer-1, a chemical which only stains the nuclei of dead cells (Haskew-Layton et al., 2011). However, the MTT assay measures cellular NADPH-reducing activity, which could drop in response to sub-toxic oxidative stress (due to the NADPH-dependent enzymatic H₂O₂ detoxification) resulting in overstated toxicity. In contrast, we observe no evidence of H₂O₂-induced toxicity at 100 μM H₂O₂ neither in astrocytes (Bell et al., 2011a) nor in AC-neurons (Fig. 4.1D), as assessed by ATP assay and/or nuclear integrity. The discrepancies in the H₂O₂-induced toxicity results may be due to differences in the seeding density of the astrocyte cultures used. This observation was previously reported in rat neuronal and astrocytic primary cultures, whereby the degree of H₂O₂ toxicity/detoxification, at the same dose of H₂O₂, varied with cells seeding density (Dringen et al., 1999a; Song et al., 2004).

Another possible reason for the disparities between the two studies lies in the Nrf2 activity assay utilized by Haskew-Layton and co-workers, in which a luciferase reporter incorporating the ARE from the NQO1 promoter was employed. However, different AREs can have different Nrf2 dependencies for basal and/or inducible activity and unpublished data from our laboratory revealed a weak induction of Nqo1 by 100 μM H₂O₂. Therefore the ARE promoter from NQO1 is not as sensitive as other ARE promoters and may not be a good indicator of Nrf2 response to H₂O₂.

Regardless of the contradicting results, both studies confirm the importance of astrocytes in mediating neuroprotection. While our results emphasize the importance of astrocytic Nrf2-dependent pathway as a mediator of neuroprotective responses to oxidative stress, it is feasible that astrocyte-dependent neuroprotection against oxidative stress involves both Nrf2-dependent and Nrf2-independent signalling pathways. However, the contribution of one pathway or the other could depend on the developmental stage or the magnitude or nature of the oxidative insult (Bell et al., 2011a; Bell et al., 2011b).

4.3.2. Mild oxidative stress activation of Nrf2 is astrocyte specific

Previous studies aimed to determine the site of Nrf2 activation, in which genetic or pharmacological approaches were utilized to activate Nrf2, have generated contrasting results. While some studies are in support of the astrocyte-specific activation of the Nrf2/ARE pathway (Ahlgren-Beckendorf et al., 1999; Murphy et al., 2001; Kraft et al., 2004), other studies documented functional Nrf2 pathway in neurons (Johnson et al., 2002; Kraft et al., 2007; Escartin et al., 2011). Discrepancies in this regard, could be due to many factors such as the differences in the detection methods for markers of Nrf2 activation, the developmental stage, the brain region under investigation and the nature and intensity of the stimuli. Despite the disparities in the results from these studies, they collectively don't establish the locus of endogenous Nrf2 activity in response to endogenous signals such as oxidative stress.

This study provides, for the first time, *in vitro* evidence of the locus of endogenous Nrf2 activation by intrinsic stimulus such as mild oxidative stress. I have observed no induction in Nrf2-dependent gene expression in cultures devoid from astrocytes neither with H₂O₂ nor with tBHQ treatments (Fig. 4.3). On the other hand, astrocytes displayed a very robust gene expression induction in response to either treatments (Fig. 4.4). Although astrocytes are represented by a small population (~10%) in our AC-neurons, they account for the strong Nrf2 response to H₂O₂ or tBHQ. This observation was further confirmed through immunofluorescent analysis revealing an increased Hmox1 expression by H₂O₂ or tBHQ co-localized with GFAP-positive astrocytes but not with cells displaying neuronal morphology (Fig. 4.5).

Previous studies have been focused on the role of astrocytes in rodent systems in mediating a neuroprotective response to Nrf2-activating stimuli, however, this response was recently shown to be relevant to human systems. A study by Gupta *et al.* demonstrated that HESC-derived astrocytes are capable of promoting the protection of HESC-derived neurons against oxidative insults and that their neuroprotective capacity can be greatly enhanced by treatment with the Nrf2 inducer 1[2-Cyano-3,12-dioxool-eana-1,9(11)-dien-28-oyl] trifluoroethylamide (CDDO^{TFEA}) (Gupta et al., 2012). Moreover, treatment of HESC-derived neurons with CDDO^{TFEA} failed to induce Nrf2 target genes and more importantly elicited no significant

protective effect against oxidative insults. This study supports the notion that astrocytes are the locus of endogenous Nrf2 activation in rodent (Fig 4.3-4.6) as well as in human systems (Gupta et al., 2012).

4.3.3. Nrf2 pathway is inactive in neurons

Activation of ARE/Nrf2 pathway by tBHQ has been observed in neurons of mixed culture systems when in close proximity to astrocytes (Johnson et al., 2002). I therefore wanted to determine if neurons within AC-neuronal culture system could contribute to the strong Nrf2-dependent gene upregulation observed following H₂O₂ or tBHQ treatments, as a result of obligatory factors release from neighbouring astrocytes. Treatments with either H₂O₂ or tBHQ led to Hmox1 induction that was specific to GFAP-positive astrocytes, while it had no effect on Hmox1 expression pattern in the neuronal population in our cultures of AC-neurons (Fig. 4.5) and (Bell et al., 2011a). Moreover, AF-neurons failed to display an upregulation of Nrf2 target genes when treated with H₂O₂ or tBHQ (Fig. 4.3).

Primary cultures derived from *Keap1*^{-/-} mice or treated with (siRNA) directed against Keap1 display an increase in phase II mRNA levels as a result of constitutively active Nrf2 pathway (Sato et al., 2009; Williamson et al., 2012). Consistent with the previous studies, Keap1 genetic ablation led to a significant induction of Nrf2-dependent gene expression in both astrocytes and AC-neurons (Fig. 4.6). In contrast, knocking out Keap1 bore no effect on Nrf2 target genes expression in AF-neurons (Fig. 4.6). However, overexpression of Nrf2 in conjunction with eGFP revealed a co-localization of Hmox1 staining to the GFP-positive neurons as seen by immunofluorescence staining (Fig. 4.7). Similarly, Nrf2 overexpression in neurons induced endogenous *Srxn1* staining (Soriano et al., 2008). This implies that ARE/Nrf2 pathway is functional in neurons and capable of driving ARE-gene transcription in the presence of adequate levels of Nrf2. Taken these data together, it

is likely that lack of ARE/Nrf2 pathway activity is due to diminished Nrf2 expression levels in neurons. In support of this notion, enriched glial cultures were

reported to express ~12 folds more of Nrf2 protein comparing to enriched neuronal cultures (Shih et al., 2003). In addition, unpublished data from our laboratory show significantly lower Nrf2 (*Nfe2l2*) mRNA expression in AF-neurons compared to AC-neurons and astrocytes, while no difference was detected in the mRNA levels of *Keap1*. The loss of Nrf2 signalling in neurons could be a result of epigenetic regulation of Nrf2 expression. The basis for this are under investigation in our laboratory and not the focus of this thesis, however, I was interested in investigating the molecular events underlying the H₂O₂-mediated Nrf2 activation in astrocytes which I will address in the next chapter.

Chapter 5

Mild oxidative stress activates the astrocytic Nrf2 pathway via a Keap1-independent mechanism

5.1. Summary

In non-neural cells, the prevailing view has been that activation of Nrf2 by oxidative stress is achieved through allowing Nrf2 to evade Keap1-mediated suppression. This effect is attributed at least in part to chemical or oxidative modification of specific reactive cysteine residues of Keap1, which may result in conformational changes in the Keap1/Nrf2 complex and ultimately stabilization of Nrf2 (Dinkova-Kostova et al., 2005; Fourque et al., 2010).

Having established that mild oxidative stress induced by the application of sub-toxic doses of H₂O₂ stimulates the astrocytic Nrf2 pathway (Fig 4.2-4.5), I attempted in this study to investigate the molecular mechanism(s) underlying the H₂O₂-mediated activation of the astrocytic Nrf2. Given the well-defined role of Keap1 in regulating Nrf2 activity in response to oxidative or chemical stress, I sought to test the involvement of Keap1 in the activation of astrocytic Nrf2 following exposure to mild oxidative stress. Contrary to the established dogma, I found that mild oxidative stress induces the astrocytic Nrf2 pathway in a manner distinct from the classical Keap1 antagonism employed by prototypical Nrf2 inducers. The mechanism was found to involve direct regulation of Nrf2's transactivation properties and can act additively to and independently of the classical Keap1-antagonism pathway. Furthermore, I show here that mild oxidative stress enhances Nrf2 transactivation activity specifically through its Neh5 transactivation domain and that this effect is mediated by a specific previously identified redox-sensitive cysteine residue within the Neh5 domain. These findings suggest that therapeutic manipulation of Nrf2 activity may be achievable even in astrocytes suffering oxidative stress.

