DELINEATION OF DELTA FOSB'S *IN VIVO* REDOX SENSITIVITY

By

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ABSTRACT

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Many neurodegenerative diseases, including Alzheimer's disease (AD), are driven by altered reduction/oxidation (redox) balance in the brain. Moreover, cognitive decline in AD is caused by neuronal dysfunction that precedes cell death, and this dysfunction is in part produced by altered gene expression. However, the mechanisms by which redox state controls gene expression in neurons are not well understood. Delta FosB is a neuronally enriched transcription factor critical for orchestrating gene expression underlying memory, mood, and motivated behaviors and is dysregulated in AD. Delta FosB regulates gene expression by dimerizing with JunD to form activator protein 1 (AP-1) which binds the promoter regions of target genes to control transcription. In controlled *in vitro* conditions, AP-1 complex formation and DNA binding are modulated by redox-sensitive disulfide bonds and related redox-sensitive conformational changes in Delta FosB. Here, we show that the redox-dependence of the structure-function relationship of Fos-family proteins found *in vitro* is also conserved in Delta FosB in cells and in the mouse brain. Under oxidizing conditions, Delta FosB cysteine residues can form disulfide bridges, including at C222 and C172, which can stabilize its interaction with a partner protein; however, these conditions reduce complex binding to AP-1 consensus sequence DNA, specifically when C172 is oxidized. We present evidence that this effect occurs in cells and in mouse brain, altering Delta FosB target gene expression during redox stress. This evidence supports Delta FosB as an

important mediator of oxidative stress-driven changes in gene expression seen in neurological conditions and implicates Delta FosB as a possible therapeutic target for intervention in diseases of oxidative stress like AD.

To my parents, Don and Ruth Lynch, and my siblings, Kaitlyn and Joshua Lynch, whose unconditional love and support have encouraged me to persevere and strive to do what makes me happy.

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CHAPTER 1: INTRODUCTION

Reduction-oxidation (redox) balance in the cell

The human body is constantly trying to maintain homeostasis to function properly and sustain everyday life. Being that it is a dynamic environment, the body is comprised of endless chemical reactions that are constantly occurring and looking to promote this balanced state. One such reaction is the reduction-oxidation (redox) reaction[1] (Figure 1). Many normal cellular functions including detoxification of harmful substances and metabolic cycles involve the redox reaction (Table 1)[15,16]. This reaction aims to maintain an electrical balance within cells, while the reactant one is losing

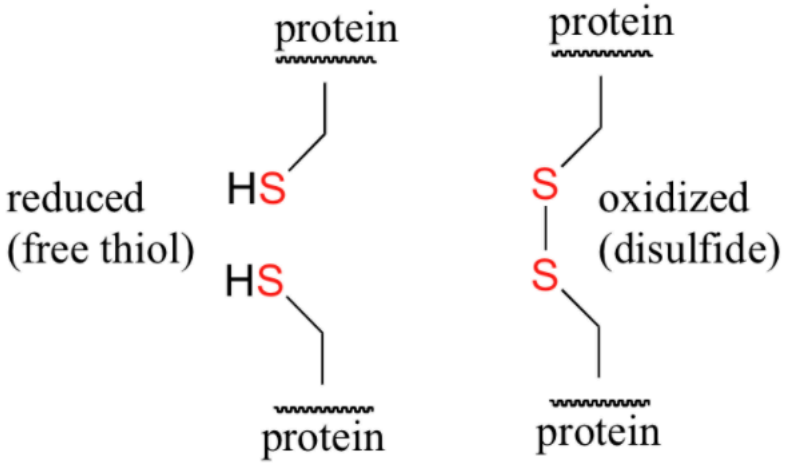

Figure 1 | Redox reaction of thiols. Two example cysteines containing thiol (-SH) groups (sulfur groups, S, in red) under reduced conditions (left) and oxidized conditions (right). Under reduced conditions the two thiol groups are part of separate structures, whereas under oxidizing conditions, there is a disulfide bridge connecting the sulfurs (S), one in the reduced and oxidized form. Reprinted from *LibreTexts*[7], copyright 2019.

electrons (becoming oxidized) and the other is gaining those same electrons (becoming reduced)[15,17]. The term “redox status” or “redox state” is used to describe the balance between oxidized and reduced species[15,17]; or the balance between the endogenous antioxidant defenses used to neutralize the reactive species continually being produced, for example, as by-products of aerobic metabolism[18]. Excess amounts of reactive

species and lack of antioxidant defenses results in a buildup of molecules called free radicals, or non-radicals that are easily converted into radicals and/or are oxidizing agents (ex: hydrogen peroxide)[19].

Table 1 | Biological roles of reaction oxygen species.[1]

Neurological	Cardiovascular	Immune Response	Cell Biology
-Mediation of learning and memory -Regulation of striatal dopamine release via glutamate	-Regulation of cardiac contractility -Regulation of vascular tone (e.g., penile erection) via NO production -Signaling involving carotid bodies (monitor arterial oxygen levels)	-Response to foreign pathogens (oxidative burst) -Production of cytokines -Wound repair	-Embryogenesis -Prevent overpopulation of cells and destroys malfunctioning cells -Cellular differentiation

Free radicals

Free radicals are molecules containing one or more unpaired electrons[20]. These free radicals are highly reactive and have several ways in which they may react with other molecules. If two radicals meet, they can form a covalent bond through sharing their unpaired electrons[20]. If a radical reacts with a non-radical, the radical can take an electron from the non-radical, donate its unpaired electron to the nonradical, or join onto the non-radical[20]. Either way, reaction of a radical with a non-radical produces another radical creating a chain[20]. These chains of reactions can damage DNA, RNA, proteins, lipids, and alter cellular processes[21] (see below for more information). This buildup of free radicals and/or non-radicals that are highly reactive and the resulting damage is referred to as oxidative stress[22]. Although there are many compounds that serve as free radicals and cause damage to cells, this dissertation will focus on those

derived from oxygen called reactive oxygen species (ROS) due to their high prevalence in the body and the surrounding environment[19] (Figure 2).

Sources of ROS

The generation of ROS can occur endogenously or exogenously (Figure 3). Endogenously, NADPH oxidases (NOXs) are a family of transmembrane cystolic enzymes and depending on which isoform is present, they can create different biological outcomes by triggering cellular transformations through ROS generation, including superoxide: a ROS[23,24]. An electron gets transferred to NADPH to a flavin

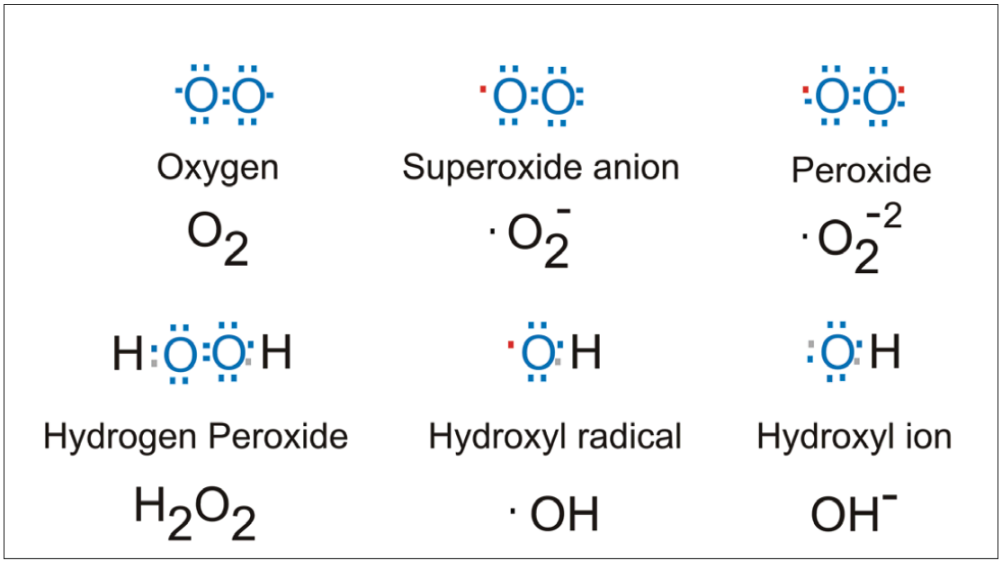

Figure 2 | **Examples of ROS**. The chemical formula and the structure of some common ROS, with the red dot representing the unpaired electron. Reprinted from *Biotek*[6], copyright 2010.

adenine dinucleotide (FAD) cofactor from the cystolic domains of NOX. The ROS radical is generated once the electron gets passed to the heme group, which then donates its electron to oxygen on the extracellular side of the membrane[24]. The mitochondria are another source of intracellular ROS and are the primary producers of ROS in many diseases consisting of oxidative stress. In the electron-transport chain,

Complex I (NADH dehydrogenase) and Complex III (ubiquinone-cytochrome c reductase) produce superoxide radicals through the transfer of an electron to oxygen[25,26]. Additionally, free radical semiquinone anion species formation is a nonenzymatic source of ROS in the mitochondria due to its ability to transfer electrons to oxygen, thereby generating the superoxide radical. This occurs as an intermediate of coenzyme semiquinone redox cycling[10]. Other endogenous sources that produce oxidants as part of their normal enzymatic function include, but are not limited to, cytochrome p450 enzymes, nitric oxide synthases, flavoenzyme ERO1 in the endoplasmic reticulum, cyclo-oxygenases, lipoxygenases, and oxidases for polyamines and amino acids[24,26]. The Fenton reaction can also create reactive oxygen species from free iron or copper ions that are released from heme groups, iron-sulfur clusters, or metal-storage proteins[27-29]. Furthermore, superoxides can form in the lipid membrane through a similar reaction involving lipid hydroperoxides[19]. These endogenous sources

all contribute to the buildup of ROS, thus causing oxidative stress; however exogenous sources amplify this problem (Figure 3).

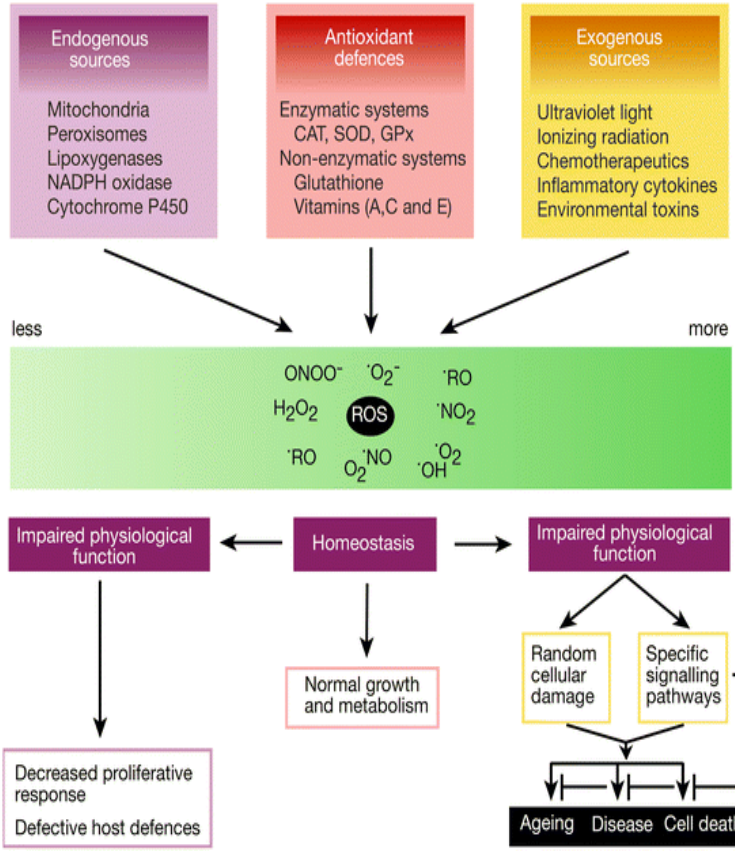

Figure 3 | Sources of ROS, antioxidant defenses, and subsequent biological effects depending on the level of ROS production. Reprinted from *Nature*[10], copyright 2000.

Exogenous sources of ROS include pollutants, ultraviolet (UV) light, and ionizing radiation. Pollutants that generate ROS include chemicals that are metabolized to radicals (ex: phenols), form ozone or peroxides, or release iron and copper that could promote the formation of hydroxyl radicals or promote the formation of superoxide (ex: bipyridylium herbicides)[10,15,30]. UV light and ionizing radiation of hydrogen peroxide can generate ROS through radiolysis of water molecules[31]. Other environmental sources that can generate ROS include xenobiotics, chlorinated compounds, metal ions, barbiturates, and non-DNA reactive carcinogens[32]. Additionally, some antibiotics

produce ROS for their bactericidal activity[33], and many chemotherapeutic cancer drugs involve ROS-mediated apoptosis[34]. Whether the source of ROS is endogenous or exogenous, maintaining a balance between their production and degradation is important for normal physiological function and calls for efficient detoxifying mechanisms.

Cellular detoxifying mechanisms

To keep the levels of ROS at homeostatic levels, they are regulated by scavengers and antioxidants that include both enzymatic and non-enzymatic systems (Figure 3)[35]. Examples of enzymes that regulate ROS, specifically hydrogen peroxide, include superoxide dismutase (SOD)[36], catalase (CAT), glutathione peroxidase (GPx), and NADH peroxidase[29]. SODs reduce superoxide to hydrogen peroxide and oxygen by catalyzing its dismutation[37-40]. CATs are another dismutase, however, they are not present in the brain mitochondria where the production of superoxides is high; thus they are not as important for the brain[25]. A family of selenium-containing enzymes, GPx decreases levels of hydrogen peroxide by coupling its reduction to hydrogen peroxide with the oxidation of the reduced/active form of glutathione (GSH)[41]. This creates two water molecules and oxidized glutathione (GSSG) that is made of two-disulfide connected GSHs and can react with glutathione reductase enzymes to be converted back into GSH[41]. NADH peroxidase is another type of peroxidase used to decrease hydrogen peroxide.

Examples of non-enzymatic systems that scavenge free radicals include alpha-tocopherol, vitamin C, and glutathione[10,42,43]. Alpha-tocopherol (TOH) is considered the most active lipid soluble antioxidant and is part of the compounds making up the vitamin E family[44,45]. TOH works by transferring a hydrogen atom to a peroxyl radical creating a hydroperoxide and tocopheroxyl radical. This radical can then react with another peroxyl radical, thereby terminating this chain reaction. Ascorbate, also known as ascorbic acid or vitamin C, can then quench the tocopheroxyl radical at the lipid-water interface[46].

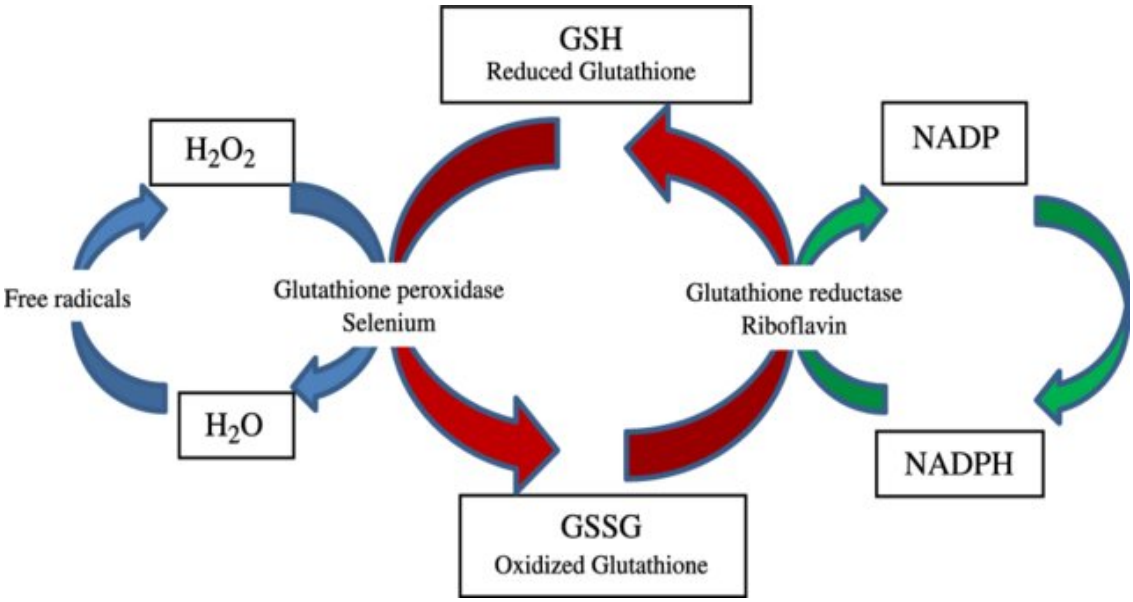

Figure 4 | **Reduction and oxidation of glutathione via free radicals**. Reprinted from *Southern medical association*[9], copyright 2014.

Ascorbate reacts with lipid peroxyl radicals and alkoxyl radicals to create lipid hydroperoxides, lipid hydroxides, and ascorbyl radicals. Disproportionation of the ascorbyl radicals yield no secondary free-radical byproducts[35]. Glutathione is another antioxidant that can react with radicals to form a thyil radical, which then reacts with a second glutathione forming a disulfide bond with the thiolate (Figure 4). Oxygen can then react with this disulfide radical ion, creating an inert disulfide[30]. Despite best efforts

to maintain adequate levels of ROS through antioxidant defenses, damage may occur if levels become too high.

Types of ROS-inflicted damage

There are a wide range of biological targets that ROS can react with to inflict damage in the body[35]. The level of reactivity of the ROS will determine the specificity it has for a particular target; ROS with high toxicity (ex: hydroxyl radical) will not be very specific relative to ROS that are less reactive (ex: hydrogen peroxide)[35]. This selectivity is also based on the amounts of different substrates available to react with[35]. Additionally, the more reactive the shorter the lifetime in solution[35]. The targets that ROS may react with can be divided into atomic and molecular targets[47]. There are only a few atomic targets within macromolecules that ROS may react with and lead to covalent protein modifications[48]. For example reaction with sulfur, found in cysteine and methionine, and selenium, found in selenocysteine, are common atomic targets of ROS[24]. Hydrogen peroxide favors reacting with thiols, specifically in their anionic form[49-51]. The iron in iron-sulfur clusters[52] within heme is also a target[35]. Superoxide radical is one ROS that prefers to react with iron-sulfur clusters[48]. Another ROS atomic target is carbon in polyunsaturated fatty acids[53] and carbon in either amino acids (ex: arginine, threonine, and proline) or nucleosides[54].

Molecular targets of ROS include DNA, proteins, and lipids[35]. ROS can cause damage to both nuclear and mitochondrial DNA[35], consequently altering transcription[24]. Mitochondrial DNA is especially prone to ROS-mediated damage since mitochondria are a main source of ROS within cells[35]. In terms of proteins, reactions with ROS can

induce conformational changes in the protein and alter its activity[35]. Specifically, many proteins contain cysteine residues that can be oxidized, resulting in structural and activity associated changes[55,56]. In these instances, cysteine residues have been thought to behave as redox switches[57]. The cell proteome can also be affected by alterations in phosphorylation of proteins via oxidation of cysteine residues in phosphatases[35]. Furthermore, carbonyl derivatives form on a protein's backbone because of protein oxidation and can be used as a marker of oxidative stress[10,58]. Reaction of lipids with a free radical (ex: hydroxyl radical) creates lipid carbon-centered radicals[59-63]. The trapping of oxygen by these lipid carbon centered radicals forms lipid peroxyl radicals[64,65], which are able to induce lipid chain autooxidation[35]. The mitochondria are particularly vulnerable to ROS-mediated oxidative damage due to their mitochondrial membrane containing polyunsaturated fatty acids[66]. No matter the target of ROS, the buildup of ROS- inflicted damage can lead to an array of pathological conditions.