5.2. Results

Although I have shown in section 4.2.3-4.2.5 that Nrf2 is active only in astrocytes, all our experiments were performed in AC-neurons rather than astrocytes. The reason for this is that astrocytes when grown in isolation from neurons have been reported to display altered morphology and changes in specific genes expression. Previous studies revealed that astrocytes *in vitro* undergo morphological alteration from an epithelial cell-like (or polygonal) shape to a process-bearing shape, that is more characteristic of astrocytes *in situ*, when co-cultured with neurons (Matsutani and Yamamoto, 1997; Swanson et al., 1997). These morphological changes were also associated with changes in gene expression pattern of the astrocytic glutamate transporters such as the excitatory amino acid transporters (EAAT) (Matsutani and Yamamoto, 1997).

5.2.1. Mild oxidative stress upregulates Nrf2-dependent gene expression even in the absence of Keap1

Previously, I have shown that sub-toxic doses of H₂O₂ upregulate *Srxn1*, *Hmox1* and *xCT* expression specifically in astrocytes in an Nrf2-dependent manner (Fig. 4.2-4.5), however, the mechanism behind the upregulation is not clear. Studies of Nrf2 regulation in non-neural cells suggest that oxidative stress induces Nrf2 activity through antagonizing Keap1-dependent suppression (Dinkova-Kostova et al., 2005; Fourque et al., 2010). I therefore sought to determine whether H₂O₂-mediated Nrf2 activation occurs through the antagonism of Keap1. To test this, WT and *Keap1*^{-/-} AC-neurons were treated with either a range of sub-toxic doses of H₂O₂ or tBHQ, which exemplifies a Keap1-dependent mechanism of Nrf2 activation (Zhang and Hannink, 2003), for 6 h after which RNA was harvested and Nrf2 target gene expression was evaluated. Real-time qPCR analysis revealed a significant induction of Nrf2 target genes expression following H₂O₂ regardless of Keap1 status (Fig. 5.1A, C, E). Consistent with previous studies (Wakabayashi et al., 2003; Satoh et al.,

2009; Gan et al., 2010), the absence of Keap1 elevated the basal expression of Nrf2 target genes. Approximately

three-fold increase in *Srxn1* and *xCT* basal expression was observed in *Keap1*^{-/-} cultures (Fig 5.1 A, E). Given the important role of Nrf2 in controlling the basal expression of *Hmox1*, reflected by the dramatic decrease of *Hmox1* basal expression in *Nrf2*^{-/-} cultures (Fig 4.2 B), I predicted a large induction in *Hmox1* basal levels in *Keap1*^{-/-} cultures. To my surprise, the absence of Keap1 resulted in a modest yet significant increase in *Hmox1* basal expression. The reason for this is not clear, however, one possible explanation is the contribution of other Nrf2-related factors in addition to Keap1 in the regulation of *Hmox1* basal levels.

Intriguingly, application of H₂O₂ produced further induction in *Srxn1*, *xCT* and *Hmox1* genes expression in the absence of Keap1 (Fig. 5.1A, C, E), while the absence of Keap1 as predicted, abolished the tBHQ-mediated induction of *Srxn1* and *xCT* expression (Fig 5.2 B, F). On the other hand, the absence of Keap1 didn't completely occlude the tBHQ-mediated induction of *Hmox1* expression as a small yet significant increase was observed in *Keap1*^{-/-} cultures (Fig. 5.2D). Although a number of studies have shown that tBHQ-mediated Nrf2 activation occurs through post-translational modification of Keap1 Cys 151 residue (Zhang and Hannink, 2003; Zhang, 2006; Abiko et al., 2011), a recent study has demonstrated the contribution of calcium in the tBHQ-induced expression of *HMOX1* in human hepatoma and colon carcinoma cell lines (Cheung et al., 2011). The reported involvement of calcium in the tBHQ-induced expression of *HMOX1* could provide a possible explanation for the induction of *Hmox1* expression observed in *Keap1*^{-/-} cultures following the application of tBHQ (Fig. 5.2D).

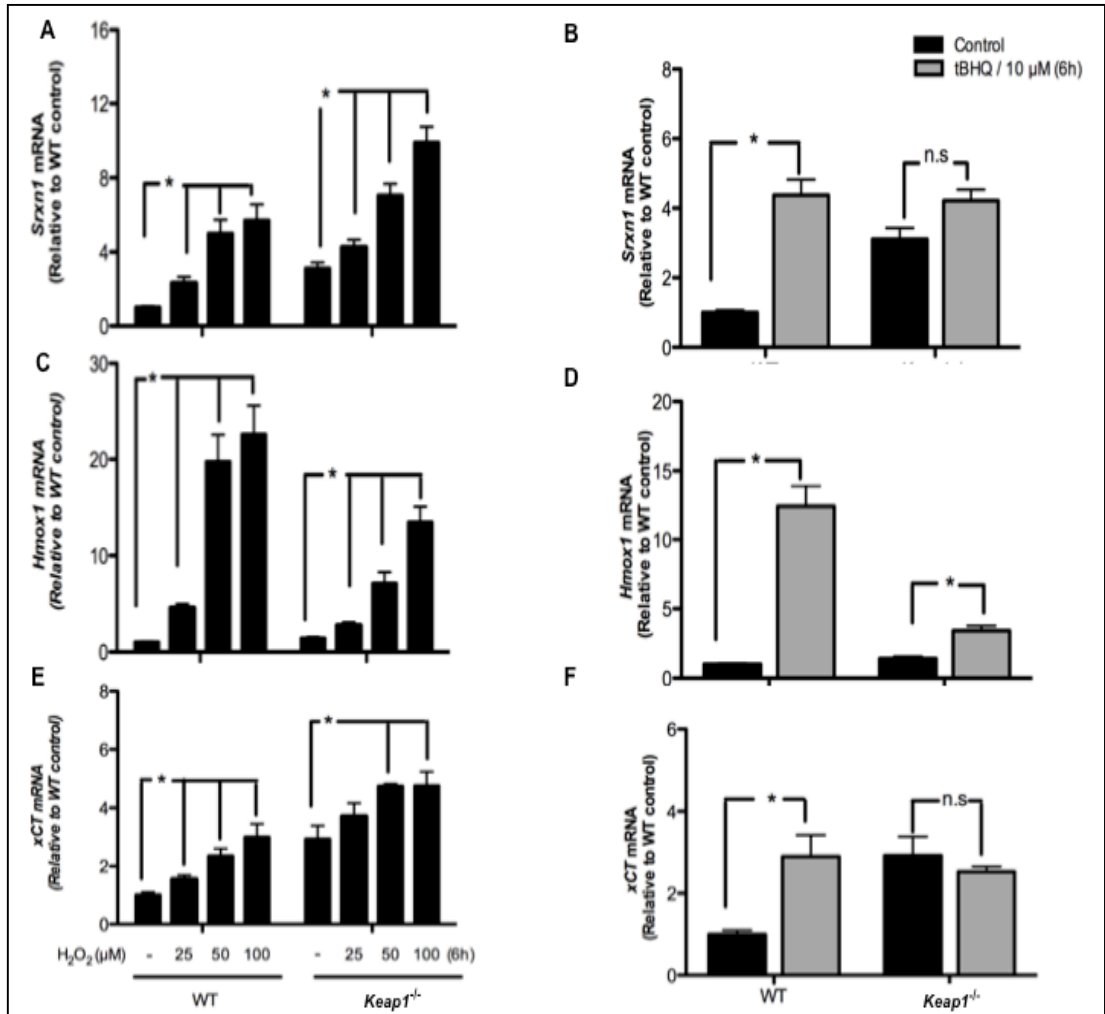


Figure 5.1. Mild oxidative stress induces Nrf2-regulated gene expression in Keap1 deficient cultures.