Redox imbalance in disease

Evidence has shown that oxidative stress plays a role in a wide range of diseases throughout the body, including neurological disorders. Neuropsychiatric disorders including schizophrenia, bipolar disorder, depression, and anxiety all show signs of having imbalanced levels of ROS[16,67-69]. In patients with schizophrenia it has been shown that there are increases in ROS and decreases in antioxidants[70]. Antipsychotic medications increase levels of the antioxidant superoxide dismutase, which is correlated with improved symptoms of the disease[71]. Additionally, other

antioxidants including vitamin E, vitamin C, or the mixture of fish oil have been shown to be beneficial for symptoms of schizophrenia[72]. ROS and antioxidants are also thought to play a role in bipolar disorder due to studies showing that these patients have alterations in nitric oxide, lipid peroxidation, and antioxidant enzyme levels[73]. Furthermore, treatment of patients with N-acetylcysteine (NAc), an antioxidant, showed improvement in bipolar symptoms[74,75]. In terms of depression, post-mortem brain samples of depressed patients were found to have increased levels of ROS[76-78] and altered levels of the antioxidant glutathione (GSH)[79]. There is also an association between polymorphisms in superoxide dismutase (SOD) and catalase (CAT), two important antioxidant enzymes, and this disease[78]. It has been hypothesized that antidepressants provide a therapeutic effect by decreasing ROS production and/or increasing antioxidant defense[80]. Animal studies have linked anxiety disorders to oxidative stress as well. Animal studies have shown that genetically or pharmacologically inducing oxidative stress increases anxiety-like behavior in rats, suggesting a causal role for oxidative stress in anxiety[81-84]. Additionally, use of a social defeat model of psychological stress induced anxiety-like behavior in rats as well as levels of oxidative stress[85]. Moreover, antioxidant enzymes, including glyoxalase and glutathione reductase, were found to be reduced in the brains of rats displaying anxiety-like behaviors[86-88]. From schizophrenia to anxiety disorder, oxidative stress plays a role in neuropsychiatric disease.

In addition to the role of oxidative stress in neuropsychiatric disorders, it also plays a role in neurodegenerative disorders including Parkinson's disease (PD), Huntington disease (HD), and Alzheimer's disease (AD). All of these disorders involve

the neurotoxic buildup of specific proteins in the brain: alpha-synuclein builds up in PD, mutant Huntington protein (mHtt) in HD, and amyloid beta also called beta amyloid or a-beta) and misfolded tau in AD[89]. The accumulation of these proteins is thought to be affected by ROS production, which also accompanies aging[90]. In fact, a popular theory called the free radical theory of aging suggests that accumulation of oxidative damage to macromolecules (DNA, lipids, and proteins) by ROS causes age-associated functional losses that can be seen in neurodegenerative disorders[91]. In PD, alpha-synuclein has been found to increase ROS levels, and aggregation of this protein is further enhanced by oxidative stress[92]. HD studies provide evidence that free radicals play a role in the misfolding and accumulation of mHtt, and this accumulation decreases antioxidant activity[93]. Research shows that a-beta buildup in AD is driven by ROS production, leading to impaired learning and memory[94-97]. Altered membrane permeability due to lipid peroxidation is also driven by ROS-induced a-beta accumulation, leading to increased calcium influx and excitotoxicity[89]. Although oxidative stress is not limited to neurological diseases, non-neurological diseases go beyond the scope of this thesis. It is especially important to look at oxidative stress in the context of AD, one of the world's one of the world's leading causes of death[98].

General introduction to AD

AD is the sixth leading cause of death in the United States, and the fifth leading cause in Americans 65 years and older[11]. Whereas this disease currently affects 5.8 million Americans, it is expected to increase to 13.8 million people by mid-century if better treatments are not found to combat the disease[99]. In addition to the physical

burden placed on people with AD, there are significant negative effects on their mental health and added financial struggles. Medicare-provided care for beneficiaries that have AD and are older than 65 costs more than triple that of people without AD for Medicare; and the costs for Medicaid are 23 times as great[11]. Additionally, it is estimated to cost $305 billion in total payments for health care, long-term care, and hospice service for this population. It is expected to grow to $1.1 trillion by 2050[100]. Moreover, the burden of this disease does not just affect people with AD, but also people directly or indirectly contributing to the care of people with AD including family, friends, and caretakers. This calls for better understanding of the disease so better therapeutics can be developed to prevent; treat; or at the very least, slow the progression of AD.

The stages of AD

AD is a degenerative disease, meaning it worsens over time[11]. It is also the most common cause of dementia, an umbrella term encompassing symptoms that include difficulty with language, problem-solving, memory, and other thinking skills that are needed to perform daily activities[11,101]. As times passes, more and more changes occur in the brain and damage is done to neurons, causing degradation. This results in a person being unable to form basic bodily functions and the need for around-the-clock care until the disease eventually kills them[11]. Unfortunately, many times initial changes in the brain are unnoticed by the affected person and are thought to arise more than 20 years before symptom onset[102-104]. Brain changes include the formation of A-beta plaques and neurofibrillary tangles (accumulation of an aberrant form of tau protein which assists in microtubule formation). The plaques are thought to interfere with

interneuron signaling at synapses, whereas the tangles hinder the transport of nutrients and other essential molecules within neurons[11]. Other changes that are believed to be driven by accumulation of plaques and tangles include neuroinflammation, brain atrophy, and oxidative stress (which will be discussed more later)[11,105]. The progression of these changes creates an AD continuum (Figure 5) that divides AD into three phases: preclinical AD, mild cognitive impairment (MCI), and dementia due to AD. This final stage is further divided into mild, moderate, and severe[106-109]. During the preclinical

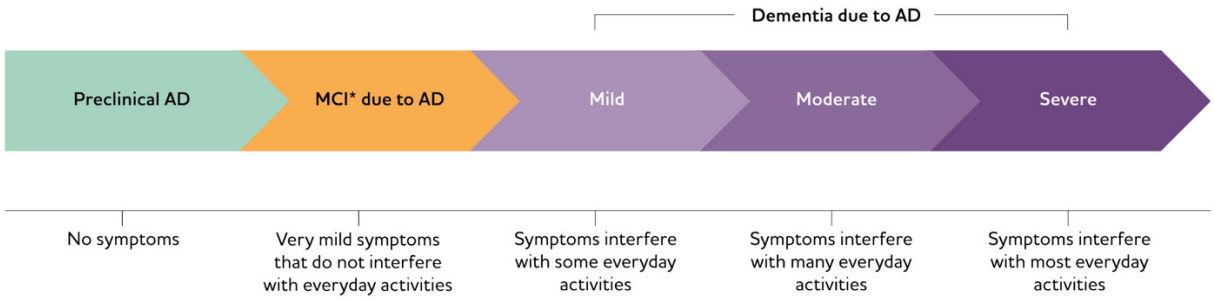

Figure 5 | Continuum of the progression of AD. (MCI=mild cognitive impairment). Reprinted from *Alzheimer's & Dementia*[11], copyright 2020.

phase no symptoms have developed, however, there are measurable brain changes and biomarkers, including abnormal a-beta accumulation[11]. The next phase, MCI, reveals subtle problems with memory and thinking that do not interfere with a person's ability to carry out daily activities, although may be noticeable by close family and friends[11]. Once cognitive issues progress towards interfering with a person's everyday life and there is extensive damage to the brain, they have progressed to dementia due to AD[11]. The division of the final phase is based on how quick the symptoms advance. People with mild AD may require assistance to maximize independence and remain safe but are able to function independently in many areas[11]. In what is often the longest sub-phase, moderate AD, there is difficulty performing routine tasks accompanied by personality and behavioral changes[11]. People with severe AD require 24/7 care and

need help performing daily activities. At this point the effects of the disease become apparent and may lead to a person becoming bed-bound[11]. This stage is also linked to common comorbidities including blood clots, skin infections, and sepsis[11].

AD diagnosis and treatment

Diagnosis and treatment of AD is not easy. Doctors use a combination of approaches to determine if a person has AD including cognitive tests, interviews with family members, medical history and family history, and tests to determine levels of a-beta[110]. Risk factors including age[99,111], genetics[112,113], sex[99], and family history of AD[114,115] may also be used to determine a person's likelihood of developing AD. The problem with these tests is that although they can reveal if a person has dementia, it is hard to determine if it is AD dementia, another type of dementia, or mixed[116,117]. In terms of medications available to treat AD, five drugs have been approved by the U.S. Food and Drug Administration (FDA): memantine, donepezil, memantine combined with donepezil, galantamine, and rivastigmine[11]. Glutamate receptors called N-methyl-D-asapartate (NMDA) receptors in the brain are blocked by memantine to prevent excess stimulation caused by glutamate binding that damages neurons[11]. The other medications work to temporarily improve cognitive symptoms by increasing neurotransmitters like acetylcholine in the brain[11]. Unfortunately, none of these medication slow or stop the damage and destruction that make the disease fatal and the FDA has not approved any specific medication to treat the psychiatric and behavioral symptoms of the disease[11]. Non-pharmacological therapies including listening to music to stir recall, memory training, and exercise[118] may be used to help maintain cognitive

function[11]. The lack of better treatments calls for more research to be done on the etiology of AD, especially considering AD can ultimately only be diagnosed post-mortem after histological analysis of the brain. This dissertation, investigates the role of oxidative stress in causing and/or driving AD.

Oxidative stress in AD

Increases in oxidative stress and subsequent damage to cells has been implicated in AD and suggests this stress plays an imminent role in disease pathology[105]. A hallmark of aging, and the biggest risk factor for AD, is dysfunctional mitochondria. Uncoincidentally, mitochondria are also the main source of ROS generation in the body[105]. Although ROS themselves are hard to measure due to their short half-lives, the increased stability of their oxidized biomolecule products as well as levels of antioxidant activity allow for detection of the levels of oxidative stress in the body and provide evidence of oxidative stress in AD[105]. For example, lipid peroxidation products resulting from attack of lipids by ROS are elevated in parts of the brain of patients with AD compared with age-matched controls[119]. There is also an increase in the oxidation of proteins as shown through examining protein carbonyl levels[120]. Damage to DNA and RNA is another sign of increased oxidative stress in AD. Damage to DNA involves DNA or base modifications and/or DNA/protein crosslinking; all of which are elevated in AD [121-123]. Increased levels of oxidized rRNA and mRNAs have also been shown[124-126]. To further exacerbate the problem of ROS-induced damage ensued in AD, there are decreases in antioxidant levels[127,128] as well as decreased activity of antioxidant enzymes[129-132]. It has been shown that markers of oxidative

damage occur early on in AD, around the MCI phase, even before evidence of A beta or tau can be detected in cerebral spinal fluid[133-135]. Additionally, decreased antioxidant levels have been shown in people with MCI[135], and deficiency in antioxidants alone is

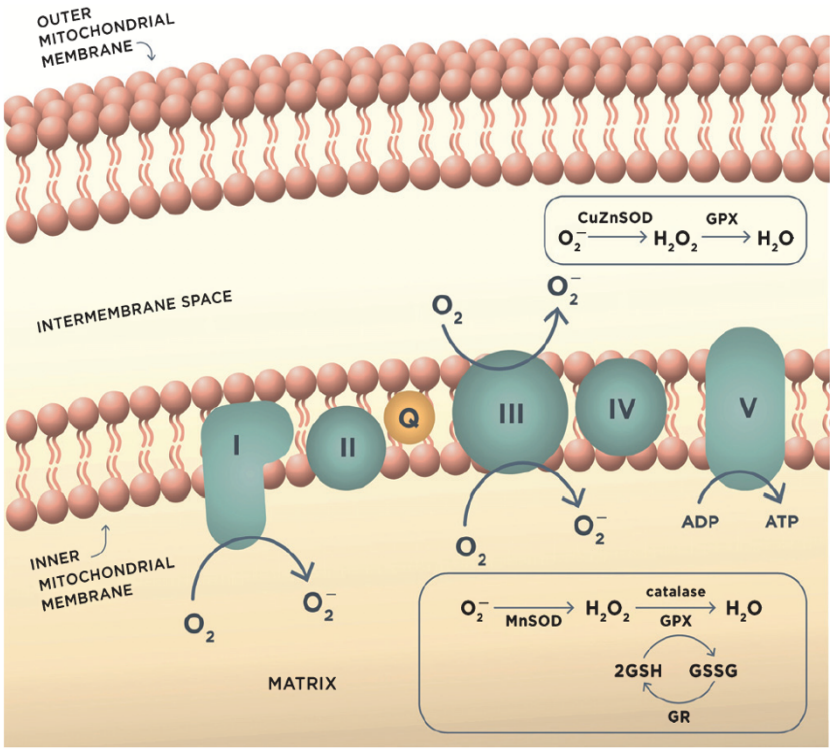

Figure 6 | Production of mitochondrial ROS during oxidative phosphorylation and responding antioxidant defenses. During aerobic respiration the superoxide anion is produced in excess by the mitochondrial electron transport chain, specifically by Complex I and II. In the intermembrane mitochondrial space, CuZnSOD and MnSOD convert the anion to H_2O_2. Detoxifying mechanisms further reduce H_2O_2 to water via glutathione peroxidase or catalase. These reactions occur in the matrix. Reprinted from *Oxidative stress, synaptic dysfunction, and Alzheimer's disease*[14], copyright 2017.

enough to drive cognitive deficits as seen in AD[136]. The fact that evidence shows oxidative stress occurs early on before AD pathology suggests that oxidative stress plays a key role in the progression of AD.

Dysfunctional mitochondria in AD

As mentioned previously, a key driver of oxidative stress early on in AD is dysfunctional mitochondria[137]. ATP production results in unavoidable leakage of electrons, thus leading to constant production of ROS[123]. In fact, the mitochondria are responsible for 90% of endogenous ROS (Figure 6) [123]. Evidence of dysfunctional mitochondria in AD include low glucose (energy) metabolism[138], alterations in key enzymes in oxidative phosphorylation[139], decreased calcium homeostasis due to impaired buffering capacity[140], mutations in mitochondrial DNA (mtDNA)[141], and changes in the apoptotic pathway[142]. It is thought that this dysfunction could be driven by the structural damage[141], decreased mitochondrial size[141], decreased mitochondrial number[141], and changed in expression of fission and fusion proteins[143] seen in AD. The abundant evidence of oxidative stress driven by mitochondrial dysfunction in early AD brings attention to its role in AD pathology. Much of what we know about AD including the connection to oxidative stress and dysfunctional mitochondria, has been possible by using animal models of AD[144].

Preclinical animal models of AD

The development of mouse models is based on the genetics associated with AD[145]. There are two categories of AD-associated genes: genes in which mutations cause autosomal dominant AD or genes in which polymorphisms serve as risk factors[145]. Autosomal dominant means that the gene is located on a non-sex chromosome and that only a single copy of a disease-associated mutation is sufficient to cause the disease. Mutations in amyloid precursor protein (*APP*)[146], and the

presenilin genes (*PSEN1* and *PSEN2*)[147] can cause autosomal dominant AD. AD-associated mutations in any of these three genes will affect APP processing, thus affecting a-beta production[148,149]. This occurs because a-beta is created by beta-secretase removing the extracellular domain of APP and the remaining fragment being cleaved by γ-secretase, which is partly composed of presenilin[150]. Two different a-beta peptide lengths, 40 or 42 peptides, can occur depending on where gamma-secretase cleaves APP. The more toxic isoform is a-beta 42 because it is more prone to oligomerization[151]. Mouse models containing *APP* point mutations or increasing *APP* gene copy number can lead to AD-like phenotypes[152]. Examples of these models include hAPP transgenic models, which overexpress wild type[153] or mutant[145] forms of human APP and display memory deficits, amyloid pathology, and synaptotoxicity; although they do not usually show significant loss of neurons[145]. Although the functions of the proteins encoded by *PSEN1* and *PSEN2* are similar, *PSEN1* mutations are more common and more severe thus making mouse models containing *PSEN1* mutations more popular[154]. Mutations in these two genes can lead to increased production of a-beta 42 compared to a-beta 41[155], or loss of other presenilin functions including apoptosis, cell adhesion, calcium homeostasis, trafficking/localization, transport, and cleavage of other γ-secretase substrates[156,157]. Models containing one[158] or multiple[159] presenilin gene mutations (mainly *PSEN1*) have been utilized. Since single transgenic presenilin mutant mice only show increased a-beta 42[160] with no other cognitive problems or AD pathology[161,162], they are usually crossed with hAPP mice so that plaque deposition and behavioral deficits occur[163]. Even though autosomal dominant AD driven by mutations in these three genes is only a small percentage of AD cases[164],

models expressing these mutations are the most common AD mouse models because the symptoms and pathology have profound similarities with sporadic AD[164].

Other models of AD include transgenic models that overexpress a-beta 42 leading to increased a-beta secretion in cells[165] and development of amyloid pathology[151]. A caveat of this model is that no resulting cognitive deficits have been published. Another model focuses on tau's role in AD. Although hyperphosphorylated tau protein leading to the formation of neurofibrillary tangles is a key hallmark of AD, models overexpressing mutant tau protein do not cause AD[145]. However, crossing mice expressing mutant human tau with mice expressing hAPP and/or mutant presenilin reveal tau pathology that is lacking in the previous discussed models while also containing a-beta deposition and behavioral deficits[145]. Additionally, since most cases of AD are sporadic and late onset and autosomal forms of AD are relatively rare, there are other genes that can be utilized in mouse models to modulate the risk of the more common form[145]. *APOE,* which encodes apolipoprotein E (apoE), is the strongest risk factor gene for AD. There are three alleles for this gene, with ε2 being protective, ε3 being the most common, and ε4 being associated with earlier onset and increased risk of AD[166]. Each copy of the ε4 allele lowers the age of onset by roughly 10 years[166]. ApoE4 causes increased a-beta in the brain and increased plaque deposition[167]. It also elevates mitochondrial dysfunction[168], and tau phosphorylation[169]. Mouse models overexpressing, usually knock-in, *APOE* (encoding for apoe4) show cognitive deficits[170] and, when crossed with hAPP mice, higher plaque deposition[171,172]. Although they have a relatively small effect compared to *APOE*, other genes that have been associated with

the late-onset, sporadic form of AD include, but are not limited to CLU*, CR1,* and *ABCa7*[173-175].

Whether the mouse models display mutations in genes that cause autosomal dominant AD or genes in which polymorphisms serve as risk factors, there are limitations to using these as models of AD. Since most models more closely resemble autosomal dominant AD, it might be hard to apply data from these models to sporadic AD in humans[145]. These AD mice might also model different stages of the disease than that which has been focused on in clinical trials[145]. Despite these and other caveats that apply to using mouse models to recapitulate human disease, the amount of knowledge that has been obtained and can be obtained from studying them cannot be ignored. This includes the knowledge that can be obtained from assessing behavioral deficits of these AD mice.

AD-related cognitive measures in mouse models

The use of mouse models is only as good as their level of validity in recapitulating the disease. The AD models discussed previously have all been shown to uphold a certain level of validity, which has in part been determined by assessing how well the models mimic the cognitive deficits seen in AD. These assessments involve the use of behavioral paradigms that measure hippocampal memory[176] . The focus of these tests on the hippocampus is due to its vulnerability in early AD[177] and its role in memory[178]. Memory can be divided into explicit, or declarative, and implicit, or non-declarative, memory[2]. Memory that can be consciously and intentionally recalled is explicit memory, whereas memory that cannot be consciously recalled and is an

experiential or functional form of memory is implicit memory. Explicit memory can further be divided into episodic or semantic memories which includes the ability to recall events or facts, respectively. Implicit can further be divided into non-associative and associative learning[2]. Non-associative memory involves habituation, the decrease in response to a stimulus when it is repeatedly presented, and sensitization, increased response to a variety of stimuli after experiencing an intense one. Associative memory involves associating two stimuli with each other or understanding that a response is associated with a given consequence or event. Associative memory includes classical and operant conditioning. Classical conditioning involves creating a relationship between a neutral stimulus (later becoming a conditioned stimulus) to cause a particular response and an unconditioned stimulus, which is a stimulus that elicits a certain behavioral response. Operant conditioning occurs when an animal learns that it will get a reward if it does something. Despite the different levels of memory, all are affected when the there are problems with the hippocampus as seen in AD. Three validated examples of behavioral protocols used to assess cognitive deficits, including problems with memory, in mice include fear conditioning (FC), the Morris Water Maze (MWM), and novel object recognition (NOR).