Gene expression was assessed using qPCR in AC-neurons derived from WT or Keap1^{-/-} mice and stimulated as indicated for 6 h. *Srxn1* mRNA levels in response to **A)** H₂O₂ or **B)** to tBHQ. *Hmox1* mRNA levels in response to **C)** H₂O₂ or **D)** to tBHQ. *xCT* mRNA levels in response to **E)** H₂O₂ or **F)** to tBHQ. (n=5-6), **p*<0.05.

5.2.2. H₂O₂ and tBHQ induce Nrf2-dependent gene expression via separate mechanisms

As another approach to confirm the existence of a Keap1-independent mechanism of H₂O₂-mediated Nrf2 activation, I explored the effect of combined treatment with tBHQ and H₂O₂ on Nrf2-dependent gene expression. Since tBHQ acts through antagonizing Keap1 (Fig. 5.1B, F) and (Zhang and Hannink, 2003; Zhang, 2006; Abiko et al., 2011), I hypothesized that H₂O₂ and tBHQ co-treatment would additively induce Nrf2 activity. To examine this, I co-applied 10 μ M tBHQ and 100 μ M H₂O₂ to AC- neurons, then harvested RNA for qPCR analysis 6 h later. As revealed by the qPCR analysis of *Srxn1* and *Hmox1* mRNA levels, tBHQ and H₂O₂ combined treatment produced a significant upregulation of Nrf2 target genes expression greater than that achieved by the either treatment alone (Fig 5.2).

As a further test of the Keap1-independent mechanism of H₂O₂-mediated Nrf2 activation, I utilized a firefly luciferase reporter fused to the human Neh2 domain of Nrf2, responsible for Keap1-dependent Nrf2 degradation, referred to here and after as (Neh2-luc). This Neh2-luc reporter system is a recently developed tool that allows for real-time monitoring of the direct effect of a particular compound on Nrf2 stability (Smirnova et al., 2011). The principle behind it is that under control conditions, Neh2-luc should undergo Keap1-mediated proteasomal degradation, which would result in a low luciferase activity. However, treatments with Nrf2 activators such as tBHQ, which disrupt the Keap1-mediated Nrf2 degradation (Zhang and Hannink, 2003), would stabilize the Neh2-luc leading to a measurable increase in luciferase activity as illustrated in (Fig. 5.3A) (Smirnova et al., 2011). One advantage of this system, is that it would allow discrimination between Keap1-dependent and -independent mechanism of action of Nrf2 inducers. I hypothesized that if H₂O₂ activates Nrf2 via a mechanism distinct from tBHQ, I then should observe no induction in Neh2-luc activity. To examine this, AC-neurons were transiently transfected on DIV02, using the astrocyte transfection protocol, with Neh2-luc reporter plasmid along with pTK-renilla normalization vector. Then on DIV08 the transfected cells were exposed to either tBHQ or H₂O₂ for 8 h prior to

measurements of luciferase activity. Parallel experiments were performed with cells transfected with a luciferase reporter (not fused to Neh2) along with pTK-renilla normalization vector, where they serve as a negative control. Consistent with the previous report (Smirnova et al., 2011), tBHQ stimulated Neh2-luc activity (Fig. 5.3B), while, as predicted no change was observed in Neh2-luc activity following H₂O₂ treatments (Fig. 5.3B). Taken together, these findings suggest that mild oxidative stress activates the Nrf2 pathway via mechanism(s) separate from the classical Keap1-antagonism mode of action employed by tBHQ.

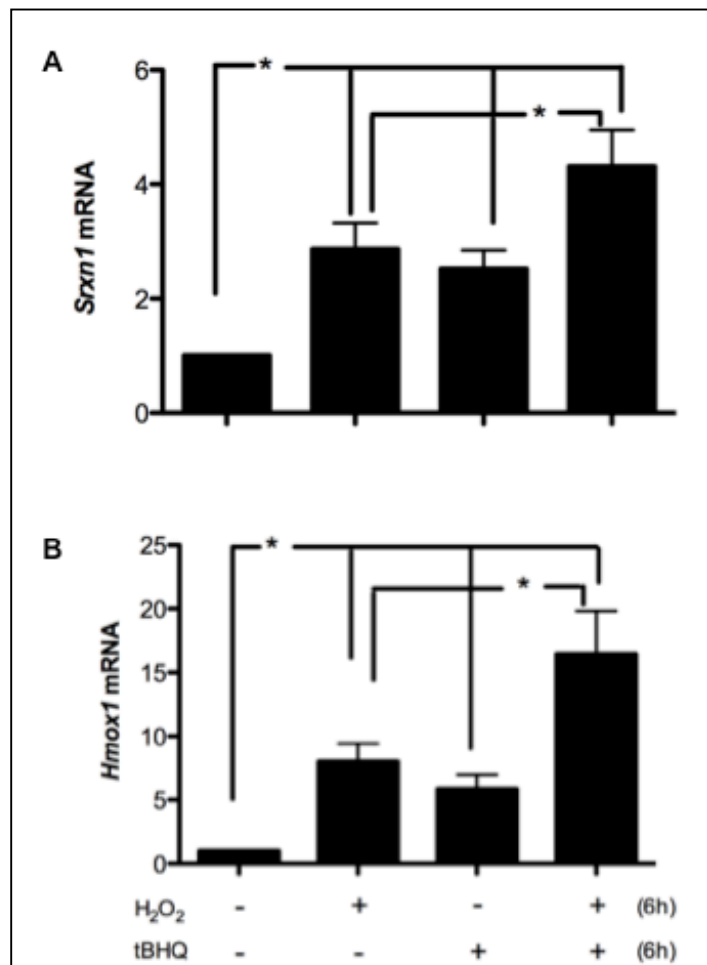


Figure 5.2. H₂O₂ acts additively to the classical Keap1-dependent mechanism employed by tBHQ.

Concomitant treatment of AC-neurons with 10 μ M tBHQ and 100 μ M H₂O₂ for the indicated time followed by RNA isolation and qPCR analysis. **A)** *Srxn1* gene expression. **B)** *Hmox1* gene expression. (n=6), **p*<0.05.

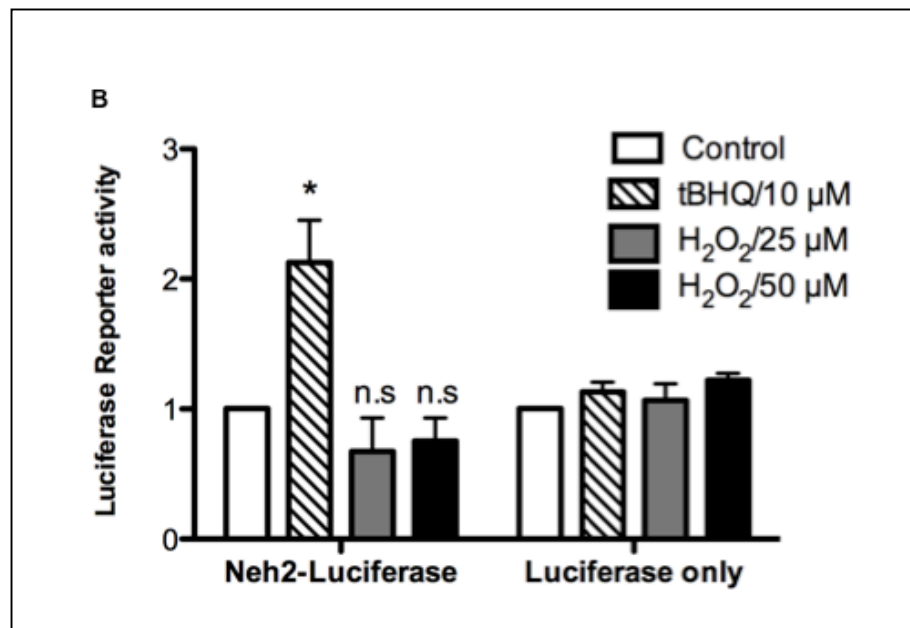
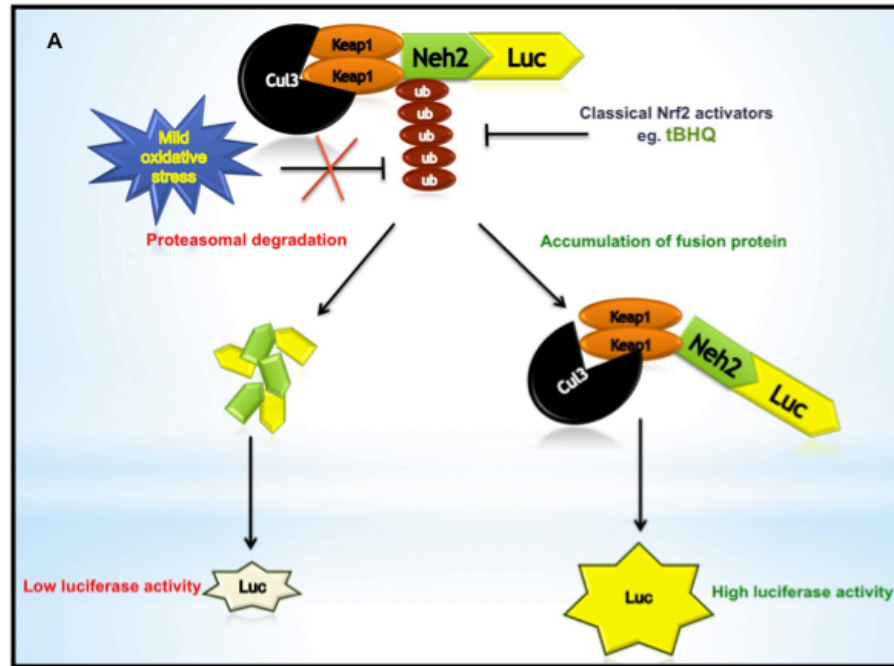