FC is one behavioral paradigm that can be used to assess associative memory. FC involves the use of the hippocampus, which is involved in learning the context that triggers the fear, and the amygdala, which is involved in the formation and storage of emotional memories that can be elicited by fear[179-181]. FC is a form of associative learning based on classical conditioning[2]. In patients with AD, there are impairments in conditioned fear responses[182]. When testing animal models of AD, typically this protocol

involves pairing a light or sound (conditioned stimulus) to a foot shock (unconditioned stimulus) during an initial learning trial[2]. This results in the mouse associating the

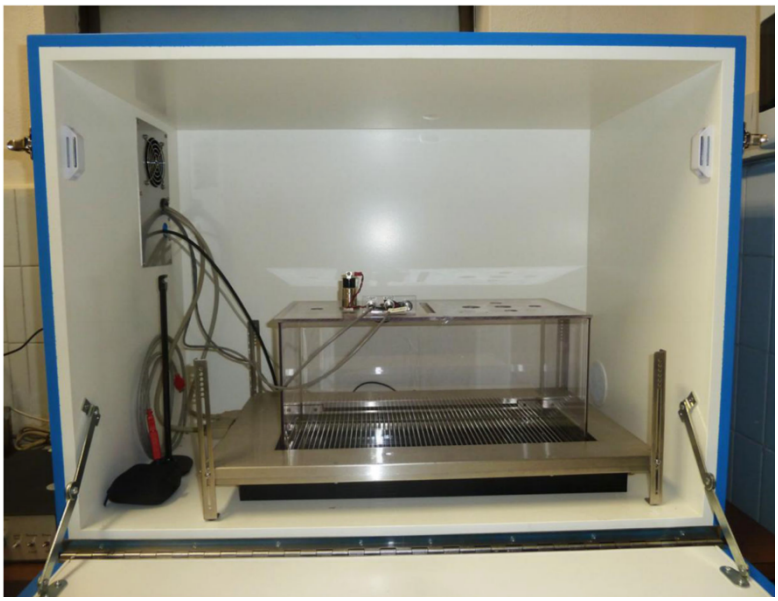

Figure 7 | Example of the apparatus used for fear conditioning (FC) paradigm. Includes the chamber that the mouse is placed in, the shock grid on the floor of the chamber, and the wires through which a shock is sent through to the grid. Reprinted from *Biological Pharmacology*[2], copyright 2014.

light/sound with the foot shock in subsequent trials because the mouse will remember that the foot shock followed the appearance of the light/sound[2]. The mouse will also be able to remember the place or context where they received the foot shock (contextual fear)[2]. Due to the foot shock being an aversive stimulus, the conditioned response will be fear[183] and accompanying behaviors will involve trying to escape the perceived danger[184]. Physiologically this will include activation of the fight or flight sympathetic response[184] and inhibition of parasympathetic function[2]. The behavioral response to this will be freezing, which demonstrates a mouse's defensive response to fear[185]. This set up typically involves placing individual mice in a conditioning chamber that has a shock grid along the floor for two minutes (Figure 7)[2]. A tone/light is then delivered for 30s and during the last 2s a shock is administered. Following cessation of the tone/light, an

additional 30s will pass before putting the mouse back into its home cage. Contextual fear learning is assessed by placing the mouse back in the conditioned chamber 24 hours later and measuring the amount of time spent freezing over 5 minutes[2]. A mouse with intact contextual memory will remember that this is the environment that it received the shock from the previous day and will spend more time freezing. To assess cued fear learning, the mouse is placed in a new, contextually distinct cage, the same tone as the training day is used, and freezing is measured over the course of three minutes[2]. Normal memory formation will cause increased freezing when the tone is played. With mice that do not have intact memory as with the AD mice, the mouse will show relatively less/no freezing when the mouse is placed back in the conditioned context or when the tone is administered, demonstrating impaired contextual fear conditioning and cued fear learning respectively[186]. Advantages of FC include that it is faster than other behavioral tests, it can test emotional and contextual memory, it assesses the integrity of involved brain structures, and its able to confine the moment at which contextual learning occurs to a very short amount of time[2].

The MWM is another popular method used to assess cognitive function in mice, specifically long-term memory, which is also altered in AD[2]. This test is used to specifically assess hippocampal-dependent spatial memory and long-term spatial memory[187]. Although there can be differences in the training procedures and/or apparatus (Figure 8)[188] the most common way uses a large plastic pool filled with water and made opaque by using beads or paint[2]. A platform is added to the pool so that it lies just below the surface of the water and stationary visual cues are placed in the

surrounding area of the pool so that they are visible by the mouse when it is in the pool. A camera is then placed above the pool so that it captures the entire arena, and the arena is divided into four quadrants. Once the room and apparatus are set up, mice individually undergo a training period that can last from two to ten days and allows determination of the mouse's ability to learn relative to spatial cues[2]. The number of

Figure 8 | Example of apparatus used for the Morris water maze paradigm. Includes the pool, geometric visual cues surrounding the area, and there will be a platform submerged under the surface of the water (not shown). Reprinted from *Biological Pharmacology*[2], copyright 2014.

trials per day can be variable, but each trial involves placing an individual mouse in the water starting from a randomly chosen quadrant that does not contain the platform and allowing 60s for the mouse to find the platform. Since swimming requires substantial amount of energy to keep the mouse from drowning, the mouse will want to find the platform to escape the water. The amount of time it takes for the mouse to find the platform (escape latency) is recorded. If the mouse cannot find the platform during the

60s , the experimenter may guide the mouse to the platform. With each trial, the mouse should be placed at a different starting position and the platform should remain in the same place. Over the course of the training days, mice with intact long-term and spatial memory should be able to find the platform faster. After the training period, a probe test day is performed. For this day the platform is completely removed from the pool. The number of trials for this can also vary, but each trial involves placing the mouse in a random part of the pool and recording how much time the mouse spends swimming in the target quadrant (quadrant of the pool that originally contained the platform), and non-target quadrants. A mouse with intact long-term memory will typically spend more time in the target quadrant relative to the other areas of the pool. This protocol has proven to successfully show spatial memory impairment in models of AD[189] as evidenced by these mice taking longer to find the platform during the training period and spending less time in the target quadrant during the probe test day. The advantages of the MWM include that it is fast, a low number of animals are required relative to other paradigms, it allows differentiation between long-term spatial memory and spatial learning, testing of non-spatial functions like motor and visual performance can be done, the mice are not put through any painful procedures, there is a reduction in olfactory interferences that rely on the hippocampus, and the cost is low[2].

Another form of explicit memory that can be affected in AD is recognition, or the ability to recall personal history, events, and facts[3]. The NOR behavioral task is another test that can be used to test cognition by measuring a mouse's ability to recognize a previously presented stimulus compared to a novel one[190]. During this task a mouse is placed into a square or rectangular, high-walled arena that lacks any distinguishing

contextual or spatial cues (Figure 9)[3]. Two identical objects are placed in the arena and the mouse can explore the arena and these objects during the training, or sample, session[3]. The mouse is then removed from the arena for a chosen period, allowing the object memory to be consolidated[3]. During this time one of the two objects in the arena is

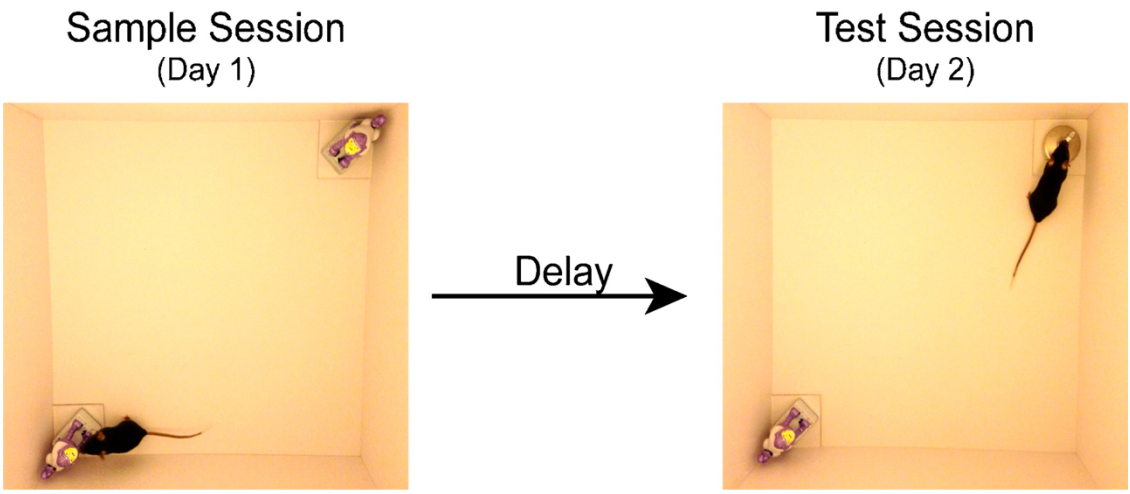

Figure 9 | Example of apparatus used for the novel object recognition paradigm. Includes the chamber containing two identical objects for the sample session (left) and the same chamber containing one of the same objects from the test session and one novel object (right). Reprinted from *Behavioural Brain Research*[3], copyright 2015.

replaced with a new object[3]. Afterwards, the mouse is placed back into the arena during the test session and is once again allowed to explore[3]. Mice have a natural tendency to spend more time exploring novel, non-threatening objects; thus, mice with intact object memory retrieval will prefer spending more time with the new object compared to the familiar one[191]. With AD mice, they will show no preference for the novel object due to the impairments in cognition[192]. Advantages to this paradigm include that it does not involve putting mice through stressful conditions, it does not require external motivation, reward, or punishment; and no extensive training is needed for the mice[3].

FC, MWM, and NOR are just a few of the options experimenters may use to test cognitive function in mice and have been used specifically to show cognitive deficits in AD mouse models. Now that these models have been validated using cognitive behavioral tasks, it begs the question of what is causing these deficits. One possible answer is that changes in gene expression encoding proteins that control these behaviors drive AD pathology.

Altered gene expression in AD

Alterations in gene expression are a common occurrence in disease pathology including AD[12]. These changes in gene expression can elicit the AD phenotype by altering levels of proteins encoding for certain behaviors or certain hallmarks associated with the disease. Identifying which genes are changed in AD could help identify potential therapeutic targets that can help address disease symptomology and could lead to slowing the progression of and/or curing the disease. Studies have shown that there are thousands of genes that are changed in AD[12]. These genes control a variety of physiological functions including hormone response, intracellular signaling (ex: G proteins), neurotransmission and synaptic function/formation, inflammatory and immune response, metal metabolism, cholesterol metabolism, stress response (like oxidative stress), constitution of blood plasma, a-beta formation, a-beta oligomerization, a-beta clearance, APP processing, kinases, phosphatases, and neurotransmitter receptors (Table 2)[12].

In AD, the direction and degree of change is different among genes; genes may be upregulated or downregulated compared to healthy controls[12]. An example of a gene

that is upregulated in AD includes a member of the family of membrane water channels called aquaporin 1 (AQP1)[12,193]. Since previous studies have shown that AQP1-expressing astrocytes tend to be located in close proximity to a-beta[194], it suggests that expression of this gene may be involved in AD pathogenesis; possibly through APP processing or aberrant ion or water fluxes[12]. Genes that encode for heat shock proteins or Metallothionein 2a can be upregulated due to cellular stress responses or neuroinflammatory reactions, respectively. These in turn

Table 2 | Gene expression changes in AD. Reprinted from *Journal of Neuroscience*[12], copyright 2010.

TABLE IV. Gene Expression Changes in AD II: Pathways and Functions

Transcipt	Title	Symbol	Assignment	Fold-Change	p-Value
	Genes Involved in APP Processing, Aβ Formation, Oligomerization and Clearance				
3311832	ADAM metallopeptidase domain 12 (meltrin α)	ADAM12	NM_003474	1.2871	0.0004438★
3360826	APP-binding, family B, member 1 (Fe65)	APBB1	NM_145689	−1.3226	0.01593
3356115	Amyloid β precursor-like protein 2	APLP2	NM_001642	−1.3901	0.003174★
3695268	Amyloid β precursor protein binding protein 1	APPBP1	NM_001018159	−1.703	0.001118★
3921933	β-site APP-cleaving enzyme 2	BACE2	NM_012105	1.6107	0.0000547★
3187686	Gelsolin (amyloidosis, Finnish Type)	GSN	NM_000177	1.5558	0.001119★
3337516	Low density lipoprotein receptor-related protein 5	LRP5	NM_002335	1.4026	0.01994
2868044	Proprotein convetase subtilisin/kexin type 1	PCSK1	NM_000439	−3.2093	0.0000887★
2383312	Presenilin 2	PSEN2	NM_012486	−1.2321	0.005727
	Neurotransmitter Receptors and Related Molecules				
2716328	Adrenergic α2C receptor	ADRA2C	NM_000683	1.5084	0.004252
2360346	Cholinergic receptor, nicotinic, β2	CHRNB2	NM_000748	−1.5061	0.007486
3315181	Dopamine receptor D1 interacting protein	DRD1IP	NM_015722	−2.3635	0.000269★
2838416	γ-aminobutyric acid (GABA) A receptor, α1	GABRA1	NM_000806	−3.0541	0.007112
2768056	γ-aminobutyric acid (GABA) A receptor, α4	GABRA4	NM_000809	−1.881	0.01044
2838462	γ-aminobutyric acid (GABA) A receptor, γ2	GABRG2	NM_198904	−3.0101	0.006973
2514969	Glutamate decarboxylase 1	GAD1	NM_000817	−2.5767	0.000833★
3239667†	Glutamate decarboxylase 2	GAD2	NM_000818	−2.6511	0.001929★
2749222	Glutamate receptor, ionotropic, AMPA 2	GRIA2	NM_001083619	−1.638	0.00421
3989448	Glutamate receptor, ionotropic, AMPA 3	GRIA3	NM_007325	−1.7843	0.006842
3347118	Glutamate receptor, ionotropic, AMPA 4	GRIA4	NM_001077243	−1.5147	0.01339
3928211	Glutamate receptor, ionotropic, kainate 1	GRIK1	NM_175611	−1.5080	0.002385★
3352618	Glutamate receptor, ionotropic, kainate 4	GRIK4	NM_014619	−1.2882	0.00241★
3119945	Glutamate receptor, ionotropic, NMDA protein 1	GRINA	NM_000837	−1.2517	0.009204
3679812	Glutamate receptor, ionotropic, NMDA 2A	GRIN2A	NM_000833	−1.6882	0.003855
3837759	Glutamate receptor, ionotropic, NMDA 2D	GRIN2D	NM_000836	1.4119	0.01053
2950970	Glutamate receptor, metabotropic 4	GRM4	NM_000841	1.3634	0.02147
2961647	Serotonin receptor 1B	HTR1B	NM_000863	1.4660	0.000654★
3513147	Serotonin receptor 2A	HTR2A	NM_000621	−2.773	0.00325★
3349660	Serotonin receptor 3B	HTR3B	NM_006028	−1.8462	0.000733★
2701081	Purinergic receptor P2Y, G-protein coupled, 12	P2RY12	NM_022788	−1.5569	0.02420
3884793	GABA vesicular transporter	SLC32A1	NM_080552	−1.9936	0.02264
2709750	Somatostatin	SST	NM_001048	−4.0153	0.004206
	Genes Which Play a Role in, or are Targets of, Inflammatory/Immune Responses				
3753860	Chemokine (C-C morif) ligand 5	CCL5	NM_002985	1.3947	0.02310
2902958	Complement component 4A	C4A	NM_007293	3.3404	0.006772
2501075	Interleukin 1 family, member 7, ζ	IL1F7	NM_014439	1.2156	0.01315
2496962	Interleukin 1 receptor, type I	IL1R1	NM_000877	1.4885	0.000211★
3261643	NF-κB 2	NFKB2	NM_001077493	1.4198	0.003011★
3863021	Transforming growth factor, β1	TGFB1	NM_000660	1.4257	0.000647★
3572517	Transforming growth factor, β3	TGFB3	NM_003239	2.2245	0.009089
3847989	Tumor necrosis factor superfamily, member 7	TNFSF7	NM_001252	1.2445	0.02296
	Kinases, Phosphatases, G-proteins and Related Signaling Molecules				
2464005†	Protein kinase B, γ	AKT3	NM_181690	−1.3273	0.01809
2881300†,a	Calcium/calmodulin-dependent protein kinase IIα	CAMK2A	NM_015981	−1.4086	0.01081
3294854†,a	Calcium/calmodulin-dependent protein kinase IIγ	CAMK2G	NM_172171	−1.5189	0.001013★
3944758	CDC42 effector protein (Rho GTPase-binding) 1	CDC42EP1	NM_152243	1.5911	0.004288
3079313	Cyclin-dependent kinase 5	CDK5	NM_004935	−1.7195	0.02336
3894228	Casein kinase 2, α1	CSNK2A1	NM_177559	−1.5143	0.000273★
3035892	Guanine nucleotide binding protein α12	GNA12	NM_007353	1.701	0.00145★
3624448	Guanine nucleotide binding protein β5	GNB5	NM_016194	−1.4719	0.00445
2420427	Guanine nucleotide binding protein γ5	GNG5	NM_005274	1.4239	0.01454
2691014†	Glycogen synthase kinase 3β	GSK3B	NM_002093	−1.6315	0.002391★
3788097	Mitogen-activated protein kinase 4 (p63MAPK)	MAPK4	NM_002747	1.5849	0.002427★
3594129	Mitogen-activated protein kinase 6 (p97MAPK)	MAPK6	NM_002748	−1.7727	0.004307
3245682	Mitogen-activated protein kinase 8 (JNK1)	MAPK8	NM_139049	−1.5007	0.01855
2890605	Mitogen-activated protein kinase 9 (JNK2)	MAPK9	NM_002752	−2.0394	0.008664

Table 2 (cont'd)

Transcipt	Title	Symbol	Assignment	Fold-Change	p-Value
3875642	Phospholipase C, β1	PLCB1	NM_182734	−2.0937	0.000883*
2876046[†,b]	PP2A, catalytic subunit, α isoform	PPP2CA	NM_002715	−1.8637	0.01105
2779638	Calcineurin A α	PPP3CA	NM_000944	−1.7756	0.00736
3294499	Calcineurin A β	PPP3CB	NM_021132	−1.5832	0.01255
3391029	PP2A, regulatory subunit A (PR65), β isoform	PPP2R1B	NM_002716	−1.2438	0.001672*
3653123	Protein kinase C, β1	PRKCB1	NM_002738	−1.8913	0.02471
2624291	Protein kinase C, δ	PRKCD	NM_006254	−1.5229	0.001888*
2480168	Protein kinase C, ε	PRKCE	NM_005400	−1.9124	0.002823*
4054481	Protein kinase C, ζ	PRKCZ	NM_002744	−2.1125	0.000434*
3365601	Striatal enriched protein tyrosine phosphatase	PTPN5	NM_006906	−1.9004	0.001528*
2364381	Regulator of G-protein signaling 4	RGS4	NM_005613	−3.2707	0.003146*
	Genes Involved in Cholesterol Metabolism				
3218528	ATP-binding cassette, sub-family A, member 1	ABCA1	NM_005502	1.5402	0.003758
3392986	Apolipoprotein A-I	APOA1	NM_000039	1.3025	0.01799
2413907	24-dehydrocholesterol reductase	DHCR24	NM_014762	−1.9255	0.01202
2815965	3-hydroxy-3-methylglutaryl-coenzyme A-reductase	HMGCR	NM_000859	−1.9735	0.02370
3337516	Low density lipoprotein receptor-related protein 5	LRP5	NM_002335	1.4026	0.01994
3147985	Low density lipoprotein receptor-related protein 12	LRP12	NM_013437	−1.6325	0.02137
3160175	Very low density lipoprotein receptor	VLDLR	NM_003383	−1.4553	0.004487
	Oxidative Stress-Related Genes				
2793054	Carbonyl reductase 4	CBR4	NM_032783	−1.4614	0.01451
2835715	Glutathione peroxidase 3 (plasma)	GPX3	NM_002084	−1.4404	0.009762
3009399	Heat shock 27 kDa protein 1	HSPB1	NM_001540	2.3517	0.001487*
3348765	Heat shock 27 kDa protein 2	HSPB2	NM_001541	1.6985	0.000614*
2809579	Heat shock 27 kDa protein 3	HSPB3	NM_006308	−1.6824	0.000996*
3540136	Heat shock 70 kDa protein 2	HSPA2	NM_021979	1.6361	0.01272
3308397	Heat shock 70 kDa protein 12A	HSPA12A	NM_025015	−1.5244	0.0205
3646199	Heme oxygenase 2	HMOX2	NM_002134	−1.4171	0.02337
	Genes Involved in Synaptic Function and Formation				
3367231	Brain-derived neurotrophic factor	BDNF	NM_170732	−1.2751	0.001809*
3127156	GDNF family receptor α2	GFRA2	NM_001495	−1.8788	0.000212*
2637112	Growth-associated protein 43	GAP43	NM_002045	−1.7562	0.00586
2940145	Neuritin 1	NRN1	NM_016588	−3.6924	0.000316*
3854756	RAB3A, member RAS oncogene family	RAB3A	NM_002866	−1.673	0.02125
3432394	Rabphilin 3A homolog	RPH3A	NM_014954	−4.4059	0.0001086*
3876245	Synaptosomal associated protein, 25 kDa	SNAP25	NM_003081	−1.8249	0.003025*
3876245	Syntaxin 12	STX12	NM_003081	−1.8249	0.003025*
2434139	Synaptic vesicle glycoprotein 2A	SV2A	NM_014849	−2.0692	0.01512
3608638	Synaptic vesicle glycoprotein 2B	SV2B	NM_014848	−2.3551	0.01086
3929325	Synaptojanin 1	SYNJ1	NM_003895	−1.8513	0.01571
3317024	Synaptotagmin VIII	SYT8	NM_138567	1.3243	0.02429
2361154	Synaptotagmin XI	SYT11	NM_152280	−1.5259	0.001213*
3336652	Synaptotagmin XII	SYT12	NM_177963	−1.537	0.0000698*
3371114	Synaptotagmin XIII	SYT13	NM_020826	−2.1484	0.01641
3441941	Synaptobrevin 1	VAMP1	NM_199245	−2.0496	0.0031*

Genes listed are a selection from 5485 annotated, differentially expressed genes with False Discovery Rate (FDR) of ≤ 10%, of which 1196 genes had FDR ≤ 5% (marked *).

[†] Gene whose product has corresponding immunoblotting measurements (Fig. 3).

[a] Measured with a pan-isoform CAMKII antibody.

[b] Antibody also recognizes β isoform of PP2A c subunits.

can drive tau hyperphosphorylation and microtubule disruption[195], thereby exacerbating the disease. Interestingly, there is almost universal upregulation of genes encoding for

inflammatory processes which have been found to be implicated in AD[196,197]. An example of a gene that is thought to play a role in APP biogenesis and processing includes beta–site APP-cleaving enzyme (BACE2), which contributes to a-beta production[12,198]. Examples of downregulated genes and their encoded proteins include pentraxin II (NPTX2) and neuronal pentraxin II receptor (NPTXR), which mediate long-term synaptic plasticity[199], another aspect of cognitive function affected in AD. There is also an abundance of genes whose products are important for synapse formation, synaptic vesicle release, and maintenance that are downregulated (ex: synaptobrevin) and could play a part in what makes AD a synaptic disease[12,200]. Additionally, decreases in expression of genes encoding ionotropic glutamatergic receptors could drive loss of pyramidal neurons, thereby altering cortical excitatory neurotransmission and cognitive processes[12,201]. All these changes in gene expression bring attention to the regulation of the transcription of these genes and specifically how the function of transcription factors could be mediating changes in gene expression in AD.

Transcription Factors

As their name suggests, transcription factors are proteins that regulate transcription. Based on the protein structure which they use to mediate DNA binding or cause dimerization, transcription factors can be grouped into families[202]. Transcription is the process of producing a primary RNA transcript from the DNA of a certain gene and is the first stage of gene expression. This RNA can then undergo a variety of processing, including RNA splicing, after which it is translated into a functional protein. This step plays a crucial role in gene selectivity because it is the deciding step of which

genes will be expressed and which will not and without transcription, the protein product of a gene will not be expressed[203].

Transcriptional activation or repression

Transcription factors regulate this process by binding to DNA sequences in gene regulatory regions. Once bound to DNA, they are able to activate or repress transcription of the target gene[202]. Transcription factors can activate transcription of a gene by binding to activation domains[204], which are rich in glutamine residues, proline residues, or acidic amino acids[205]. In addition to a single transcription factor, the activation domain may also interact with other proteins, including RNA polymerase[206] or multiple transcription factors thereby creating a transcriptional complex that can interact with RNA polymerase[202]. In terms of gene repression, there are multiple ways that a transcription factor may work to prevent transcription. One way to repress transcription is if the transcription factor binds to DNA, thereby blocking the ability of an activating transcription factor to bind[202]. A repressing transcription factor can also bind to an activating transcription factor in solution, creating a complex that can no longer bind DNA[202]. Additionally, once an activating transcription factor is bound to DNA, the repressing transcription factor may bind to an adjacent site on DNA and mask the activation domain, thereby preventing transcription. This phenomenon is called quenching[202]. Finally, there is also the possibility that there is a domain on DNA that a transcriptional repressor can directly bind to and can affect the formation or stability of the transcriptional complex, there by inhibiting trasnciption[202]. Although transcription

factors can be identified as activators or repressors for certain gene targets to alter transcription, there are other ways by which these factors can be regulated.

Transcription factor regulation

In addition to gene expression being regulated through the interplay of activating and repressing transcription factors, it can also be regulated by controlling the synthesis and activity of the transcription factor. Synthesis can be controlled by only having the genes that encode for specific transcription factors in certain cells[202]. Activity can be controlled in response to a signal if a transcription factor changes conformation in response to a certain signal or ligand binding to one of its receptors, thereby allowing its binding to DNA[202]. Activity may also be controlled if the activation of a transcription factor is mediated by an inhibitory protein-protein interaction that must be disrupted for the factor to become active[202]. Moreover, activation of a transcription factor may occur with specific protein modifications such as phosphorylation[202]. Regardless of how the synthesis and activity of a transcription factor is regulated, this ensures that transcription factors are only present in a specific cell type at a given time and are only active in specific cells in response to specific signals[202].

Knowing that transcription factors play a key role in regulating gene expression, it is no surprise that aberrant transcription factor activity can create alterations in gene expression that drive disease pathology. One way this can occur is if there is a problem with the transcription factor itself that can lead to certain genes not being expressed or being overexpressed[202]. This can occur if there is a mutation in the transcription factor so that it does not transcribe the correct genes or if the transcription factor is present,

but is unable to bind the gene promotor because there is a mutation in the DNA sequence that the transcription factor normally binds[202]. In addition to failure of transcription factor function, dysregulation of transcription factor activity can also lead to problems. This can occur if the transcription factor is synthesized or becomes active in the wrong place and/or at the wrong time[202]. An example of how transcription factors can be altered in disease is if they are redox-sensitive, meaning diseases accompanied by oxidative stress have influence on transcription factors that can alter their function and/or activity[207].

Redox-sensitive transcription factors

Although initially the idea that transcription could be regulated by redox reactions was questioned, it quickly became apparent that this was an important aspect of gene regulation with the discovery that transcription factor NF (nuclear factor)-kappa beta[208-211] is activated by the same molecules the trigger the production of certain ROS[212] or by the oxidants themselves. Additionally, its activation is inhibited by antioxidants[212,213]. This opened a whole new level of transcriptional regulation. There are a variety of ways in which oxidants can affect transcription including oxidative activation or inactivation of phosphatases or kinases that act on transcription factors, redox-dependent complex formation between antioxidants with transcription factors, and alterations of proteins that form complexes with transcription factors through thiol modifications[214]. These redox modifications have been thought to influence two major steps in transcription[215]. The first step is the redistribution of the transcription factor to the nucleus. An example of this occurs with the previously mentioned NF-kappa beta. The latent form of NF-kappa

beta resides in the cytoplasm and is bound to a cytoplasmic inhibitor, such as I kappa beta-alpha. Activation of NF-kappa beta involves its dissociation from the cytoplasmic inhibitor, allowing it to translocate into the nucleus where is can initiate transcription of target genes[215]. This activation of NF-kappa beta can be driven by an array of agents and conditions that usually disrupt intracellular redox homeostasis by promoting a prooxidant state[216,217]. Conversely, its activation is prevented by increased levels of antioxidants[218]. The second step of transcription that is affected by redox state is the binding of the transcription factor to the promotor region of genes of interest[215]. Specifically, oxidation of cysteine residues in the protein sequence of the transcription factor can result in inter- or intramolecular disulfide bond formation thereby making the transcription factor conformationally unfavorable to bind to the DNA binding domain on target genes[215]. An example of this occurs with the activator protein 1 (AP-1) transcription factor. This transcription factor complex, which will be discussed more in the next section, is a dimeric factor composed of Fos and/or Jun proteins. The ability of this complex to bind to target genes is affected by redox state. Moreover, studies have shown that oxidizing agents weaken the ability of the complex to bind DNA[219]. This is the result of the complex depending on the reduction of a conserved cysteine in the DNA binding domain of the two proteins to bind the target gene[220]. Another transcription factor that is regulated by redox state is HSF1, a heat shock factor whose DNA binding and activation is regulated by stressors that disrupt thiol homeostasis[215]. This transcription factor is dually regulated by oxidants, meaning that although oxidants drive nuclear translocation of the protein, oxidants also alter its ability to bind to DNA[215]. Although these are not the only transcription factors that are regulated by redox state,

they provide good examples to support the idea that redox state can regulate transcription, thereby regulating gene expression. The transcription factor that is particularly important for this dissertation is AP-1.

AP-1 transcription factors

AP-1 transcription factors are involved in responding to a wide range of physiological and pathophysiological stimuli in the brain including redox state. They are composed of proteins encoded by immediate early genes (IEGs)[221]. These genes are unique in that they do not need new protein synthesis to respond to stimuli and, therefore, are able to respond rapidly to stimuli. These genes connect cytoplasmic biochemical changes resulting from substrates binding to cell surface receptors to an activating response. When these IEG protein products become activated, they are then able to modulate downstream gene expression that controls the response of the cell to a stimulus[222,223]. The IEG products that make up the AP-1 transcription factor include members of the Fos, Jun, Maf, and ATF families that dimerize, and in addition to mediating gene expression, control cellular events including apoptosis, proliferation, and differentiation[224-226]. These AP-1 complexes include Fos/Jun or Fos/Fos hetero- and homomers[8,227]. These protein products contain a basic DNA binding domain residing closer to the N-terminal relative to the hydrophobic amphipathic alpha-helix. This domain contains a leucine zipper, which is a region of four to five leucine residues that are spaced seven amino acids apart and allows heterodimeric complex formation to occur with structurally similar proteins[228]. This basic region in combination with the leucine zipper make the Fos and Jun protein families part of the basic region/leucine

zipper (bZIP) class of proteins[229]. In Fos/Jun AP-1 complexes, the bZIP domains form a fork and two helical basic regions form that can insert into the major grooves of DNA[227](6). This fork is formed due to the two inter-coiled helices that are held together by a leucine zipper, found in the bZIP domain[227].

Members of the *Jun* family include *c-Jun*[230], *JunB*[231,232], and *JunD*[233,234]. Specifically, in their DNA-binding domain and leucine zipper regions, there is 75% amino acid homology within this family[234]. They are differentially induced by agents including growth factors, cAMP, UV light, and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)[233,235]. Additionally, their protein products are able to form complexes with other members of their family[233,236] or with members of the *Fos* family[237]. Members of the *Fos family* include *c-Fos, Delta FosB* (a truncated form for *FosB*), *Fos*-related antigen-1 (*fra*-1), and *Fos*-related antigen-1 (*fra*-2)[237-241]. Although the size of their untranslated regions is variable, these genes have the same number of introns and exons and their amino acid sequence is conserved, particularly in the leucine zipper and basic regions[240]. Unlike the members of the *Jun* family, the *Fos* family protein products are unable to bind DNA alone and require dimerization with a Jun, or possibly another Fos[242], protein in order to activate AP-1 containing promoter regions in target genes[243,244]. Like *Jun* family members, a variety of stimuli can induce *Fos* family member expression including growth factors, UV light, cAMP, neurotransmitters, calcium, metal ions, and phorbol esters[229]. As with the *Jun* family, there is differential expression of *Fos* family members in response to stimuli and the response is kinetically distinct for each member[229].

The distinction in the induction of the members of the *Fos* and *Jun* family members indicate that these AP-1 members must be regulated differently. This level of regulation

may occur at the transcriptional, post-transcriptional, and/or post-translational levels[229]. At the transcriptional level, the particular transcription factor and/or accessory proteins driving transcription of *Fos* and *Jun* genes could alter the expression of these genes relative to other transcription factors[229]. Post-transcriptionally, the stability of the mRNA transcript determines how fast the mRNA will degrade, thereby altering the amount of mRNA available to be translated into functional protein; and alternative splicing can determine the function of the end protein product[229]. An example of alternative splicing is the removal of a 101 amino acid region of the C-terminus of FosB creating the splice variant Delta FosB[238], which will be discussed in the next section. In terms of post-translational regulation, the ability of the protein product to be transported back into the nucleus to perform transcription is one aspect. Another aspect is phosphorylation of the AP-1 proteins; hyperphosphorylation or dephosphorylation can alter the DNA binding and transactivation capability of the proteins.

Despite the level of regulation of AP-1, the availability of the component proteins allows formation of the AP-1 complex and subsequent binding to target genes. As mentioned previously, Fos proteins are unable to bind to DNA alone because they lack a transactivation domain, therefore, they must bind to another protein in order to function as a transcription factor[229]. Once formed, AP-1 acts by binding to TPA response element (TRE) sites in the promotor region of target genes[229]. The palindromic sequence 5' TGAC/GTCA 3' has been identified as the AP-1 consensus binding site[245]. Fos/Jun protein dimers are the preferentially formed AP-1 complex compared to other dimer combinations due to the monomers having opposite charges so any electrostatic destabilization evident with homodimers is removed[246]. The

formation of the Fos/Jun heterodimer creates a fork, due to the coiled coil a-helical dimer region being joined to the basic binding domains[247]. The basic residues are able to recognize AP-1 consensus sequences in no preferred orientation suggesting that this protein fork is flexible in that it may interact with other proteins next to DNA sites or present within the basal transcriptional machinery[229]. AP-1 transcription factors can also interact with other bZIP transcription factors through their leucine zipper regions, which can also influence DNA binding[229].

Once bound to DNA, AP-1 can transcribe thousands of genes that encode proteins with diverse functions. Much information has been gathered about the function of AP-1; however, lots can be said about the individual components as well. This dissertation in particular focuses on one component of AP-1 complexes that has been found to play a pertinent role in a wide range of redox-sensitive diseases, including AD: Delta FosB.

Delta FosB

As mentioned previously , Delta FosB is part of the Fos family of transcription factors and encoded by the *FosB* gene[248]. Delta FosB is a neuronally enriched transcription factor critical for orchestrating gene expression underlying memory, mood, and motivated behaviors[249]. This being said, it has been found to play a role in many pathological states including addiction[250], depression[251], epilepsy[5], and AD[5]. Studies have shown that overexpressing Delta FosB in the nucleus accumbens leads to enhanced sensitivity to the rewarding effects of drugs of abuse[250] and promotes drug seeking behavior[252]. Additionally, Delta FosB overexpression in the nucleus accumbens

of mice has been shown to promote resilience to chronic stress and prevent depressive-like behaviors[251]. Evidence for Delta FosB's role in depression is further enhanced by the knowledge that antidepressants exert their effect by inducing Delta FosB in this brain region[13]. Delta FosB is also induced in the hippocampus by seizures in mouse models and could play a role in the disruptions in cognition seen in epilepsy[5]. Additionally, Delta FosB is upregulated in the hippocampus of rodent models of AD and post-mortem hippocampi collected from patients with AD[4]. These animal models of AD have also shown that levels of Delta FosB in this region are negatively correlated with cognitive function[5]. A major reason that Delta FosB can play a role in these diseased states whereas other *FosB* isoforms do not, is because it features a unique characteristic that other isoforms lack, stability.

Delta FosB's unique stability

Like other Fos family proteins, Delta FosB is induced by a variety of activities including chronic exposure to drugs of abuse[253], seizures[5], stress[254], and learning [255] among other perturbations. As is characteristic of Fos family members, induction of Delta FosB by acute stimuli leads to a rapid and transient increase in Delta FosB levels[13]; however, these *FosB* protein products differ when it comes to chronic stimulation (Figure 10). Compared to other *FosB* gene isoforms, Delta FosB's unique stability allows it to accumulate with chronic stimulation and remain elevated in brain regions, such as the hippocampus for weeks after the final insult, whereas other isoforms show resilience[256-258]. This stability is due to two reasons. The first is that alternative splicing of *FosB* removes two degron domains that are near the C-terminus

of full-length FosB and all other Fos family proteins, thereby removing the targeting of Delta FosB for rapid degredation[259]. One of these degron domains targets Delta FosB for ubiquitination and degradation while the second domain induces degradation through proteasome-independent mechanism[259]. The second reason is that Delta FosB

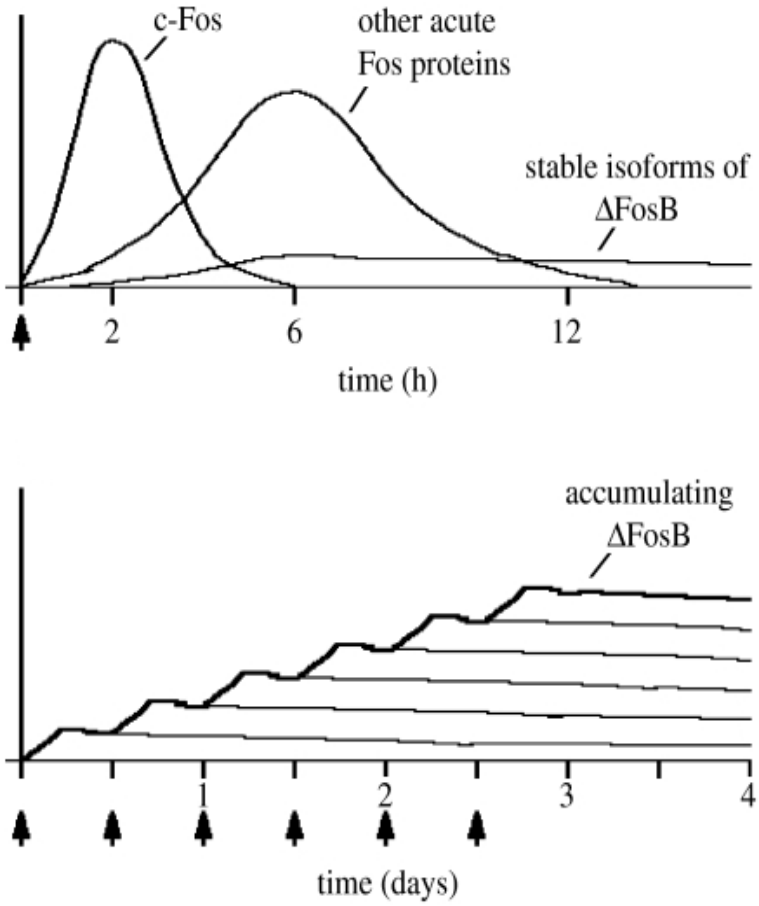

Figure 10 | Induction of Delta FosB relative to other FosB Isoforms. (Top) Audiogram displaying gradual induction of Delta FosB relative to the transient and rapid accumulation of other Fos family members in response to an acute stimulus. (Bottom) Audiogram showing increasing accumulation of Delta FosB with chronic stimulation. Reprinted from *Philosophical Transactions of the Royal Society*[13], copyright 2008.

phosphorylation by protein kinases (i.e. Casein kinase 2) at its N terminus enhances protein stability[260,261] (Figure 11). This unique stability allows Delta FosB to mediate

long-term changes in gene expression seen in diseased states; however, the ability of Delta FosB to regulate gene expression is dependent on its ability to form the AP-1

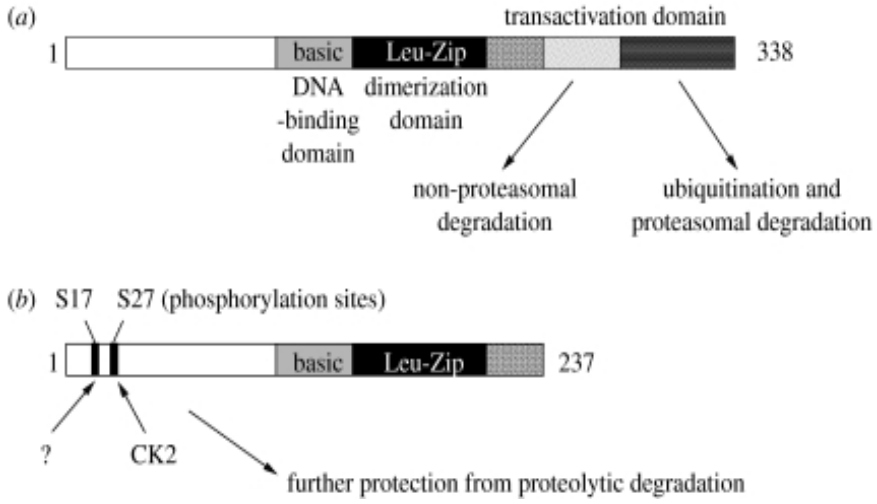

Figure 11 | Illustration of the unique stability of Delta FosB. Full-length FosB (338 amino acids) (top) and Delta FosB (237 amino acids) both encoded by the *FosB* gene. Lack of two degron domains near to the C terminus, one targeting the protein for ubiquitination and degradation, and the other for proteasome-independent degradation; in Delta FosB increases its stability relative to other Fos proteins. This stability is further enhanced by a phosphorylation near the N terminus of Delta FosB. Reprinted from *Philosophical Transactions of the Royal Society*[13], copyright 2008.

complex and bind DNA.