Figure 5.3. Neh2-luc responds to tBHQ-mediated activation but not H₂O₂. Transient transfection experiments were carried out on AC-neurons employing astrocyte transfection protocol. On DIV02, cells were transfected with Neh2-luc or luciferase only reporter plasmid along with pTK renilla transfection control. 6 days post transfection (DIV08) cells were treated with tBHQ or H₂O₂ at the indicated doses for 8 h before assessing luciferase reporter activity. Luciferase expression was normalized to Renilla control. (n=3), **p*<0.05.

5.2.3. Inhibition of GSK-3 β potentiates the inductive effect of H₂O₂ on *Hmox1* gene expression but not *Srxn1*

Apart from Keap1, GSK-3 β was described as a negative regulator of Nrf2 promoting its nuclear exclusion and inactivation (Salazar et al., 2006; Rojo et al., 2008; Rada et al., 2011). However, under strong oxidative stress conditions, GSK-3 β and Nrf2 are subject to biphasic regulation. Short-term exposure to oxidative stress activates Akt, which in turn leads to inhibitory phosphorylation of GSK-3 β and eventually Nrf2 nuclear translocation. On the other hand, long-term exposure results in GSK-3 β activation and limits Nrf2 activity by expelling it from the nucleus (Salazar et al., 2006; Rojo et al., 2008). I therefore hypothesized that if GSK-3 β inhibition is the mechanism by which mild oxidative stress activates the Nrf2 pathway, then I should observe: 1) an increase in the basal expression of Nrf2 target genes, following treatment with GSK-3 β inhibitor CT-99021, similar to that achieved following H₂O₂ 2) pre-treatment with CT-99021 would occlude H₂O₂ effect on Nrf2 target gene expression. To test this hypothesis, AC-neurons were treated for 6 h with H₂O₂ in the presence or absence of CT-99021. Then RNA was isolated and converted to cDNA for subsequent qPCR analysis. The data obtained from qPCR analysis revealed no significant change in *Srxn1* or *Hmox1* basal expression following the application of CT-99021 (Fig. 5.4). In addition, GSK-3 β inhibition didn't occlude the inductive effect of H₂O₂, as demonstrated by the significant upregulation of *Srxn1* and *Hmox1* gene expression by H₂O₂ in the presence or absence of CT-99021. Co-treatment of H₂O₂ and CT-99021 results in a modest insignificant increase in *Srxn1* mRNA expression (Fig. 5.4A). However, GSK-3 β inhibition appears to potentiate the inductive effect of H₂O₂ on *Hmox1* gene expression (Fig. 5.4B). The mechanism involved requires further studies, in which the effect of H₂O₂ on GSK-3 β activity could be evaluated.

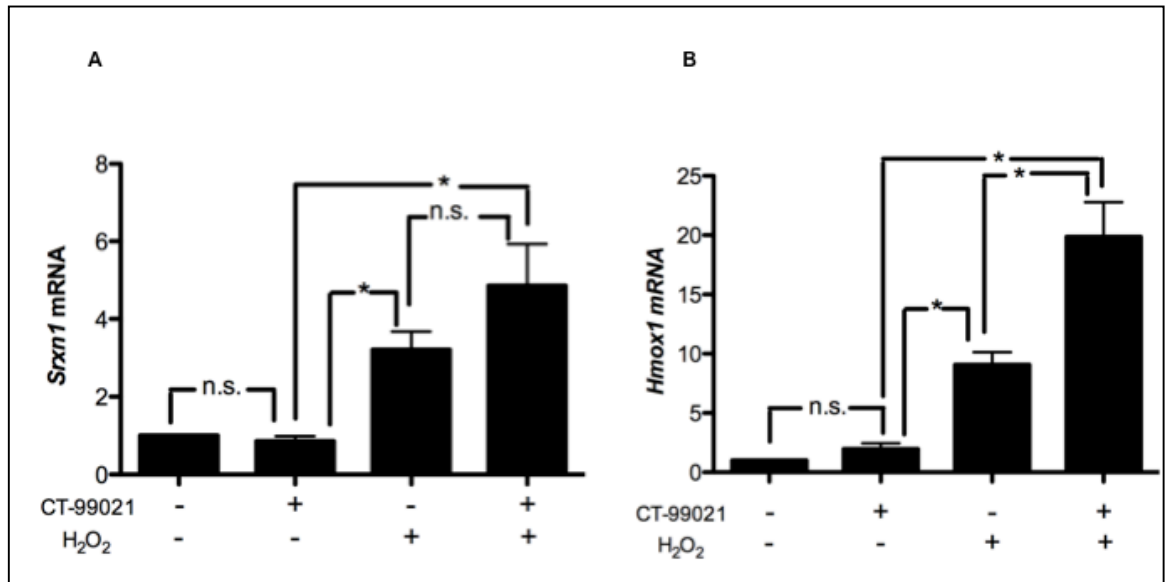


Figure 5.4. Inhibition of GSK-3 β potentiates the inductive effect of H₂O₂ on *Hmox1* gene expression but not *Srxn1*

AC-neurons were pre-treated with 2 μ M CT-99021 for 1 h prior to treatment with 100 μ M H₂O₂ and then 6 h later RNA was isolated for subsequent qPCR analysis. **A)** *Srxn1* mRNA expression. **B)** *Hmox1* mRNA expression. (n=4), * p <0.05.

5.2.4. Mild oxidative stress enhances Nrf2 transactivation activity via the Neh5 domain

I next sought to explore if H₂O₂ elicits its activating effect through direct regulation of Nrf2 transactivation potential. To address this, a firefly luciferase reporter gene, linked to four Gal4 sites (Gal4-luc), was used to evaluate transcriptional activation by Nrf2 or by either of its two transactivation domains Neh4 and Neh5 following H₂O₂ application (Fig. 5.5A). For this purpose, I utilized constructs of Gal4-DNA binding domain (GBD) -Nrf2 functional domain fusion proteins previously generated by Katoh and co-workers (Katoh et al., 2001), in which GBD-Nrf2 contains the full length protein, GBD-Neh (2-4) contains both Neh2 and Neh4 domains (1-156 amino acids) and GBD-Neh5 contains the Neh5 domain (153-227 amino acids) of Nrf2 (Fig. 5.5A) (Katoh et al., 2001). GBD-Nrf2 fusion proteins mediate a strong activation of the reporter gene, compared to the GBD alone (Fig. 5.5B). Remarkably, H₂O₂ stimulation generated a significant induction in GBD-Nrf2 full length and GBD-Neh5 mediated luciferase reporter activity (Fig. 5.5B). Moreover, the H₂O₂-induced transactivation activity seems to be attributable to the Neh5 transactivation domain, as demonstrated by the lack of reporter activity response to H₂O₂ stimulation when using GBD-Neh (2-4). These results demonstrate that mild oxidative stress-mediated Nrf2 activation involves a direct regulation of Nrf2 transactivation property.