Delta FosB complex formation and DNA binding

As an alternative splice variant of *FosB*, Delta FosB contains a disordered N-terminal domain (residues Met^{1}-Glu^{156}) and a bZIP domain composed of a basic region (Lys^{157}-Arg^{177}) containing a leucine zipper (Thr^{180}-His^{218}) that forms a coiled-coil interaction with a dimerization partner and a DNA-binding motif[254]. The lack of the 101-residue transactivation domain found in full-length FosB (Val^{238}-Leu^{338}) renders Delta FosB unable to bind to DNA alone. Thus, Delta FosB must bind to a partner protein, either a different protein or another Delta FosB, thereby forming an activator-protein-1

(AP-1) complex. Moreover, to calrify, Delta FosB monomers are unable to bind, and Delta FosB must heteromize with either itself or another Delta FosB protein to bidn to DNA. Complex formation occurs with dimerization of the leucine zippers of Delta FosB

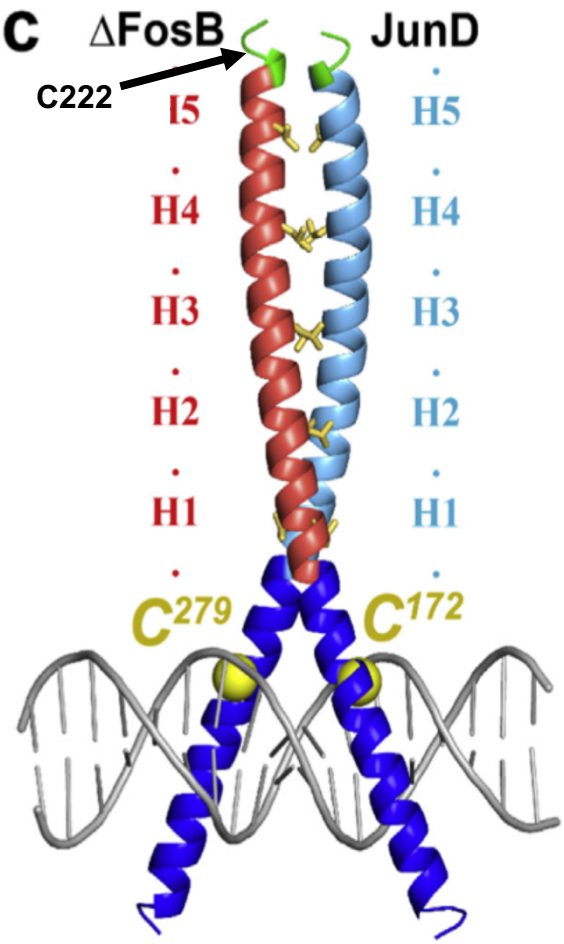

Figure 12 | Canonical arrangement of /Delta FosB/JunD AP-1 complex. DNA-bound Delta FosB/JunD heterodimer. Heptad repeats are indicated by the letter "H". The Delta FosB leucine zipper is shown in red whereas it is light blue in JunD. The DNA binding regions of the proteins are shown in dark blue and the C-terminal residues are green. Yellow depicts the leucine side chains at the d-position. Reprinted from *Current research in structural biology*[8], copyright 2020.

and its partner protein, clamping the basic regions on either side of the DNA strand into the major groove like a pair of forceps (Figure 12)[8]. This complex binds DNA via AP-1 consensus sequences (TGA C/G TCA) in the promoter region of genes of interest [247]. Although JunD is presumed to be the primary binding partner *in vivo* [257,262], *in vitro*

evidence suggests Delta FosB homodimers may also allow Delta FosB alone to bind DNA [242,263]. Looking at a Delta FosB/JunD complex, a series of heptad repeats creates a long helix making up each bZIP domain[8]. Specific leucine residues within these repeats use coiled coil geometry to form a leucine zipper[8]. A hydrophobic core aligning the two helices in a parallel and symmetric manner is then formed by the hydrophobic portions of the leucine side chains[8]. This arrangement allows the heptad of one protein to interact with the facing helix's heptads[8]. Whereas there is significant interaction in the bZIP regions of Fos and Jun proteins, DNA-binding sites found in the basic regions do not interact, thus leaving it open to bind the DNA of target genes[8]. Although this canonical arrangement is the most studied, recent evidence reveals that the Delta FosB bZIP domain can form an array of different molecular arrangements including dimers and tetramers with Jun proteins or with itself[8,227]. In addition to this level of complexity, evidence suggests that the ability of this complex to bind to DNA is redox-sensitive[220,227].

Delta FosB's *in vitro* redox sensitivity

As mentioned previously, Fos and Jun family proteins that interact to form AP-1 transcription factors are redox-sensitive and Delta FosB is no exception[220, 258]. Early on it was discovered that Fos and Jun cysteine residues are important for DNA binding, and that this complex is unable to bind DNA under oxidizing conditions *in vitro*[220]. Later studies delving into the mechanism of this redox sensitivity revealed that in a FosB/JunD complex, only JunD is in a conformation that allows DNA binding[258]. FosB, specifically, must undergo a redox-sensitive conformational change to bind DNA[258]. This

change is under control of a ‘redox switch’ that is centered on a redox-sensitive intermolecular disulfide bond, that occurs via oxidation of cysteine residues found in Delta FosB and its partner protein [220]. FosB has a total of four cysteine residues: two in the disordered region C15 and C44, one near the DNA binding domain C172, and the fourth near the top of the leucine zipper region C222. Whereas C222 may act in a “zip-lock” fashion to stabilize the leucine zipper interaction in a FosB complex (unpublished) due to its proximity to the DNA binding domain, C172S is the most probable candidate for mediating FosB DNA binding. Further investigation confirms this and identifies C172 as the “redox-switch” that mediates the conformational change of FosB that is needed to bind DNA[258]. This conformational change is necessary because FosB is swiveled preventing its ability to interact with AP-1 sites on target genes[258]. This state is stabilized by a conserved intermolecular disulfide bond between C172 on FosB and C285 on JunD[258]. When the redox switch is ‘on’, reduction of C172S removes the disulfide bridge stabilizing the improper conformation of FosB and the FosB bZIP undergoes a coil-to-helix transition that is localized to that area and so that the interrupted, kinked helix becomes a continuous helix that extends the leucine zipper helix so that FosB can insert into the major groove of DNA (Figure 13) [258]. When the switch is ‘off’, under oxidizing conditions, the disulfide bridge connecting FosB/JunD at C172 remains and the structural transition is prevented, thus FosB is locked into a kinked confirmation that is unable to bind DNA (Figure 13) [258]. This kinked position is a problem because it sterically hinders the Jun consensus motif from binding to its AP-1

half-site[258]. This evidence of redox sensitivity was found using purified protein derived from insect cells and crystalline studies, however, there is no evidence as to whether

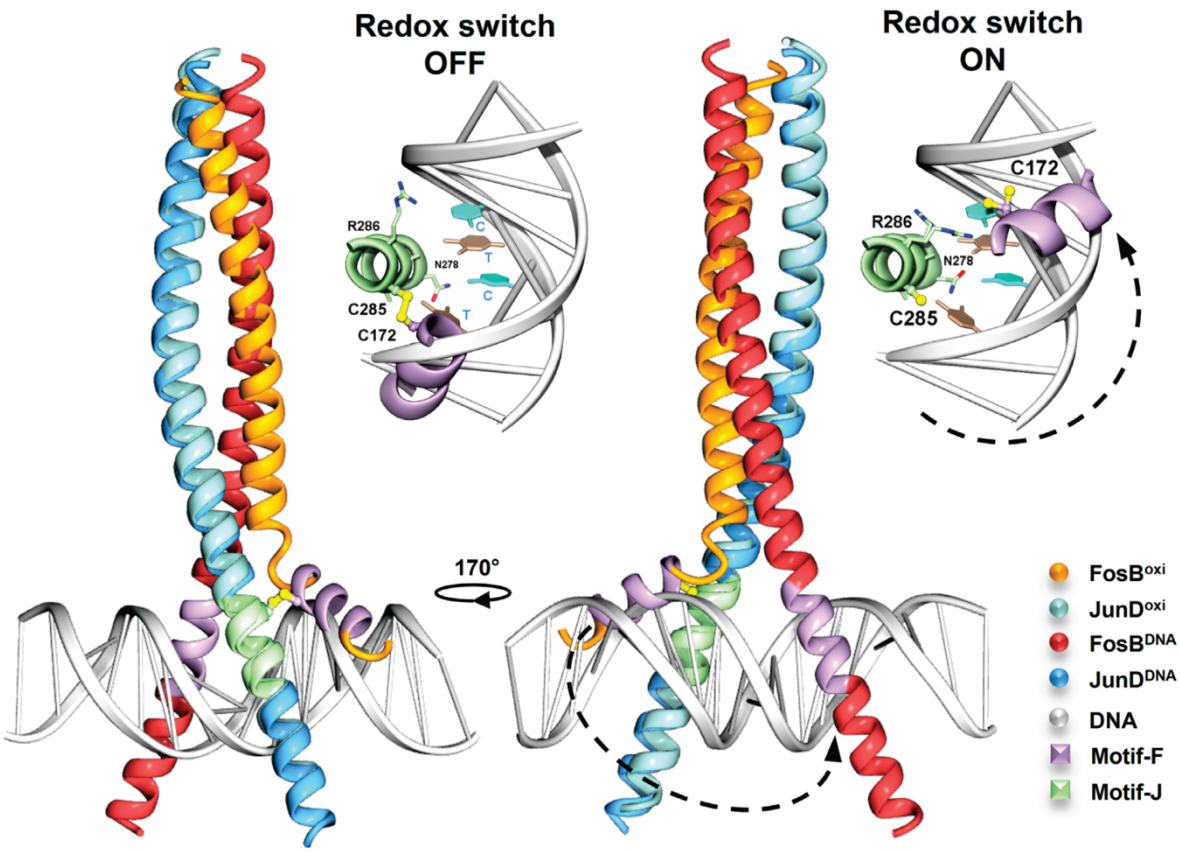

Figure 13 | Structural mechanism of Delta FosB redox switch. Fos/Jun^{oxi} is superimposed onto Fos/Jun-DNA by matching the JunD molecules. Left image is rotated 170 degrees to get the right image, and the dashed lines represent conformational change controlling the redox switch. Zoom-in view of DNA-binding consensus motifs of FosB (Motif-F) and JunD (Motif-J) show the redox switch in the on verses off states. Reprinted from *Nucleic acids research*[258], copyright 2017.

this effect is conserved *in vivo*. Understanding the *in vivo* mechanism of redox sensitivity of Delta FosB could shed light on how pathological states of oxidative stress could drive changes in gene expression. Thus, it is necessary to investigate the types of Delta FosB-target genes that are prevalent under basal conditions.

Delta FosB-target gene regulation

Regardless of the arrangement of the Delta FosB-containing AP-1 complex, once formed and bound to DNA Delta FosB exerts transcriptional regulation. Both *in vitro* and *in vivo*, Delta FosB can behave as a transcriptional activator or repressor. At long-term, higher levels Delta FosB behaves predominantly as an activator. Conversely, it mainly acts as a repressor at short-term lower expression levels[264]. Interestingly, some genes that are down-regulated by chronic Delta FosB expression may be initially upregulated by low levels of Delta FosB[265]. This bidirectional transcriptional regulation occurs in different parts of the brain and in different cells types[265] then translates to different behavioral patterns depending on the target gene and the direction or regulation. Examples of Delta FosB-target genes include glutamate receptor-2 (GluR2), N-methyl-d-aspartate receptor 1 ($NMDAR_1$), dynorphin, cyclin-dependent kinase 5 (cdk5), and NF-$\kappa\beta$. GluR2 is an AMPAR glutamate receptor subunit whose expression is driven by Delta FosB overexpression in the nucleus accumbens[266]. Increased expression of GluR2 elevates the rewarding effects of cocaine that is seen with chronic Delta FosB overexpression[250]. NMDA is another glutamate receptor that is increased in the frontal cortex and nucleus accumbens by Delta FosB overespression[265]. It is thought that Delta FosB binding to AP-1 sites in the $NMDAR_1$ may play a role in the induction of this receptor in response to chronic seizures[267]. CDK5 is another protein whose expression is increased with increasing levels of Delta FosB in the hippocampus, nucleus accumbens, and dorsal striatum. It is linked to increases in dendritic spine density[268], and changes in the phosphorylation state of various synaptic proteins[269] seen in the nucleus accumbens. It also accompanies chronic cocaine administration[270]. NF-kappa

beta is also induced in the nucleus accumbens by Delta FosB overexpression and chronic cocaine and is thought to be implicated in the neurotoxic effects of methamphetamine in the dorsal striatum[264]. An example of a protein whose expression is repressed by increased Delta FosB is dynorphin[265]. Dynorphin is an opioid peptide whose expression is increased or decreased in response to certain drugs of abuse and treatment conditions used[265]. On one hand Delta FosB induction has been found to attenuate dynorphin levels in the striatum; however, in other contexts it can also induce dynorphin expression. Other targets that are regulated by Delta FosB include, but are not limited to, Ca^{2+}/calmodulin-dependent protein kinase 2α (CaMKIIα), heat shock-protein 40, adenosine 2A receptor, and kruppel-like factor 5[264]. Despite the direction and level that these target genes are altered by Delta FosB, the resulting changes in gene expression led to different behavioral outcomes including those affecting learning and memory[255].

Delta FosB's role in learning and memory

Hippocampal synaptic plasticity is imperative for memory consolidation and Delta FosB has been found to be a major player in this process[271,272]. Not only is Delta FosB critical for proper hippocampal formation and neurogenesis[273], but it also regulates learning and memory[255]. Delta FosB is induced in the hippocampus, specifically in the CA1 and dentate gyrus sub-regions, by spatial learning and environmental exposure, and there seems to be a "sweet spot" level that is necessary for learning and memory. In other words, transcriptionally silencing Delta FosB using a dominant negative form of binding partner JunD, Delta JunD, impairs learning and memory, but so does

overexpressing Delta FosB[255]. Additionally, too much Delta FosB function increases immature dendritic spines on CA1 pyramidal cells, and too little reduces the number of immature and mature spine types[255]. This supports the conclusion that Delta FosB could be exerting behavioral effects through alterations of hippocampal synaptic function[255]. It is thought that groups of cells encoding an engram for a particular association express Delta FosB and during exposure to a novel stimulus or during learning, expression of Delta FosB in these cells mediates downstream signaling that alters memory consolidation by preparing the cell for the associated physiological and synaptic morphological changes[255]. This notion is supported by the fact that in other brain regions, like the nucleus accumbens, Delta FosB is known to directly regulate the expression of genes pertinent to learning and hippocampal synaptic plasticity[274], such as CaMKII, GluA receptors[249], and CDK5[275]; which were mentioned previously. The genes have already been studied in the hippocampus and have been found to play roles in learning and memory. CaMKII's activity is necessary for long-term potentiation induction[276], structural remodeling of synapses[277], and AMPA receptor trafficking and function[278]. In the hippocampus it is known to be a molecular substrate of synaptic plasticity and memory[279]. As mentioned before, GluA subunits make up AMPA receptors which are involved in learning and memory. CDK5 regulates glutamatergic neurotransmission[280], neurodevelopment and neurogenesis[281,282], and synaptic plasticity[282-284]. Although Delta FosB is necessary for proper learning and memory, it is hypothesized that too much of this could disrupt the signal to noise ratio involved with activating *FosB* gene products in hippocampal cells. Moreover, it is thought that these gene products accumulate in only a few hippocampal cells that encode a memory, but

non-specific overexpression to increase Delta FosB throughout the entire hippocampus can mask the effects of Delta FosB on the specific cells encoding the engram and losing their signal in the “noise” created by Delta FosB overexpression[255]. Furthermore, studies have shown that Delta FosB overexpression or silencing alter the excitability of CA1 pyramidal neurons, which in turn would alter these neurons ability to allow learning and memory formation[285]. Since Delta FosB plays a role in learning and memory, it makes sense that it is receiving attention as being a potential therapeutic target in diseases associated with impairments in cognition, like AD.

Delta FosB’s role in AD

Knowing that Delta FosB plays a role in learning and memory, it is not surprising that there is differential expression of Delta FosB in mouse models and patients with AD compared with wild-type and healthy controls (Figure 14). Specifically, Delta FosB is upregulated early in the AD continuum and is apparent as early the MCI stage and persists into severe AD[4]. This is also apparent in animal models of AD[4]. Interestingly, there is a negative correlation between Delta FosB levels and Mini-Mental State Exam (MMSE) scores in MCI, but not AD[4]. This is likely due to the exacerbated level of deficits found throughout the brain and not just the hippocampus, which the MMSE primarily tests, in AD relative to MCI[4]. This suggests that AD patients should receive therapeutic interventions as early as possible to maximize their effectiveness. As a result, Delta FosB can contribute to AD pathology, including seizure activity seen early on in AD and the alterations in gene expression evident in the disease.

Delta FosB and AD-associated epileptiform activity

Cognitive decline because of neural and network alterations is a key feature of AD. Network dysfunction in AD can manifest as seizures and epileptiform activity, which is thought to drive cognitive dysfunction in the disease[5]. Interestingly, there is a 5- to 10-fold increase in seizures for patients with AD[286-288], although it is likely this number is

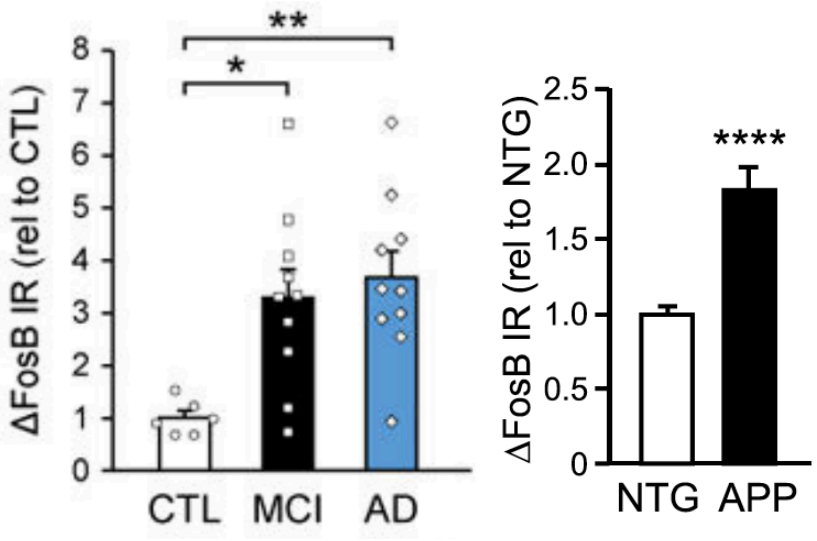

Figure 14 | Increased hippocampal Delta FosB be in patients with and models of AD. Left: Levels of Delta FosB in posdt-mortem hippocampal samples collected with patients who served as healthy controls (CTL), who had mild cognitive impairment (MCI), or who had AD. Right: Levels of Delta FosB in nontransgenic (NTG) control mice compared to the APP mouse model of AD. Reprinted from *Nature medicine*[4], copyright 2017; and *Cell Reports*[5], copyright 2017.

underestimated due to the incidence of non-convulsive seizures and subclinical epileptiform activity seen early on in AD[289]. This is supported by a study that showed that 42.4% of patients with AD showed subclinical epileptiform activity, despite no clinical history of seizures. This seizure incidence was further associated with increased rates of cognitive decline[290]. Seizures have even been found in mouse models of AD that exhibit cognitive dysfunction[291-294]. The mechanism connecting increased seizure incidence with cognitive decline in AD[291] remains unresolved, and research into this is

what has led to Delta FosB being a potential answer. Additionally, studies have shown that Delta FosB is increased in the hippocampus of mouse models of AD relative to non-transgenic wild-type controls, and Delta FosB levels return to that of the control mice when AD mice are treated with antiepileptic drugs[5]. In these AD mice, Delta FosB expression inversely correlates with performance in tasks measuring cognitive function[5]. Due to the unusually long half-life of Delta FosB, induction of Delta FosB by even sporadic seizures would be enough to maintain increased levels of Delta FosB; thereby allowing long-term modulation of gene expression that can lead to cognitive deficits seen in AD mice both during periods of epileptiform activity and during seizure free periods[5].

AD-related Delta FosB gene targets

As mentioned previously, Delta FosB has an abundance of hippocampal gene targets, resulting in regulation of the expression of proteins with diverse functions. Interestingly, there is a shift in these gene targets in AD mouse models compared to wild-type littermates[295]; in AD mice the amount of target genes and the amount of over-represented gene ontology categories are increased[295]. 1,197 total genes have been identified as putative Delta FosB gene targets in the hippocampus of AD and/or wild type mice[295]. 524 of these genes are targets in wild type mice whereas 725 are targets in AD mice[295]. Surprisingly, one study showed that only 53 Delta FosB-target genes were shared between AD and wild type mice[295], leaving 427 genes to be unique to wild type mice and 637 to AD mice[295]. Those shared between wild type and AD mice were responsible for normal cellular functions including cell-cell adhesion, cell development,

differentiation, and organelle localization[295]. Although both AD and wild type mice show Delta FosB targets genes involved in neuron development, neurogenesis, and dendrite development, these three gene ontology categories ware less enriched in the AD mice[295]. In wild type mice, genes involved in Wnt signaling, cellular component assembly, and synapse maturation were significantly enriched compared to AD mice, whereas genes involved in the regulation of membrane potential, ion transport, and glutamate receptor signaling were enriched in AD mice[295]. The increase in regulation of genes involved in neuronal excitability as well as the number of overrepresented genes involved in epilepsy-related pathway, fits in well with the notion that epileptiform activity plays a role in the pathology of AD[4,289-291,295]. Overall, when comparing the gene ontology networks of wild type and AD mice, AD mice have a diversified and expanded network, which is consistent with the increased number of target genes[295], that shifts target genes from being focused on neurodevelopment processes like in wild type mice towards those involved in regulation of excitability[295]. Although identification of Delta FosB-target genes in wild type versus AD mice has made huge progress towards deciphering Delta FosB's role in AD, specifically in epileptiform activity seen in the disease, the next step is to determine how regulation of these genes leads to an AD phenotype. This is no small feat given the breadth of Delta FosB gene targets involved in AD, however, investigation into some of these targets c-Fos and calbindin have already shed light on this regulation.

Investigation into Delta FosB's ability to alter cognitive function was supported by the discovery that it regulates expression of c-Fos, a protein critical for synaptic plasticity and hippocampal memory[296-298], through epigenetic mechanisms involving

histone modifications[299]. Specifically, in AD mice Delta FosB binds to the c-Fos promoter and promotes deacetylation of histone H4 through histone deacetylase 1 (HDAC1) recruitment[5,299], thereby decreasing the amount of c-Fos mRNA transcribed in the hippocamus[5]. Moreover, increased Delta FosB expression is negatively correlated with c-Fos expression in AD mouse models[5]. Further investigation revealed that in a mouse model of AD that exhibits seizures, Delta FosB is induced in the hippocampus and epigenetically suppresses c-Fos expression[5]. Additionally, blocking Delta FosB activity or preventing histone deacetylation, improves c-Fos induction and improves hippocampal-dependent memory in AD mice[5].

Another Delta FosB gene target that has been implicated in AD is calbindin-D28k. Expression of this protein in the dentate gyrus of the hippocampus has been found to be indicative of cognitive function in both patients and mouse models of AD[300-304]. Moreover, decreasing or removing expression of this protein in animals has led to impaired spatial memory and synaptic plasticity[305-309]. It makes sense that animal models of AD display reduced expression of calbindin compared to healthy littermates, and its expression was inversely correlated with seizure frequency[4]. Interestingly, this downregulation of expression was seen even with infrequent seizure activity[4] indicating the involvement of a long-lasting, activity-dependent mechanism, which led to the focus on Delta FosB. Delta FosB's role in this mechanism was confirmed when it was discovered that Delta FosB is not only induced in the hippocampus by seizures[4], but these higher levels are inversely correlated with calbindin expression[4]. Furthermore, Delta FosB was found to be able to bind to the calbindin gene promoter, *Calb1*, and enrichment of Delta FosB at this site is greater in AD mice compared to wild type

littermates[4]. Like Delta FosB's regulation of c-Fos, Delta FosB promotes hypoacetylation of histone H4 of calbindin through recruitment of HDAC1, more so in AD mice[4]. Long-term hypoacetylation has been found to trigger methylation, which further suppresses gene expression[299,310], and AD mice show evidence of hypermethylation of *Calb1*[4]. This upregulation of Delta FosB in AD mice as well as in patients with AD is necessary and sufficient to suppress calbindin expression, thus negatively impacting cognition[4].

As indicated in this introduction, Delta FosB's *in vitro* redox sensitivity, its ability to regulate gene expression, and its role in AD provide evidence that Delta FosB could be a potential target for AD and other diseases of oxidative stress; however, more information on the *in vivo* effects is needed. This dissertation aims to address this gap by looking whether Delta FosB is redox-sensitive *in vivo*, and how this redox sensitivity can alter gene expression associated with AD.

HYPOTHESIS AND SPECIFIC AIMS

Central hypothesis

Neuronal Delta FosB is directly affected by redox state, which leads to altered gene expression relevant to diseases like Alzheimer's Disease

Specific Aims

Aim 1. Redox regulation of Delta FosB oligomerization:

1A: Redox-dependent changes in endogenous Delta FosB disulfide bond formation

1B: Redox-dependent changes in overexpressed Delta FosB and binding partners in cultured cells

Aim 2. Delta FosB DNA binding and target gene transactivation and expression in altered redox states:

2A: Redox modification of Delta FosB DNA binding

2B: Effect of redox state on transcriptional regulation of Delta FosB-target genes

CHAPTER 2: MATERIALS AND METHODS

Animals

All experiments were approved by the Institutional Animal Care and Use Committee at Michigan State University in accordance with AAALAC. Male C57Bl/6J mice (3–5/cage, 7–8-week-old and 25-30g upon arrival from Jackson Labs) were allowed at least 5d to acclimate to the facility prior to use and maintained at a constant temperature (22°C) and humidity (50-55%) on a 12-hour light/dark cycle. The *hemagglutinin (HA)-tagged FosB* mouse strain ($FosB^{H/H}$) was a generous gift from the laboratory of Dr. Eric Nestler at the Icahn School of Medicine at Mount Sinai. All mice were group housed with ad libitum food and water.

Immunoprecipitation

Mouse brain was extracted from the HA-mice as indicated and bilateral 12G dorsal hippocampal punches, centered on dentate gyrus, were collected in a fresh Eppendorf tube on ice. Tissue was harvested with ice cold 150uL buffer (50mM Tris-HCl pH 7.5, 150M NaCl, 0.3% Tween) supplemented with protease inhibitor cocktail and phosphatase inhibitors (Sigma; St. Louis. MO); and treated with 1mM desired redox reagent (Dithiothreitol (DTT), N-ethylmaleimide (NEM), or Diamide (Sigma; St. Louis, MO)). Lysates were centrifuged at 4°C at 10,000 rcf for 10 minutes. 15uL of supernatant was added to 5x Laemmli buffer as the input Western blot sample. Remaining supernatant was separated from pellet and brought up to 800uL with buffer in fresh Eppendorf tube. After 30uL of 5/6 Protein G Sepharose, Fast Flow beads (Sigma; St. Louis, MO) were washed 3x with 0.1% PBST, resuspended in 0.1% PBST and flipped in

a vial rotator overnight with 3.5uL anti-HA (Abcam ab 18181; Cambridge, MA) at 4°C in a fresh Eppendorf tube; the antibody-bound beads were washed with 0.1% PBST and the remaining supernatant from the mouse brain lysate was added. The Eppendorf tube was flipped overnight in a vial rotator at 4°C. 12 hours later, the Eppendorf tube containing the bead/lysate mix was spun down (1000rcf), and 50uL of the supernatant was mixed with 20uL 5x Laemmli buffer for the flowthrough Western blot sample. Once the lysate/bead/antibody sample was washed 3x with 0.1% PBST, and the remaining supernatant was removed, immunocomplexes were eluted from the beads using 18uL of 1.5x Laemmli buffer (IP western blot sample). Before running, Western samples were boiled at 95°C for 1.5min. This procedure was repeated, with the exception that the Western blot samples were prepared using non-reducing Laemmli buffer. This IP protocol resulted in the specific and effective precipitation of virtually all the Delta FosB in the lysate. The samples were run as indicated in the Western blot methods and probed for FosB (1:500 FosB 5G4 2251s, antirabbit, Cell Signaling; Danvers, MA).

Co-Immunoprecipitation

The same protocol was used as in the immunoprecipitation methods; however, Delta FosB binding partners were probed for during the Western blot (i.e. 1:500 JunD 5000s, antirabbit, Cell Signaling; Danvers, MA).

Mammalian cell lines and DNA constructs

Neuro2a cells (N2a; American Type Culture Collection) were cultured in EMEM (ATCC; Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum

(ATCC; Manassas, VA) and 5% Penstrep (Sigma; St. Louis) in a 5% CO_2 humidified atmosphere at 37°C.

For the transient transfections with DNA, N2a cells were seeded onto 12-well plates to reach 90-100% confluence the next day and were then transfected with the desired pcDNA3.1 plasmid using Lipofectamine (Invitrogen; Waltham, MA). A total of 1000ng of WT or mutant Delta FosB was solely transfected, and 500ng of WT or mutant Delta FosB with 1000ng JunD were co-transfected per well.

Delta FosB, FosB, and JunD cDNAs were obtained from our own pTetop-constructs[257], and subcloned into a pcDNA3.1 vector (Invitrogen; Carlsbad. CA). These pcDNA3.1-Delta FosB/FosB constructs were used for expression in mammalian cells and as a template for site-directed mutagenesis used to create mutant Delta FosB. Recombinant HSV-Delta FosB was prepared as described previously[311], and the preparation had a titer of $\sim 1 \times 10^8$pfu/ml.

Site-directed mutagenesis

Mutation of Cys172 and/or Cys222 to Ser was accomplished using a Quick-Change Site-Directed Mutagenesis kit (Agilent; Santa Clara, CA) and following the instructions from the manufacturer. To introduce the Cys mutations in the mouse Delta FosB protein, the following mutagenesis primers were used:

Cys172 to Ser: 5'GACGGTTCCT*G**G***ACTTAGCTGC3'(forward primer)

5'GCAGCTAAG*T**C***CAGGAACCGTC3'(reverse primer)

Cys222 to Ser: 5'AGGGGATCTT*G**C**A*GCCCGGTTTG3'(forward primer)

5'CAAACCGGGC*T**G**C*AAGATCCCCT3' (reverse primer)

The mutated bases are in bold, and the Cys codons are italicized.

Intracellular protein extraction

Approximately 48 hours post-transfection cells were washed with 1 X PBS and detached from the plate using 0.3% Trypsin EDTA (Sigma; St. Louis, MO). Complete media was added to quench the trypsin and cells from each well were pipetted into a fresh Eppendorf tube. After cells were pelleted by centrifuging at 1000 rcf for 15 minutes, the supernatant was removed and 100uL RIPA buffer (10 mM Tris base, 150 mM sodium chloride, 1 mM EDTA, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% sodium deoxycholate, pH 7.4) supplemented with protease inhibitors and phosphatase inhibitor cocktail with or without redox reagent (1mM DTT, NEM, or Diamide) was added. Lysate was sonicated at 30% amplitude 10x (1s on and 1s off). 50uL of lysate was added to new Eppendorf tube with 15uL reducing or non-reducing 5x Laemmli buffer. Western blot samples were vortexed then boiled at 95°C for 1.5minutes. Western blot was performed (see methods).

Chemicals and cell culture treatment

N2a cells were plated into 12-well plates. 24 hours later (when cells were ~95% confluent) cells were transiently transfected with desired pcDNA3.1 plasmid containing WT or mutant Delta FosB with or without JunD using Lipofectamine. A total of 1000ng of

WT or mutant Delta FosB or JunD was solely transfected, and 500ng of WT or mutant Delta FosB and 1000ng JunD were co-transfected per well. 24 hrs post-transfection, cells were treated with Diamide or H_2O_2 containing media or Delta FosB-targeting compound-containing media for 2 hours. Oxidant- or compound-containing media was removed, and new oxidant- or compound-containing media was added. Protein was extracted using the intracellular protein extraction and Western blots were run (see methods).

Luciferase Assays

Cells were plated into 12-well plates. Twenty-four hours later (when cells were ~95% confluent) cells were transiently co-transfected with a combination of 4 × AP-1/RSV-Luc plasmid and pcDNA3.1 plasmid (Life Technologies; Carlsbad, CA) containing WT or mutant ΔFosB with or without JunD. A total of 1000ng of each DNA plasmid was transfected per well. Approximately 48 hours post transfection, cells were washed twice with 1ml PBS and whole-cell lysates were prepared using 150µl lysis buffer provided with ONE-Glo Luciferase Assay System (Promega; Madison, WI). 50µl of the lysate were removed for Western blot analysis. The remaining lysates were incubated on ice for 5 minutes and the luciferase activity (luminescence) present in each sample was assayed using the substrates and protocol included in the ONE-Glo Luciferase Assay System. The luminescence of each sample was detected in triplicate using luminometer (TD-20/20 set at 2s premeasurement delay and a 1s measurement period). Luminescence was normalized to total WT Delta FosB expression as assessed by Western blot.

Viral targeting

Following experimental testing, mice were perfused with 4% paraformaldehyde-PBS and brains were cryo-preserved in 30% sucrose-PBS. Brains were sectioned (30 μm) and bilateral hippocampal targeting was confirmed by GFP expression. The representative hippocampal targeting and viral expression was generated using standard immunohistochemistry techniques to label GFP (1:1000 GFP ab5450, antigoat, Abcam; Cambridge, MA). Mice with GFP expression outside the hippocampus were not included in analyses.

Surgery and viral vectors

Stereotaxic surgeries were completed as previously described[312]. Briefly, mice were anesthetized (100mg/kg ketamine, 10mg/kg xylazine) and viral vectors were bilaterally injected into the dorsal hippocampus of adult male mice. 30G needles (Hamilton) were bilaterally placed at the following coordinates for dorsal hippocampus: 10° angle, −2.2mm anteroposterior (AP), ±2.5mm mediolateral, virus was infused (0.6μl) separately (0.3μl/infusion) over 3-minute periods at two sites: −2.1mm dorsoventral (DV) and −1.9mm DV. After infusion, the needles remained at the injection site for 5 minutes to allow for diffusion of the viral particles. Previously validated viral vectors included the following: herpes simplex virus (HSV) expressing HSV2-CMV-GFP, HSV2-CMV-Delta FosB-GFP, or HSV2-CMV-JunD-GFP[313,314]. Newly created HSV2-CMV-mutant Delta FosB-GFP viruses were validated using immunohistochemistry. HSV viral vectors came from Massachusetts General Hospital (Dr. Rachael Neve, Gene Delivery Technology Core: https://researchcores.partners.org/mvvc/about).

Western blotting

Tissue punches and/or cell lysates were processed as above. After addition of Laemmli buffer, proteins were separated on 4-15% polyacrylamide gradient gels (Criterion System, Bio-Rad; Hercules, CA), and transferred to PVDF membranes. Blots were probed using a polyclonal FosB antibody (1:500 FosB 5G4 2251s, antirabbit, Cell Signaling; Danvers, MA) and enhanced chemiluminescent detection (Super Signal West Dura, Fisher Scientific; Waltham, MA). Films were scanned and band intensity was quantified using ImageJ software. The resulting histogram data were averaged for all samples of each group, and the lowest raw pixel intensity value was subtracted from all values for each graph, thus removing the background (which varied due to exposure time). For analysis of individual isoforms, a box was drawn around each of the pertinent bands (50kDa for FosB; the 35-37kDa doublet for Delta FosB) and average pixel intensity was calculated. A background value for each blot was generated from a region containing no specific bands and subtracted from all band values. Finally, membranes were stained for total protein with Swift Membrane Stain (VWR; St. Louis, MO) and all band values were normalized to total protein for each lane.

Immunohistochemistry

Adult male mice were terminally anesthetized (15% chloral hydrate) and transcardially perfused with PBS followed by 4% formalin. Brains were then postfixed overnight in formalin at 4°C and cryoprotected in 30% sucrose at 4°C until isotonic. Brains were sliced in 35μm sections on a freezing microtome and immunohistochemistry for Delta FosB expression was performed essentially as

described previously[315]. Briefly, slices were blocked for 1hour in 0.3% Triton X-100 and 3% normal goat serum at room temperature then incubated overnight at 4°C in 1% normal goat serum, 0.3% Triton X-100, and primary antibody. Sections were washed, placed for 1hour in secondary, and slices were mounted under glass coverslips for visualization on a fluorescent microscope. The images captured in both the red (FosB) and green (GFP) channels. Fluorescent images were visualized on an Olympus FluoView 1000 filter-based laser scanning confocal microscope. Immunofluorescence was performed using the following primary antibodies: Anti-FosB (1:500 FosB 5G4 2251s, antirabbit, Cell Signaling; Danvers, MA) and Anti-GFP (1:1000 GFP ab5450, antigoat, Abcam; Cambridge, MA). The following corresponding secondary antibodies were then used: Donkey anti-rabbit Cy3 (1:200, 711-165-152, Jackson Immunoresearch; West Grove, PA), Donkey anti-goat Alexa Fluor 488 (1:200, 705-545-147 Jackson Immunoresearch; West Grove, PA).

Oligonucleotides

The 19-mer *cdk5* oligonucleotide (‘*cdk5* oligo’) contains the forward and reverse strands of 5′-CGTCGGTGACTCAAAACAC-3′ (AP1 site underscored) from the AP1 site in the cyclin-dependent kinase 5 promoter. The non-specific DNA oligomer SCR (5’-GTATGCGATACGTCTTTCG-3’) contains the same nucleotides as *cdk5* but is scrambled. The oligos were made by annealing equivalent molar quantities of the complementary strands labelled with TAMRA at both 5′-ends (Sigma Aldrich) and heating them to 95 °C for 2.5 min, followed by slow cooling to room temperature

(roughly 1 min/°C) and storage at -20°C as 50 μM stocks in annealing buffer (10 mM Tris pH 8, 50 mM NaCl).

Protein expression and purification

N-terminally hexaHis-tagged mouse Delta FosB (MGHHHHHHAG followed by residues F^{2}–E^{237}, a splice form of FosB, accession number P13346) and mutants as well as N-terminally hexaHis-tagged mouse JunD (MGHHHHHH followed by residues E^{2}-Y^{341}, accession number J04509) were expressed in Sf9 cells using baculovirus mediated overexpression (Bac-to-Bac system, Invitrogen) and purified using similar methods as we have described before[242,263].