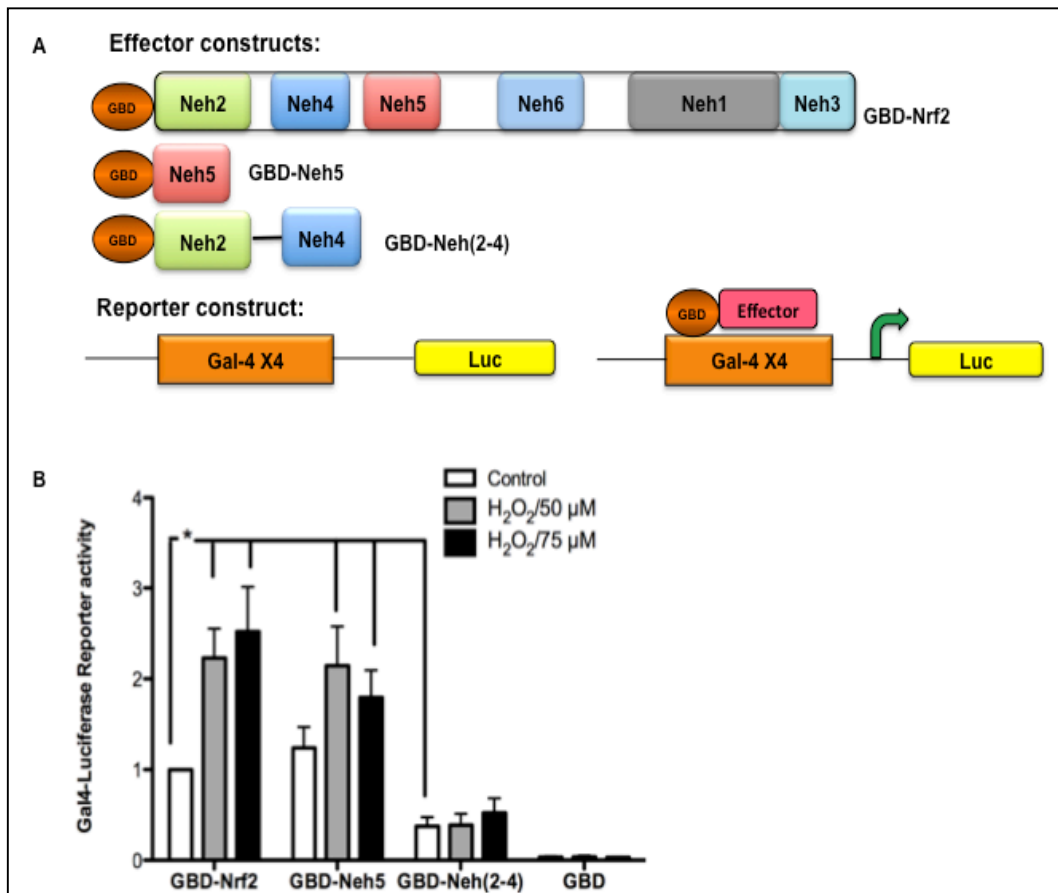


Figure 5.5. Mild oxidative stress directly regulates Nrf2 transcriptional activity through the Neh5 domain.

A) Schematic representation of the GBD-Nrf2 fusion effector constructs and Gal4-luc reporter construct. B) On DIV02 AC-neurons were co-transfected with Gal4-luc plasmid and either of the Nrf2-GBD fusion plasmids indicated above. PTK-renilla was included in all transfections to normalize for transfection efficiency. On DIV08, cells were exposed to H₂O₂ for 8 h prior to analysis of firefly and renilla luciferase activities. (n=3-9), *p<0.05 significantly different from GBD-Nrf2 control.

5.2.5. Neh5 sensitivity to H₂O₂ is mediated by the Cys-191 residue

I have previously demonstrated the redox reactivity of GBD-Neh5 to H₂O₂ (Fig. 5.5 B), however, the molecular mechanism responsible for this response is not clear. Cysteine residues have a well-defined role in sensing and transducing cellular redox status (Barford, 2004) and have been found to be the primary targets for H₂O₂ oxidizing action (Reth, 2002). In addition, Neh5 harbours a reactive cysteine residue that has been reported to mediate the increased nuclear translocation and transactivation of Nrf2 in response to H₂O₂ treatment observed in HeLa cells (Li et al., 2006). I therefore introduced a Cys to Ala mutation at position-191 (C191A) of Neh5 domain to examine whether this cysteine residue is required for the H₂O₂-induced Neh5 transactivation activity. While H₂O₂ significantly induced Gal4-luc activity mediated by wild type GBD-Neh5, the C191A mutation completely abolished the inductive effect of H₂O₂. Furthermore, mutant GBD-Neh5 (GBD-Neh5 (C191A)) displayed a reduced basal transactivation activity compared to WT (GBD-Neh5) emphasizing the key role of this cysteine residue in mediating basal and inducible Neh5 transactivational activity (Fig. 5.6A). To ensure that the effect of the mutation on Neh5 transactivity is not due to differential expression levels between the WT and the mutant plasmids, I transfected HEK-293 cells with either the GBD-Neh5 or GBD-Neh5 (C191A) and harvested the protein 24 h post-transfection for subsequent western blot analysis. The GBD expression level was comparable between the WT and mutant constructs as revealed by densitometric analysis of the bands normalized to β -actin (Fig. 5.6 B, C).

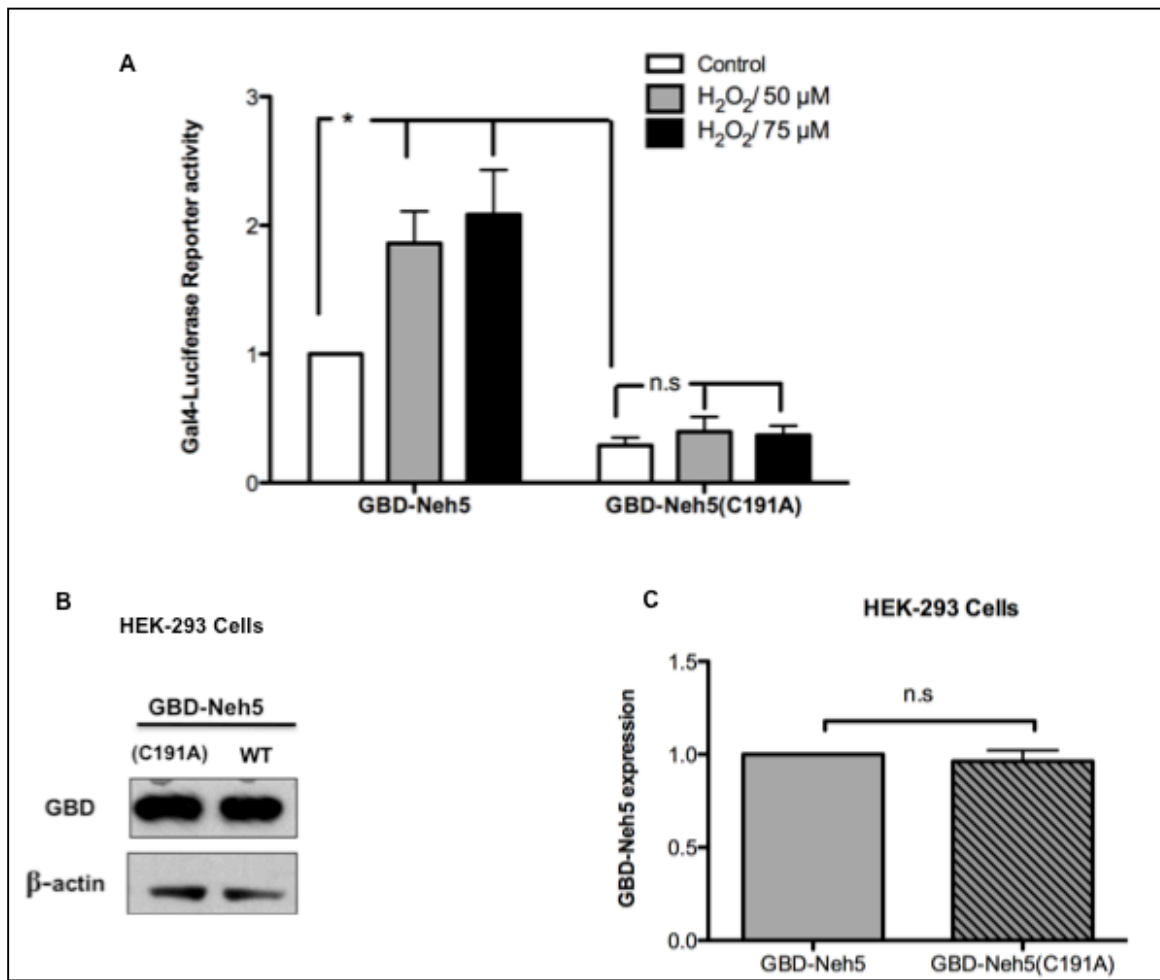


Figure 5.6. C191A mutation attenuates the redox reactivity of GBD-Neh5 domain.

A) AC-neurons were transfected on DIV02 with GBD-Neh5 or its mutant version GBD-Neh5 (C191A) along with Gal4-luc reporter and pTK-renilla plasmid. 6 days later (DIV08), cells were treated with H₂O₂ for 8 h before performing the luciferase reporter gene assay. Luciferase reporter activity was normalized to Renilla control. **B, C)** GBD-Neh5 and GBD-Neh5(C191A) expression levels **B)** GBD-Neh5 or its C191A mutant was transfected into HEK-293 cells. At 24 h post transfection, protein was harvested and analysed for GBD expression and β-actin loading control, (n=3). **C)** Quantification of GBD protein expression normalized to β-actin.

5.3. Discussion

Contrary to the established dogma, I report here a mechanism by which mild oxidative stress triggers the astrocytic Nrf2, distinct from the classical Keap1 antagonism pathway affected by small molecule Nrf2 inducers such as tBHQ. The mechanism involves direct regulation of Nrf2's transactivation properties, and can act additively to the classical tBHQ-induced pathway, suggesting that therapeutic manipulation of Nrf2 activity may be achievable even in astrocytes suffering oxidative stress.

5.3.1. Mild oxidative stress activates the astrocytic Nrf2 in a Keap1-independent manner

Prototypical activators of the Nrf2 pathway such as tBHQ and sulforaphane, induce the pathway via blocking Keap1-mediated Nrf2 ubiquitination and subsequent proteasomal degradation leading eventually to enhanced Nrf2 stability and activation of ARE-dependent gene transcription (Zhang, 2006). Herein I have presented a previously unrecognized mechanism for astrocytic Nrf2 activation by mild oxidative stress that is independent of the classical Keap1 antagonism pathway.

In this study I found that mild oxidative stress activates the astrocytic Nrf2 independently of Keap1. AC-neurons prepared from *Keap1*^{-/-} displayed a significant induction in Nrf2-dependent gene expression when treated with H₂O₂ (Fig. 5.1). On the other hand, parallel experiments, in which cells were exposed to tBHQ, were used as a model for Keap1-dependent mechanism. Although previous studies have not explored the effect of knocking out Keap1 on tBHQ-mediated Nrf2 activation, they have identified a particular cysteine residue (C151) located in the BTB domain of Keap1, required for the activation of Nrf2-regulated transcription by tBHQ (Zhang and Hannink, 2003). In addition, a more recent report showed that C151 mutations could promote and accelerate Keap1 degradation upon tBHQ treatment (Kobayashi et al., 2006). As predicted Keap1 deletion substantially abrogated the ability of tBHQ to elevate Nrf2-dependent *Srxn1* and *xCT* gene expression (Fig. 5.1B and F). While a modest yet significant tBHQ-mediated induction was observed in *Hmox1* gene

expression even in the absence of Keap1 (Fig. 5.1D). This induction could be attributable to the calcium-regulated tBHQ activation of Nrf2. A recent study described the role of calcium in mediating tBHQ-induced HMOX1 expression, whereby chelation of calcium attenuated the enhanced binding of Nrf2 to its co-activator CREB and to the ARE in the enhancer region of HMOX1 induced by tBHQ in human hepatoma and colon carcinoma cell lines (Cheung et al., 2011).

In light of the qPCR data obtained from WT and *Keap1*^{-/-} cultures exposed to either tBHQ or H₂O₂ revealing a Keap1-dependent and -independent mechanisms of action respectively (Fig. 5.1), I speculated that concomitant treatment with H₂O₂ and tBHQ would additively induce Nrf2 target genes expression. Indeed, the combined treatment resulted in an upregulation of *Srxn1* and *Hmox1* gene expression greater than that elicited by each treatment individually (Fig. 5.2) confirming the Keap1-independent mechanism of Nrf2 activation by mild oxidative stress.

Further evidence for the Keap1-independent mechanism was obtained using the Neh2-luc reporter system. Treatment with tBHQ led to a measurable increase in Neh2-luc activity (Fig. 5.3B). This observation is consistent with the data generated by Smirnova and co-workers showing an induction in Neh2-luc activity in human neuroblastoma cells exposed to tBHQ (Smirnova et al., 2011) and in line with the widely accepted model of Nrf2 activation by tBHQ (Zhang, 2006). On the other hand, no induction in Neh2-luc activity was observed with H₂O₂ (Fig. 5.3B). These data correlate well with the results presented in (Fig. 5.1 and 5.2) and together strongly suggest that mild oxidative stress activates Nrf2 in astrocytes via a Keap1-independent mechanism, however, it doesn't rule out some dependence on Keap1 as suggested by the drop in H₂O₂-mediated induction of Nrf2 target genes in *Keap1*^{-/-} cultures compared to WT (Fig. 5.1).

It is noteworthy, that in zebrafish embryos H_2O_2 induced Nrf2-dependent glutathione S-transferase gene (*gstp1*) expression but failed to cancel Keap1-mediated suppression of Nrf2. Unlike other species, zebrafish have two types of Keap1, Keap1a and Keap1b with the latter sharing higher identities (73-81%) to the functional domains of the mouse Keap1 protein (Li et al., 2008). H_2O_2 failed to cancel Nrf2 suppression mediated by either Keap1 proteins. This was thought to be due to the lack of factors additional to Nrf2 and Keap1 necessary for sensing H_2O_2 , which are down regulated or non-functional at the embryonic stage (Kobayashi et al., 2009). However, it's plausible that the failure to alleviate Keap1 suppression by H_2O_2 is not due to Keap1 impaired sensing for H_2O_2 but rather due to H_2O_2 Keap1-independent mechanism of action.

Keap1 function as a redox sensor is mainly attributed to its possession of reactive cysteine, which could be subject to oxidation by H_2O_2 residues (Zhang and Hannink, 2003; Holland et al., 2008; Fourquet et al., 2010). H_2O_2 has been shown to induce chemical modification to specific cysteine residues in Keap1. Two types of disulfide bonds could be formed upon Keap1 oxidation by various oxidants including H_2O_2 ; 1) an intermolecular, bridging two Keap1 monomers at Cys151, 2) an intramolecular disulfide bond, linking Cys 226 and Cys 613. Fourquet and co-workers reported a parallel between Keap1 oxidation and Nrf2 stabilization in HeLa cells exposed to H_2O_2 and suggested a cause-and-effect relationship between the two phenomena. In their study, H_2O_2 induced a transient stabilization of Nrf2 coinciding with Keap1 oxidation. However, this effect was very short lived lasting for not more than 20 mins and the role of Keap1 in H_2O_2 -induced Nrf2 stability was not confirmed through Keap1 knock out or knock down approaches (Fourquet et al., 2010). Nevertheless, its is conceivable that the initial response to H_2O_2 could be through Keap1 requiring pathway which then switches to an alternative Keap1-independent pathway.

Aside from Keap1, Nrf2 is subject to dual regulation by GSK-3 β , whereby the kinase controls its subcellular distribution (Rojo et al., 2008) and degradation in a Keap1-independent manner (Rada et al., 2011). The data presented here (Fig. 5.4A and B) shows that GSK-3 β pharmacological inhibition doesn't attenuate the inductive effect of H₂O₂ on *Srxn1* or *Hmox1* gene expression. However, it suggests that GSK-3 β inhibition could potentiate the inductive effect of H₂O₂ on *Hmox1* expression as demonstrated by the further induction in gene expression levels beyond that achieved with H₂O₂ alone (Fig 5.4B). This potentiating effect was observed in *Hmox1* gene expression but not *Srxn1* (Fig 5.4A) and further investigation is required to decipher the underlying mechanism and whether H₂O₂ influences the activity of GSK-3 β .