Wild-type (WT) full-length $(His)_6$-Delta FosB/$(His)_6$-JunD, $(His)_6$-Delta FosB, and the mutants $(His)_6$-Delta FosB $C^{172}S$, $(His)_6$-Delta FosB $A^{168}Y$ $A^{169}Y$, and $(His)_6$-Delta FosB Del Mut2 (which lacks residues K^{166} $L^{167}A^{168}$ $A^{169}A^{170}K^{171}$ C^{172} of the DNA binding motif) were produced as homomers or as heteromers with $(His)_6$-JunD. Complexes of Delta FosB/JunD or mutants were made either by combining the Ni-eluate of each protein in a 1:1 ratio, and then purifying the protein further by ion-exchange and gel filtration chromatography, or by co-infecting Sf9 cells with $(His)_6$-Delta FosB (MOI ~1-1.5) and $(His)_6$-JunD baculoviruses (1:1 or 1:3 with respect to $(His)_6$-Delta FosB) followed by further purification of the complex. Briefly, flash-frozen cell pellets from 3 L or 6 L insect culture were thawed, lyzed by sonication (in 20 mM Tris pH 8, 0.2% Triton X-100, 1 mM TCEP, 0.5 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin A), and then 300 mM NaCl, 5 mM $MgCl_2$ and 50 ugr/ml DNAse added. In preparation for Ni-NTA chromatography (Invitrogen), the NaCl concentration was increased to 1M, 0.5 M NaBr

and 5mM imidazole were added to the lysate, and the mixture subject to high-speed centrifugation. Following Ni-NTA chromatography, the Ni-NTA eluate of $(His)_6$-Delta FosB (WT or mutants) and $(His)_6$-JunD were mixed together (1:1 ratio) to form heteromeric complexes, diluted to 0.1-0.2 mg/ml and dialyzed overnight (in 25 mM Tris pH 9.0, 300 mM NaCl, 0.5% glycerol, 1 mM TCEP (or 5 mM DTT), 0.5 mM PMSF, 0.5% glycerol) and then dialyzed for 3 hr in a low salt buffer (25 mM Tris pH 9.0, 40 mM NaCl, 0.5% glycerol, 1 mM TCEP, 0.5 mM PMSF, 0.5% glycerol) before purification on a Resource-Q column (GE Healthcare). For $(His)_6$-Delta FosB (or mutant) homomeric complexes ion-exchange was omitted, and instead the Ni-eluate was treated with 1 mM DTT, concentrated and then subject to size exclusion chromatography. Proteins were purified on a HiLoad 16/600 Superdex 75 pg column (GE Healthcare) equilibrated with 20 mM Tris pH 8, 1 M NaCl for wild type and mutant Delta FosB:JunD heteromers. Additionally, fractions were confirmed to contain both proteins in a 1:1 complex based on SDS−PAGE prior to being pooled, concentrated and flash-frozen. $(His)_6$-Delta FosB/$(His)_6$-JunD, $(His)_6$ Delta FosB and variants with mutant forms of $(His)_6$ Delta FosB were stored in 20 mM Tris pH 8, 1 M NaCl at protein concentrations of 2 to 6 mg/ml. Protein purity was assessed by SDS-PAGE on 12% gels.

Fluorescent polarization binding assay

A fluorescence polarization (FP) based DNA-binding assay was performed as we have described before[242,263]. Briefly, $(His)_6$- Delta FosB, $(His)_6$- Delta FosB $C^{172}S$, $(His)_6$-Delta FosB $A^{168}Y$ $A^{169}Y$, and $(His)_6$- Delta FosB DelMut2, either as homomers or heteromers with $(His)_6$-JunD were plated in a concentration series (0-800 nM) and a

fixed concentration of TAMRA-labeled oligo TMR-*cdk5* or TMR-*SCR* added (25 nM). The FP buffer for $(His)_6$- Delta FosB/$(His)_6$-JunD heteromers was 20 mM HEPES pH 7.5, 180 mM NaCl and for $(His)_6$- Delta FosB homomers, 20 mM HEPES pH 7.5, 65 mM NaCl with 1-5 mM TCEP (reducing conditions) or 10 uM diamide (oxidizing conditions) end concentration added. The protein:oligo samples were mixed and then manually dispensed in quadruplicates into 384-well round bottom low-volume black microtiter plates (Corning) 20 ul per well) and incubated for 15 minutes at room temperature. The FP signal was measured on a BioTek, Synergy Neo2 plate reader (excitation 530 nm, emission 590 nm) or using a Pherastar plate reader (BMG Labs; excitation 540 nm, emission 590 nm, 100 flashes per well); the target was set to 20 mP for each individual TMR-oligo by adjusting the gain on a well with oligonucleotide in the absence of protein). The FP signal observed for the free oligo (i.e., no protein) was set as the baseline value and subtracted from the FP values measured for oligos in the presence of varying amounts of protein.

Protein Carbonylation Assay

Reagents were prepared per kit instructions (ab178020). Protein lysates were prepared by rinsing the brain tissue with 1 x PBS then homogenized in 100uL 1 x PBS for 20s using a motor pestle. 50uL of the homogenate was added to 100uL 1 x PBS. 150uL of extraction buffer was added and samples were incubated on ice for 20 minutes. After the incubation period samples were spun down at 18,000 g for 20 minutes at 4°C. Supernatants were transferred to clean tubes and pellets were discarded. 10uL of each supernatant was added to 12% SDS. 20uL of DNPH was also

added to the tubes. Tubes were incubated for 15 minutes at room temperature. 20uL of neutralization buffer was added to the tubes. 20uL of 5x Laemmli and samples were run and transferred at described in the Western blot procedure above. Membranes were blocked for 1 hour at room temperature using supplied blocking buffer, washed, and incubated overnight with primary antibody overnight at 4°C. The following day membranes were washed with 1 x PBST and incubated for 1 hour in secondary antibody in blocking buffer at room temperature, rinsed with 1 x PBS, and developed.

Statistical Analysis

All analysis was performed using Prism software (GraphPad). Student's *t* tests were used for all pairwise comparisons (indicated in Results where the *t* value is given), and one-way or two-way ANOVAs were used for all multiple comparisons (indicated in Results where the *F* value is given), followed by Bonferroni or Tukey *post hoc* tests where appropriate.

CHAPTER 3: EFFECT OF REDOX-SENSITIVITY ON DELTA FOSB DISULFIDE BOND AND COMPLEX FORMATION *IN VITRO* AND *IN VIVO*

Author contributions:

Conceived and designed the experiments: Lynch, Aglyamova, Rudenko, Nestler, and Robison

Performed the experiments: Lynch and Aglyamova

Analyzed the data: Lynch, Aglyamova, Rudenko, and Robison

Contributed reagents/materials/analysis tools: Robison, Nestler, Rudenko

**Lynch and Aglyamova contributed equally to this work, Aglyamova on the in vitro purified protein and computational studies and Lynch on all cell culture and mouse work*

Introduction

As described in the first chapter, Delta FosB's redox sensitivity[227], importance in learning and memory[255], and induction in AD[4] call for further research to be conducted on Delta FosB's role in AD pathogenesis. Its role as a transcription factor and its ability to regulate gene expression make it a prime candidate for meditating AD progression and prevention. This mediation resulting from Delta FosB's unique stability and prolonged half-life of approximately eight days *in vivo*[259,260] allow it to accumulate in the neuron and mediate long-term changes in gene expression seen in chronic diseases like AD[12,316]. To function, Delta FosB must bind to promoter regions in genes of interest. Due to its lack of a complete DNA binding region[242], this requires Delta FosB to bind to a partner protein (either itself [homomer] or JunD [heteromer]) to complete this region, form an AP-1 complex, and allow for DNA binding. This AP-1 complex is formed

through the interaction of the leucine zipper regions of the involved proteins and the supporting disulfide bridges that form between cysteine residues. These interactions allow the complex to form various conformations, some of which allow it to fit in the DNA binding sites of target genes[317] under certain conditions. One of these conditions that adds to the complexity of Delta FosB is the oxidative state of the cell; there is *in vitro* evidence that suggests that specific cysteine residues in Delta FosB respond to oxidative stress in the brain[227], a factor that is a key characteristic in many neurological conditions including AD[105].

Delta FosB contains four cysteine residues, C172, C222, C44, and C15. Since C44 and C15 are in the disordered region of the protein, it is unlikely that they play a significant role in regulating Delta FosB complex formation; however, C172S, which is near the DNA binding region of the protein, and C222S which is near the leucine zipper region, are possible pertinent players in Delta FosB's altered function in response to oxidative stress[227]. C172S has been termed the "redox switch" in previous *in vitro* studies, due to its importance in the regulation of Delta FosB's ability to bind DNA[227], which will be discussed in the next chapter. *In vitro* evidence additionally suggests that C222's position near the leucine zipper region of Delta FosB could regulate the stability of the zipper when a complex forms via disulfide bridging between Delta FosB and its partner protein; thereby stabilizing complex formation[8].

To elucidate whether Delta FosB disulfide bridge and complex formation are dependent on redox state *in vivo* as well as *in vitro*, I utilized a HA-tagged Delta FosB mouse line as well as N2a cells. I tested the hypothesis that oxidizing conditions allow disulfide bridges to form, thereby stabilizing Delta FosB complex formation, whereas,

reducing conditions remove any disulfide bridges and attenuate complex formation.

Additionally, C172, C222, or both were mutated to serine residues through a point mutation, rendering them unable to be oxidized even while under oxidizing conditions.

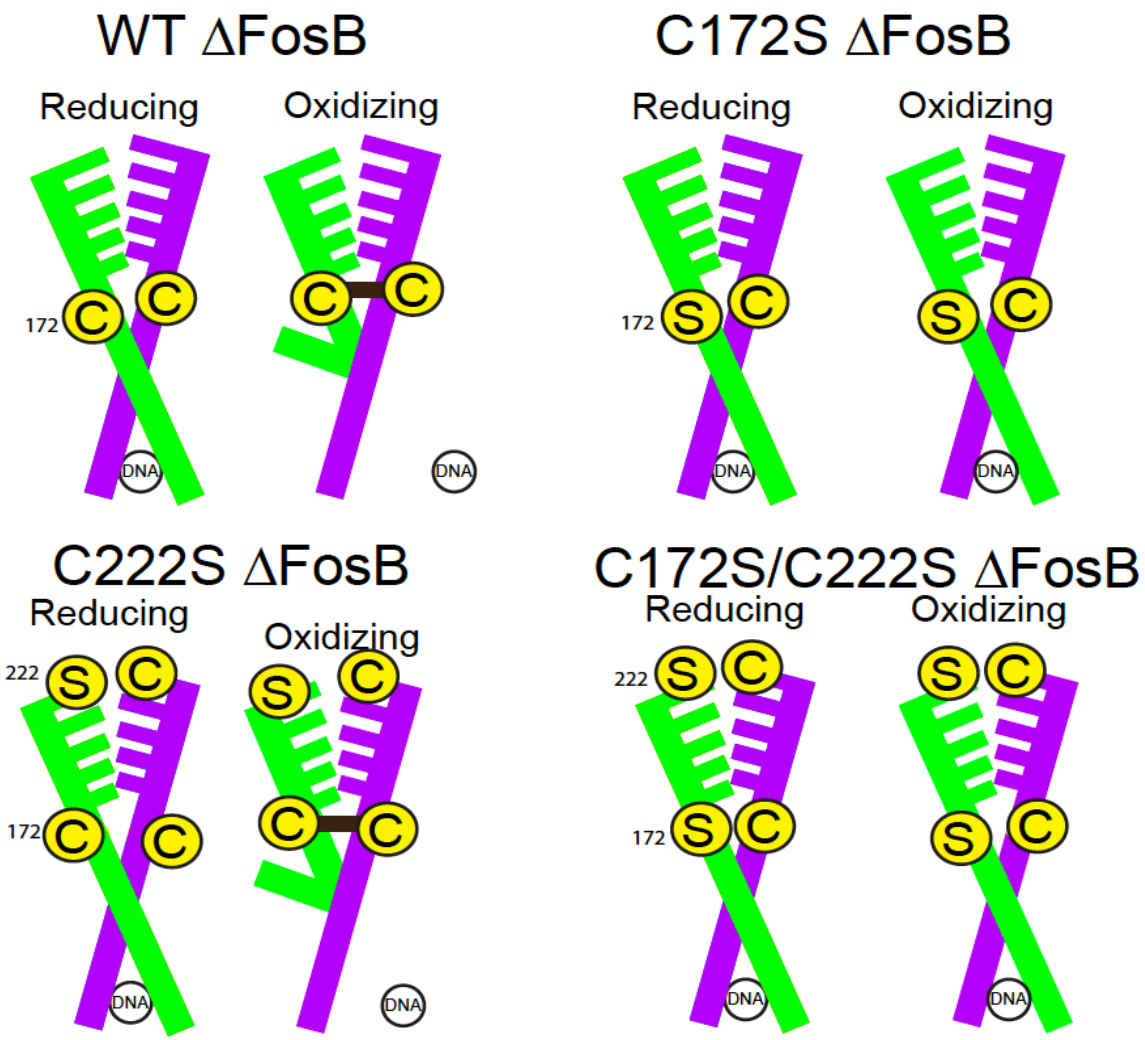

Figure 15 | Delta FosB wild type and redox mutants. Schematic of the point mutation induced in Delta FosB (green) to mutate the cysteine (C) at residue 172, 222, or both to a serine (S) in order to render the complex redox-insensitive. This FosB protein can then bind to partner JunD (purple) and form complexes via amino acid (yellow), disulfide bridging (black) under certain redox conditions.

Multiple mutants were made to assess the significance of each cysteine residue or combination of residues in Delta FosB's redox sensitivity (Figure 15). It was predicted

that C222 would play a larger role in mediating complex formation due to its location near the leucine zipper region. Conversely, we did not think C172 would have much impact on complex formation due to it playing a larger role in DNA binding.

Results

In vitro Delta FosB protein-protein interactions are mediated by specific cysteine residues

Although this dissertation focuses on the redox sensitivity of Delta FosB in mouse brain and cell culture, the recent *in vitro* and computational studies completed by Dr. Rudenko's lab set the stage for my current research and must be covered here. The Rudenko lab found that C172 and C222 and their ability to form disulfide bonds are important in the redox dependence of purified Delta FosB structure. C172 is implicated in the redox switch that regulates Delta FosB-DNA binding[227]. When Delta FosB and JunD form a complex, disulfide bonding of Delta FosB C172 to JunD C279 under oxidizing conditions causes Delta FosB to kink is a way that sterically hinders the complex from binding to DNA. Release of the disulfide bond, due to reducing conditions, triggers a different conformational change that allows the two DNA-binding motifs to insert into the major groove on either side of DNA[227] (Figure 13 in Introduction Section). Additionally, the Rudenko lab also found *in vitro* evidence that C222 may act to stabilize the leucine zippers of an AP-1 transcription factor, like Delta FosB, by locking the bZIP domains into parallel dimers that are compatible with DNA binding under oxidizing conditions when a bridge is formed[8]. This begs the question of whether reducing conditions, thus removal of the disulfide bond, would alter the stability of heteromer

oligomerization. Although there is no evidence yet to suggest how they respond to redox state, Delta FosB homomers are also capable of forming via disulfide bridges and

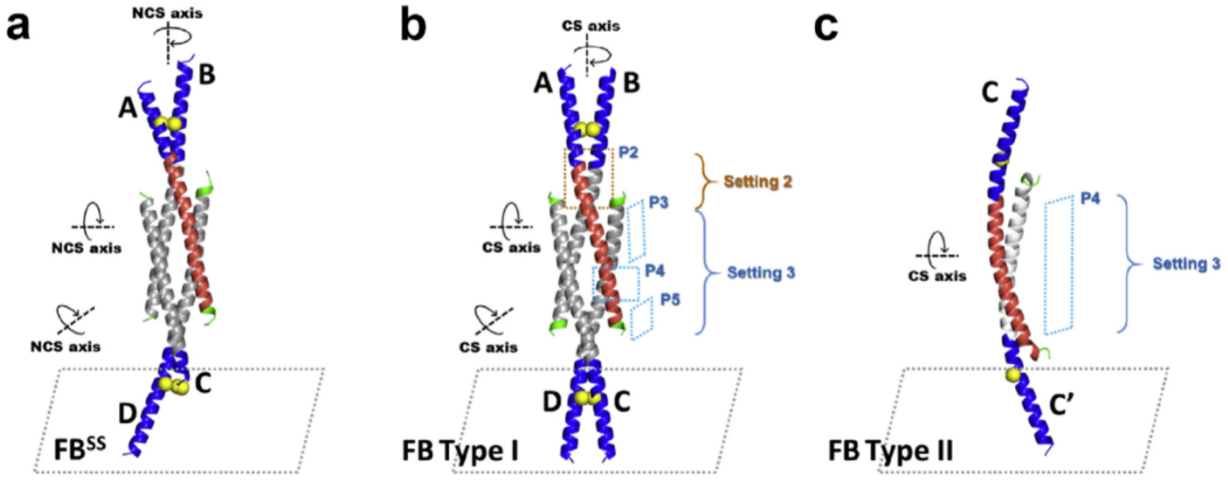

Figure 16 | 3D-structures of FosB self-assemblies. Non-canonical arrangements of Delta FosB homomers. **a)** Crystal structure FB^{SS}; **b)** Crystal structure FB Type I; **c)** Crystal structure FB Type II. In panel **a)** though **c)**, each FB subunit is shown with the basic/DNA-binding region in blue (amino acids N-terminal to residue 179), the leucine-zipper region in red or grey (residues 180–214), and the C-terminal region in green (amino acids C-terminal to residue 215). Cys^{172} is shown as a yellow sphere. Helix chains are labeled A, B, C, C′, and D, respectively. Interactions between helices that are discussed in the text are labeled (Setting 2, Setting 3, and packing P2 through P5) As described in and reprinted from *Current research and structural biology*[8], copyright 2020.

binding to DNA; however, less is known as to how this occurs[8]. Additionally, the noncanonical arrangements formed by homomers differ greatly from that of heteromers (Figure 16)[8].

Extracellular manipulation of redox state alters Delta FosB oligomerization in N2a cells in a cysteine-dependent manner

To investigate whether the *in vitro* redox sensitivity found previously by our collaborators is preserved *in vivo*, we manipulated the redox state in neuron-like cells to determine if this affects Delta FosB's ability to form complexes through disulfide bridges. This was done by transfecting N2a cells with HA-Delta FosB with or without FLAG-JunD

to test heteromers and homomers, respectively. Lysates were processed with the reducing agent DTT, the oxidation preserving reagent n-ethyl maleimide (NEM), or the

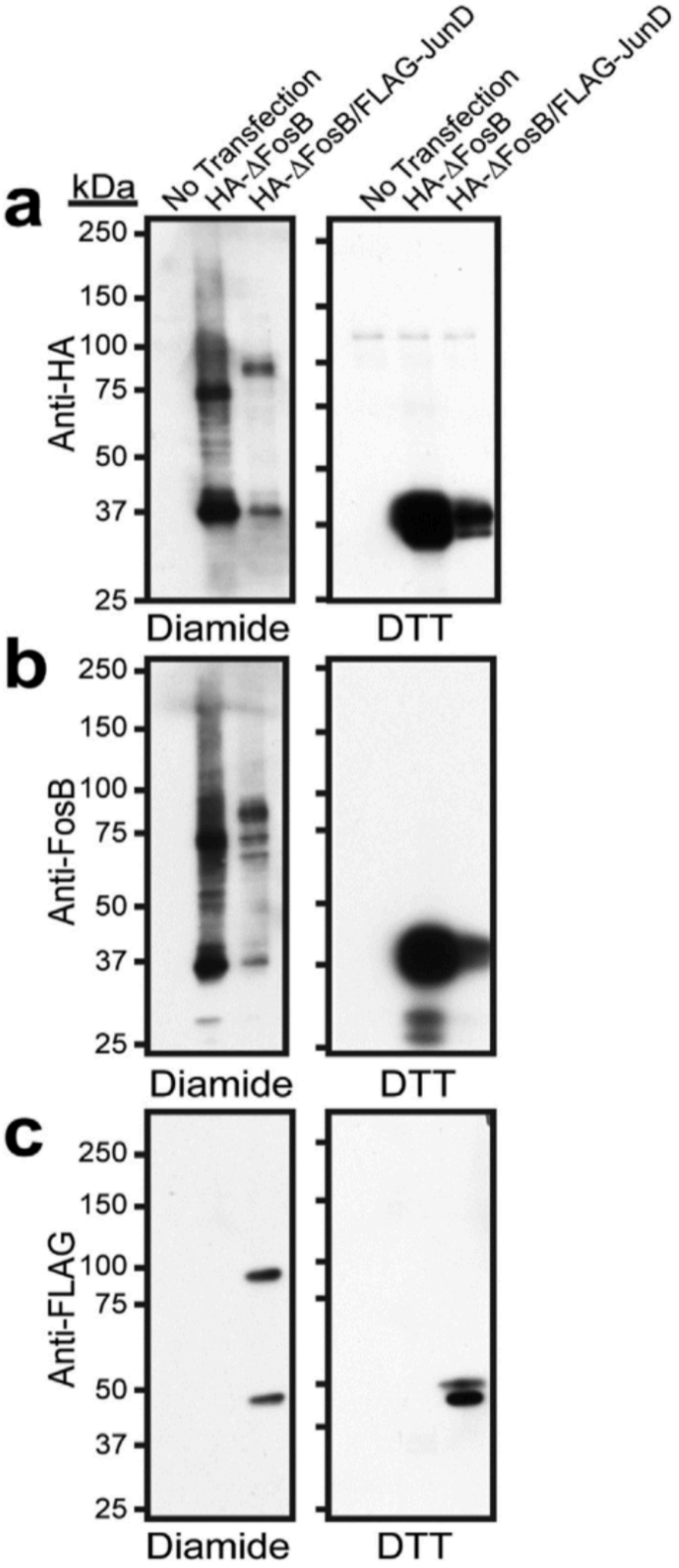

Figure 17 | Delta FosB's ability to form complexes is mediated by disulfide bridges. N2a cells were either not transfected or transiently transfected with HA-tagged Delta FosB with or without FLAG-tagged JunD. Cells were homogenized and samples prepared in mildly oxidizing (diamide) or reducing (DTT) conditions. a) Western blot analysis using anti-HA antibodies reveals HA-tagged Delta FosB species; b) Western blot analysis using anti-Delta FosB/FosB antibodies reveals Delta FosB; c) Western blot analysis using anti-FLAG antibodies reveals FLAG-tagged JunD. Reprinted from *Current research in structural biology*[8], copyright 2020.

oxidizing reagent diamide and gel samples were prepared using reducing or non-reducing Laemmli buffer. Western blot analysis revealed that under reducing conditions (DTT and/or reducing Laemmli buffer), both heteromer and homomer conditions reveal bands at the predicted molecular weight of Delta FosB (~37kDa). Under oxidizing conditions, both the HA and FosB blots reveal the same lower band but also a higher molecular weight band the reflects the combined molecular weight of a Delta FosB/JunD complex (~87kDa, heteromer) or two Delta FosB proteins (~74kDa, homomers). The FLAG-JunD blot reveals the same pattern, indicating that JunD is part of the heteromer complex (Figure 17). Because these are denaturing SDS-PAGE gels, the stabilized higher molecular weight complexes must be held together by covalent bonds, implicating the formation of disulfide bridges at cysteine residues.