5.3.2. Mild oxidative stress enhances Nrf2 transactivation activity

In the present study I have demonstrated that mild oxidative stress activates the Nrf2 pathway in astrocytes via a mechanism independent of the classical Keap1 antagonism pathway employed by Nrf2 small molecule inducers such as tBHQ. The mechanism involves a direct regulation of Nrf2 transactivation activity. H₂O₂-induced activation of the ARE promoter has been previously reported in non-neural cells. For instance in cardiomyocytes, exposure to mild doses of H₂O₂ resulted in an increase of ARE-luc reporter activity in an Nrf2-dependent manner (Purdom-Dickinson et al., 2007). Moreover, treatment with H₂O₂ induced Nrf2-mediated ARE-luc activity in HeLa cells (Li et al., 2006).

In the present study, I utilized the GBD-Nrf2 chimera constructs and Gal4-luc reporter co-transfection system (Fig. 5.5A) (Katoh et al., 2001) to analyse transactivation activity of Nrf2 and both its transactivation domains Neh4 and Neh5 individually in response to H₂O₂ stimulation. Interestingly, H₂O₂ triggered a strong GBD-Nrf2 mediated Gal4-dependent luciferase activity (Fig. 5.5B). The data also indicated that the H₂O₂-mediated increase of GBD-Nrf2 transactivation activity is attributed to Neh5 domain but not Neh4, since GBD-Neh5 had displayed similar levels of H₂O₂-induced activity compared to GBD-Nrf2 whereas GBD-Neh (2-4) showed no response to H₂O₂ stimulations (Fig. 5.5B).

Many proteins including transcription factors such as p53, Jun and Fos are subject to redox regulation occurring through particular highly conserved cysteine residues (Barford, 2004; Sun and Oberley, 1996). However the H₂O₂-induced oxidation of these transcription factors can either up- or down regulate their transcriptional activity (Reth, 2002). In HeLa cells, Li *et al.* has identified a reactive cysteine residue at position 183 of the human Neh5 transactivation domain that is when mutated results in the attenuation of the H₂O₂-mediated Nrf2 translocation and transactivation (Li et al., 2006). Consistent with the previous study, I have found that the substitution of Cys 191 for Ala in the mouse Neh5 domain not only ablates the induction of GBD-Neh5-mediated Gal4-luc activity following exposure to H₂O₂, but also significantly reduces Neh5 basal transactivation activity (Fig. 5.6A). The same mutation was shown to decrease the half life of Nrf2 when overexpressed in Nrf2 null mouse fibroblasts and was correlated with increased Keap1 binding (He and Ma, 2009). However, in my hands expression levels of Neh5 and its mutant version were comparable (Fig. 5.6B and C). The disparities between the two results could be due to differences in the cell types used. Finally, oxidative stress is a complex event and its control of the Nrf2 pathway is expected to be intricate involving regulation at different levels.

Chapter 6
Concluding statement

The research reported herein has shed light on some of the molecular mechanisms involved in the control of intrinsic antioxidant and neuroprotective pathways by synaptic NMDAR signalling and mild oxidative stress. I have presented here three key findings:

- *Foxo1* is a Foxo target gene and as a result, its expression is subject to a feed-forward inhibition by synaptic NMDAR activity, which promotes Foxo3 export from the nucleus.
- Mild oxidative stress activates the Nrf2 antioxidant pathway in astrocytes but not neurons.
- Mild oxidative stress activates the Nrf2 antioxidant pathway in a mechanism independent of Keap1, which involves the induction of Nrf2 transactivation activity via Cys 191 residue within the Neh5 domain.

The finding that *Foxo1* is a Foxo target gene, as demonstrated in sections (3.2.1-3.2.3), indicates a feed-forward regulation mechanism that may serve to reinforce the effect of Foxo-inactivating or -activating signals such as synaptic activity or oxidative stress respectively, on *Foxo1*-target gene expression. This suggests that the protective effects of synaptic activity or neurotrophic factors may last longer than previously thought. In agreement with this finding, a study on FOXO regulation in human fibroblasts also demonstrated that FOXO1 is a FOXO target gene and that FOXO3 was a stronger activator of the *FOXO1* promoter than FOXO1 itself (Essaghir et al, 2009), similar to our results in section 3.2.3. Moreover, Essaghir *et al.* identified the same proximal FOXO binding site (GTA AAC AA), which was required for the regulation by growth factors such as IGF-I and platelet-derived growth factors, and mediated a large portion of the observed FOXO responsiveness (Essaghir et al, 2009). They also found that FOXO1 and FOXO3 were associated to the *FOXO1* gene promoter in HEK293T cells as well as fibroblasts, which raises the possibility that such an association may as well exist in neurons. Although I have

shown that *Foxo1* is a Foxo target gene based on the results obtained from luciferase reporter assay experiments (see section 3.2.3), further studies should be carried out to: 1) confirm the luciferase assay results by looking into the effect of overexpressing Foxos on the mRNA and protein expression of endogenous Foxo1 2) determine whether the enhanced *Foxo1* promoter activity is a result of direct or indirect regulatory effect of Foxos, which could be assessed by performing chromatin immunoprecipitation experiments 3) determine whether Foxo3 itself is a Foxo target gene.

A large body of evidence shows that cell death is the most likely outcome of Foxos activation in neurons in response to various stresses including oxidative stress and trophic factor withdrawal (for review see sections 1.2.4 and 1.2.5). Moreover, increased Foxo1 activity was implicated in the pathogenesis of two forms of familial PD; the autosomal dominant late onset leucine-rich repeat kinase 2 (*LRRK2*)-linked PD, in which *LRRK2* has been reported to phosphorylate FOXO1 and enhance its transcriptional activity both in *Drosophila* and mammalian cells (Kanao et al., 2010), and in the autosomal recessive early onset PTEN-induced kinase 1 (*PINK1*)-linked PD, in which lack of *Pink1* was shown to increase Foxo1 activity in MEF cells via inhibiting IGF-I-induced Foxo1 nuclear exclusion (Akundi et al., 2012). In addition, a very recent microarray study in the prefrontal cortex of PD and control brain samples revealed a significant increase in *FOXO1* gene expression in PD samples compared to controls and identified a set of differentially expressed genes, which was enriched for genes regulated by FOXO1 (Dumitriu et al., 2012). Therefore, the protective effects of Foxo inactivating signals such as neurotrophic factors and synaptic NMDAR activity are thought to be mediated at least in part through promoting Foxos export and the subsequent suppression of Foxo-mediated gene expression (Gan et al., 2005; Maiese et al., 2007; Soriano et al., 2006; Papadia et al., 2008; Martel et al., 2009; Al-Mubarak et al., 2009).

Finally, in light of the data presented here and in previous studies by our group (Soriano et al., 2006; Papadia et al., 2008; Martel et al., 2009), it is conceivable that the activity-dependent suppression of Foxos could confer neuroprotection by fighting oxidative stress on two fronts: 1) boosting antioxidant defenses through the inhibition of Foxo-target gene, *Txnip* (Papadia et al., 2008) and 2) blocking the Foxo-mediated pro-apoptotic signalling (Fig. 6.1).

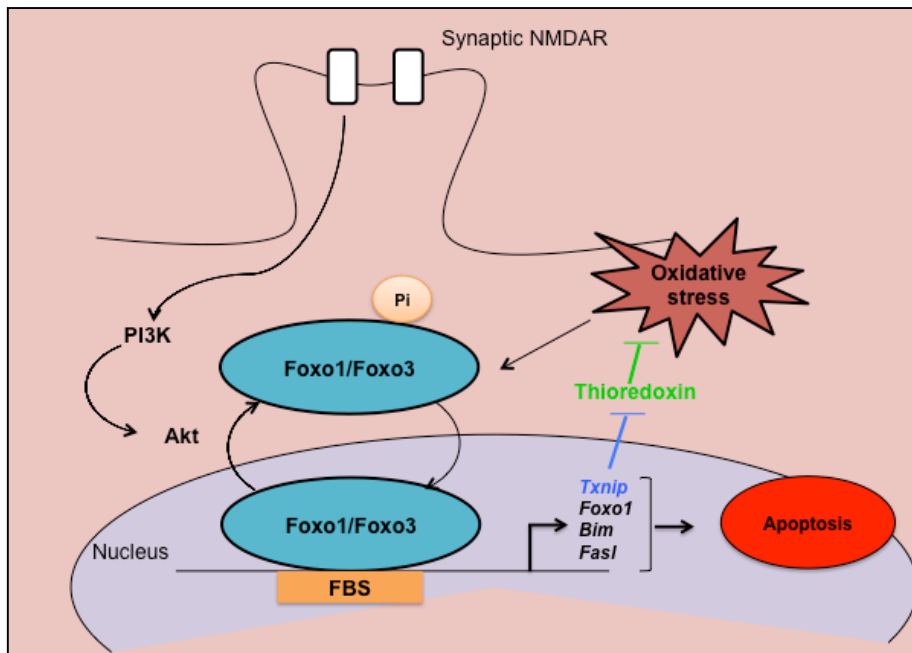


Figure 6.1. Mechanisms by which activity-dependent suppression of Foxos could combat oxidative stress.

This simplified diagram, adapted from (Hardingham and Bading, 2010), illustrates how synaptic activity-dependent suppression of Foxos could promote neuroprotection through turning off Foxo pro-apoptotic target genes including Foxo1 itself and the thioredoxin antioxidant system inhibitor, *Txnip*. For further review on the mechanism of Foxo3 activation by oxidative stress see David-Dávila and Aleman, 2005. (FBS) Foxo binding sequence.

Despite the well-established role of oxidative stress in the etiology of neurological disorders, antioxidant intervention based on the administration of free radical scavengers and spin-traps have met with limited success in clinical trials (Kamat et al., 2008). This could be due to various factors including failure to maintain the drug in the brain at levels sufficient to neutralize ROS as and when they appear. However, enhancing the activity of intrinsic antioxidant pathways such as the Nrf2 is an alternative antioxidant approach. Emerging evidence has shown that genetic or pharmacological activation of Nrf2 in astrocytes confers neuroprotection against oxidative stress in cell and animal models of neurodegeneration (for review see section 1.6). In line with this, I have demonstrated in sections 4.2.2-4.2.5, that the endogenous Nrf2 is activated by physiologically relevant stimulus such as mild oxidative stress in an astrocyte-specific manner. The responsiveness of endogenous Nrf2 to mild oxidative stress was recently shown by our group to contribute to the neuroprotective effect of ischemic preconditioning *in vitro* and *in vivo* (Bell et al., 2011a; Bell et al., 2011b). However, whether human astrocytes are capable of mediating a neuroprotective response to Nrf2 activating-stimuli, was addressed in a recent study by Gupta *et al.*, in which HESC-derived astrocytes were shown to promote survival of HESC-derived neurons following oxidative injury and that their neuroprotective capacity can be greatly enhanced by treatment with the Nrf2 inducer CDDO^{TFEA}. This neuroprotective effect was restricted to astrocytes as HESC-derived neurons fail to respond to CDDO^{TFEA} either in terms of Nrf2 activation or by conferring neuroprotection (Gupta et al., 2012). This study is consistent with the results from rodent cultures (refer to section 4.2.3) and (Bell et al, 2011a) showing that astrocytes represent the major locus of Nrf2 activation, triggered by small molecules and mild oxidative stress. Moreover, the loss of Nrf2 signalling in neurons could be a result of epigenetic regulation of Nrf2 expression, which is currently under investigation in our laboratory

Nrf2 activation by oxidative stress/Nrf2 inducers in non-neural cells such as HeLa and NIH3T3 is believed to occur through Keap1 antagonism (Itoh et al., 1999; Zhang and Hannink, 2003; Fourquet et al., 2010). In the study on Nrf2 stabilization in

NIH3T3 cells, Keap1 C151 residue was found to be uniquely required for inhibition of Keap1-dependent degradation of Nrf2 by sulforaphane and tBHQ-induced oxidative stress (Zhang and Hannink, 2003). However, the involvement of oxidative stress in the tBHQ-induced Nrf2 activation is controversial. While tBHQ was shown to lead to ROS formation, in human hepatoma cells (Pinkus et al., 1996), activation of the ARE by tBHQ in human neuroblastoma cells (Lee et al., 2001) or in primary cortical cultures derived from ARE-hPAP reporter mice (Johnson et al., 2002) was independent of oxidative stress (for review see section 4.2.1). Therefore, it is important to utilize an accurate model of oxidative stress when investigating the mechanism of Nrf2 regulation in response to oxidative stress. In another study, where H₂O₂ was utilized to model oxidative stress in HeLa cells, a transient stabilization of Nrf2 protein coinciding with the oxidation of multiple cysteine residues on Keap1 was observed suggesting a cause-and-effect relationship between the two phenomena (Fourquet et al., 2010). However, this effect was very short lived lasting for not more than 20 mins and the role of Keap1 in H₂O₂-induced Nrf2 stability was not confirmed through either Keap1 knock out or knock down approaches or through immunoprecipitation experiments.

Contrary to the established dogma, this study provided evidence, presented in section 5.2, suggesting that oxidative stress activates astrocytic Nrf2 via a mechanism independent of the classical Keap1 disinhibition pathway. The mechanism involves direct induction of Nrf2 transactivation activity via Cys191 within the Neh5 domain. This is in agreement with a previous study in HeLa cells, in which a reactive cysteine residue located in the human Neh5 domain was shown to attenuate the H₂O₂-mediated Nrf2 translocation and transactivation (Li et al., 2006). However, some aspects of the Keap1-independent mechanism reported in this thesis require further investigation these include: 1) assessing the influence of C191A on the oxidative stress-induced transactivational activity of the full length Nrf2 2) determining the effect of oxidative stress on the stability and subcellular distribution of astrocytic Nrf2.

Two important points could be inferred from identifying the existence of a Keap1-independent mechanism of H₂O₂-mediated Nrf2 activation: 1) The fact that oxidative stress and Nrf2 inducers such as tBHQ activate Nrf2 via two independent mechanisms, suggests that Keap1 antagonism alone doesn't saturate the Nrf2-driven antioxidant defences and that there is potential for further enhancement of Nrf2 activity in astrocytes suffering oxidative stress (Fig 6.2). 2) Although oxidative stress activates Nrf2 independent of cell type, the underlying mechanism could be cell type-specific. The concept of cell type-specific regulation of Nrf2 was also observed in the context of kinase dependence of Nrf2 activation, whereby PI3K inhibition was shown to attenuate tBHQ-mediated ARE activation in IMR-32 (Lee et al., 2001) cells but not in HepG2 cells (Zipp and Aktas, 2006). Understanding the cell type-specific mechanisms of Nrf2 activation may be valuable in designing therapies aimed at cell type-specific Nrf2 activation.

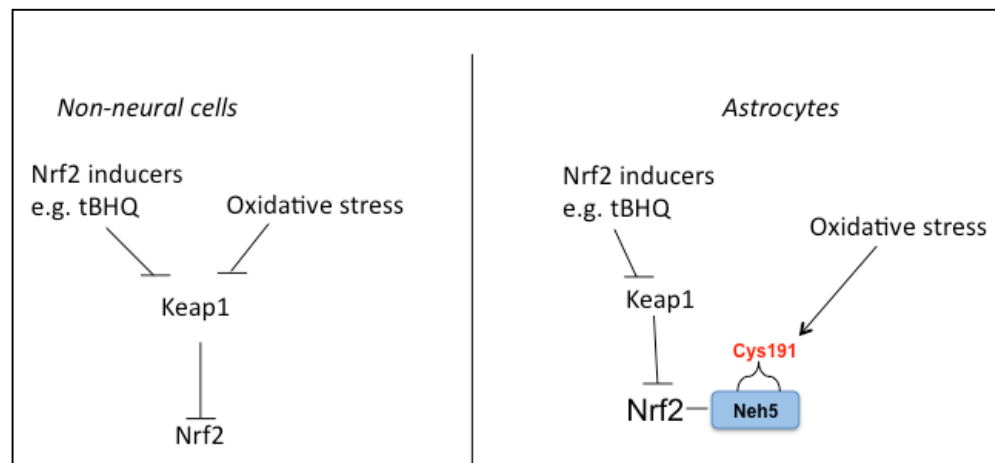


Figure 6.2. Nrf2 inducers could further enhance Nrf2 activity in astrocytes suffering oxidative stress.

This diagram illustrates that oxidative stress activates the Nrf2 pathway in astrocytes independently of the classical Keap1 antagonism previously reported in non-neural cells. The two independent mechanism of Nrf2 activation by oxidative stress and Nrf2 small molecule inducers such as tBHQ, suggest that Nrf2 small molecule inducers could further enhance Nrf2 activity even when astrocytes are suffering oxidative stress.

In summary, these results advance our knowledge of the mechanism(s) underlying the control of endogenous antioxidant and anti-apoptotic pathways by mild oxidative stress or synaptic activity. Such knowledge could be of value for designing therapies involving the manipulation of intrinsic defenses.

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