To investigate whether specific cysteine residues were driving these SDS-impervious complexes, we examined the formation of heteromeric or homomeric complexes when we manipulated the redox state by utilizing the redox insensitive cysteine mutants (C172S and C222S). N2a cells were transfected with WT or mutant Delta FosB cDNA with or without JunD cDNA, processed, and added to reducing or non-reducing Laemmli buffer. Reducing conditions would remove all disulfide bridges regardless of which Delta FosB cDNA was present, thereby attenuating WT Delta FosB's ability to form SDS-stable complexes with other proteins. Thus WT Delta FosB and the mutants remained at Delta FosB's predicted molecular weight of 37kDa during reducing Western blot procedures (data not shown). Under non-reducing, oxidizing conditions (using non-reducing Laemmli buffer), when WT Delta FosB can form SDS-stable heteromers or homomers through covalent disulfide bridges between cysteine

residues, transfecting cells with WT Delta FosB and JunD results in a shift in the molecular weight of the protein's predicted 37kDa to a combined molecular weight of Delta FosB and its perspective partner (Figure 18), like what was seen previously (Figure 17). However, mutating C172 to a serine deters the increase in the higher molecular weight band (HMW) compared to the lower molecular weight band (LMW). This decrease was exacerbated when both C172 and C222 were mutated to serines, thereby preventing oxidation at these cysteine residues. Solely mutating C222 did not significantly affect the level of oxidation relative to the WT heteromer (Figure 18).

Looking at homomers, when WT Delta FosB is transfected alone, the lack of disulfide bridging under reducing conditions (reducing Laemmli buffer) prevents WT Delta FosB

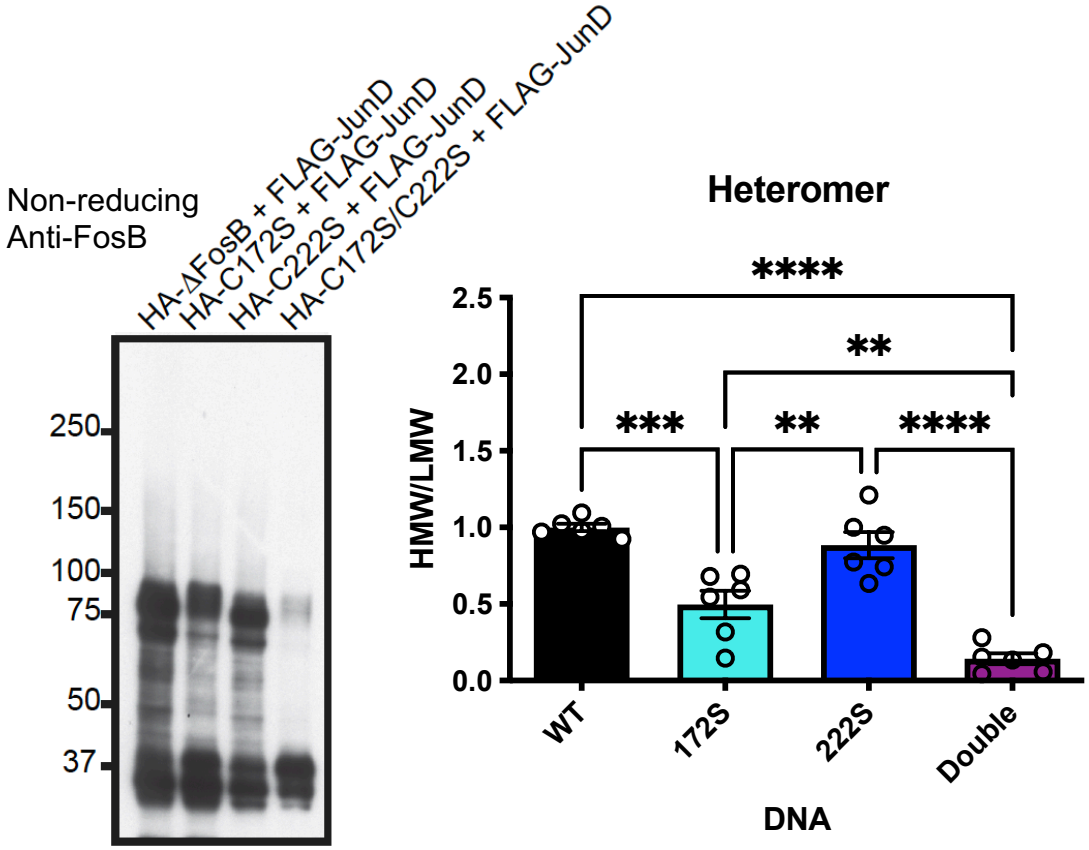

Figure 18 | Delta FosB heteromer formation is mediated by cysteine-dependent disulfide bridges. N2a cells were transiently transfected with WT or mutant HA-tagged Delta FosB with FLAG-tagged JunD. Cells were homogenized and samples prepared in non-reducing or reducing (not shown) conditions. Western blot analysis using anti-FosB antibodies reveal Delta FosB species at the predicted molecular weight (lower molecular weight band, LMW) for reducing conditions, and increased molecular weight (higher molecular weight band, HMW) for oxidizing conditions. The ratio of HMW to LMW (i.e. the level of oxidation) was quantified. Significant differences were determined by one-way ANOVAs. **p<0.05, ***p=0.0001, ****p<0.0001

from forming SDS-stable complexes even with other Delta FosB proteins, just like was seen with the heteromer (data not shown). Under oxidizing conditions (non-reducing Laemmli buffer), WT Delta FosB can form SDS-resistant complexes with other Delta

FosB proteins, despite which cysteine is mutated. Once again there is a shift from 37kDa, Delta FosB's predicted molecular weight, to ~74kDa, the combined molecular weight of two Delta FosB proteins (Figure 19). The homomer maintains the shift in molecular weight under oxidizing conditions even if one or both cysteines are mutated, although there is a trend toward a decrease in the double mutant. These data indicate that additional cites of disulfide bond formation may exist in the homomeric complex.

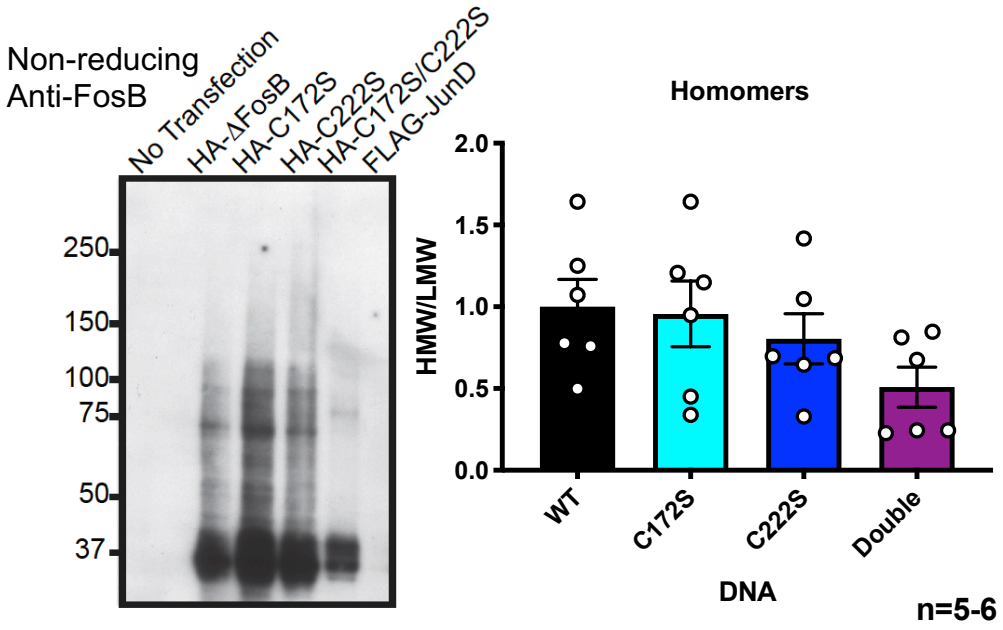

Figure 19 | Delta FosB's homomer formation does not depend on C172 and/or C222. N2a cells were either not transfected (leftmost lane) or transiently transfected with WT or mutant HA-tagged Delta FosB. Cells were homogenized and samples prepared in non-reducing or reducing conditions. Western blot analysis using anti-FosB antibodies reveals Delta FosB occurred at the predicted molecular weight for reducing conditions(not shown) (lower molecular weight band, LMW) and increased molecular weight (higher molecular weight band, HMW) for oxidizing conditions. The ratio of the HMW to LMW was graphed.

Although these data support Delta FosB's redox sensitivity in cells, the redox conditions were manipulated after cell homogenization. To determine whether these effects occur within the intact cell, we manipulated redox states in the medium of the living cells.

Delta FosB disulfide bridges form in living cells

The previous experiments tested the redox sensitivity of Delta FosB's ability to form complexes via addition of non-reducing or reducing Laemmli buffer after the cells had been homogenized. We next sought to determine the redox sensitivity of Delta FosB heteromer while inside the cell. Transfected cells were treated with media containing diamide (no, 0.01mM, 0.1mM, 1.0mM, or 10mM) or H_2O_2 (no, 0.01mM,

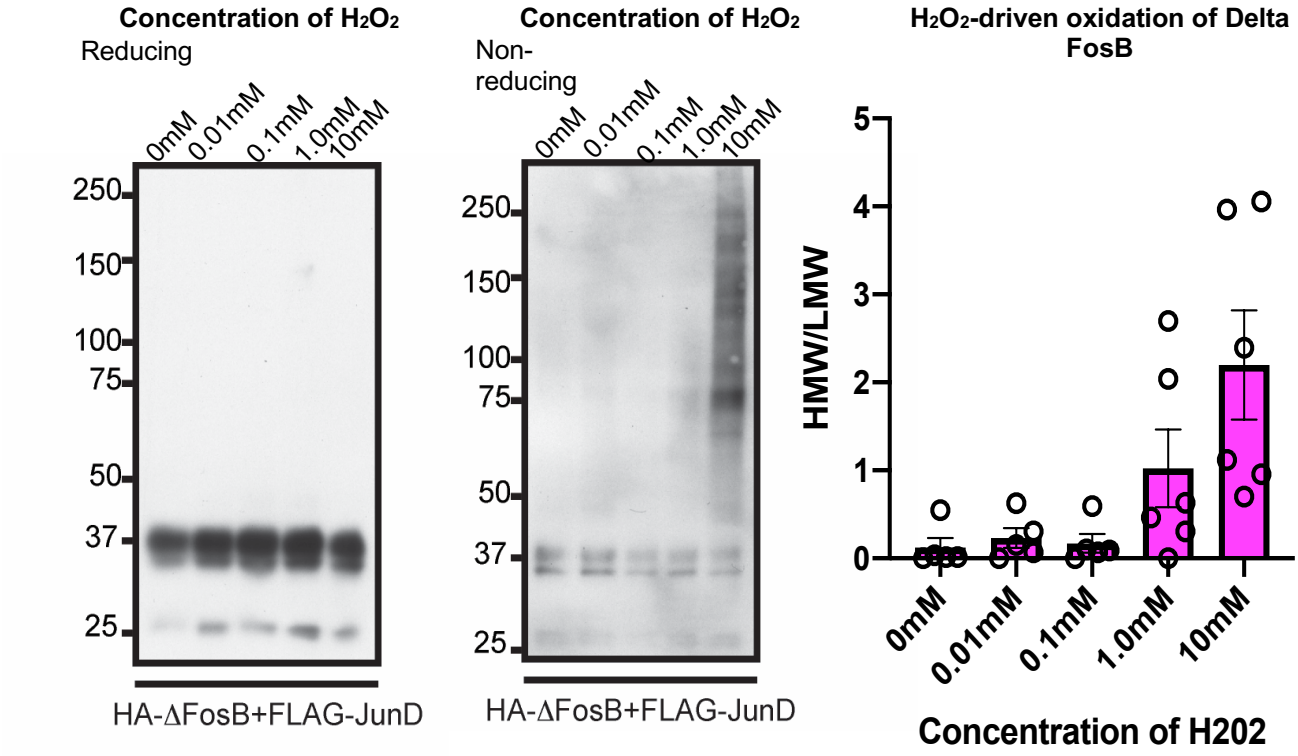

Figure 20 | H_2O_2 drives Delta FosB complex formation. N2a cells were transiently transfected with HA-tagged Delta FosB with FLAG-tagged JunD. Cells were treated with H_2O_2 and prepared using reducing or non-reducing Laemmli buffer. Western blot analysis using anti-FosB antibodies reveals Delta FosB species at the predicted molecular weight for reducing conditions, and increased oxidation for oxidizing conditions. Non-reducing condition were graphed (n=5-6).

0.1mM, 1.0mM, or 10mM), to mimic the state of oxidative stress seen in neurological disorders like AD. Diamide was used to draw parallels between the cell culture redox sensitivity testing already conducted, whereas H_2O_2 was chosen because it is more

physiologically relevant seeing as it is one of the natural byproducts of energy production in the cell[14]. Post-treatment cells were removed from the oxidant-containing media 48 hours after transfection and processed using homogenizing buffer (with no added redox reagents) and added to Laemmli buffer with or without reducing reagents.

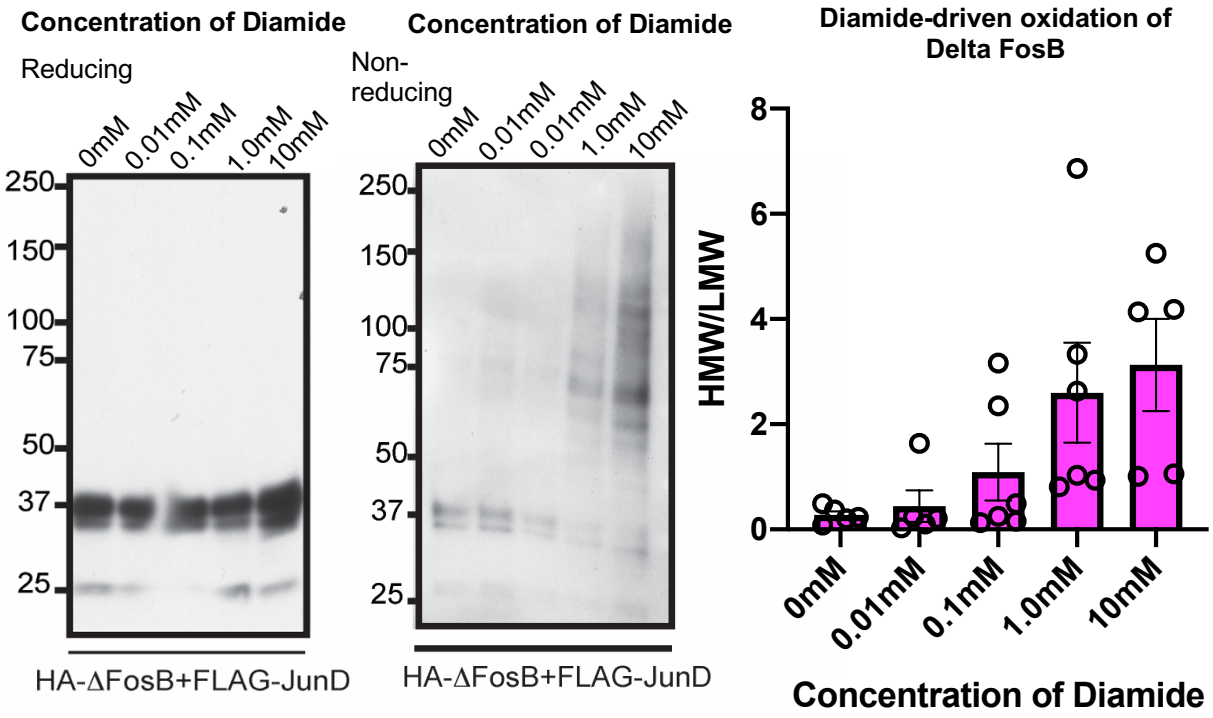

Figure 21 | Diamide drives Delta FosB complex formation. N2a cells were transiently transfected with HA-tagged Delta FosB with FLAG-tagged JunD. Cells were treated with diamide and prepared using reducing or non-reducing Laemmli buffer. Western blot analysis using anti-FosB antibodies (Cell Signaling 2251S; 1:500) reveals Delta FosB species at the predicted molecular weight for reducing conditions, and increased oxidation for oxidizing conditions. Non-reducing condition were graphed (n=5-6).

The samples processed in reducing Laemmli buffer served as the control, reducing conditions. Treating cells with oxidant-containing media and transfecting with WT Delta FosB and JunD results in increasing presence of dimer-like bands with increasing concentrations of H_2O_2 or diamide when processed with non-reducing Laemmli buffer

(Figure 20 and 21). Reducing Laemmli buffer removed all trace of these bands, indicating that they depend on oxidizing conditions.

Mouse brain redox state alters Delta FosB oligomerization

Although N2A cells are an important model for neuronal function, we wanted to examine whether Delta FosB complex formation was also regulated by redox state in the brain itself. To do this, we used *in vivo* manipulation of redox state to investigate the effect of oxidative stress on Delta FosB complex formation in the mouse brain. We wanted to assess the preclinical relevance of redox state on Delta FosB oligomerization by using a transgenic HA-tagged Delta FosB mouse line that was graciously given to us by one of our key collaborators, Dr. Eric Nestler at Mount Sinai. We extracted the brains from the mice, collected 12G bilateral samples of the dorsal hippocampus (dHPC), and conducted an immunoprecipitation. This involved pulling down the HA-tagged Delta FosB using an anti-HA antibody and probing for Delta FosB with non-reducing Western blot. When processing the samples, brain punches were homogenized in buffer containing DTT, NEM, or diamide. These processed brain lysates were then added to reducing or nonreducing Laemmli buffer to prepare for Western blot. Brain lysate that was homogenized in DTT-containing buffer and/or added to reducing Laemmli buffer consequently had all disulfide bonds removed. As a result, in the Western blot the only bands visible were those at the lower, 37kDa band of Delta FosB (Figure 22). When NEM or Diamide were used to process the brain samples, and added to non-reducing Laemmli buffer, the 37kDa band of Delta FosB is no longer seen. Rather, there is a shift

in the band to higher molecular weights indicative of the combined molecular weight of

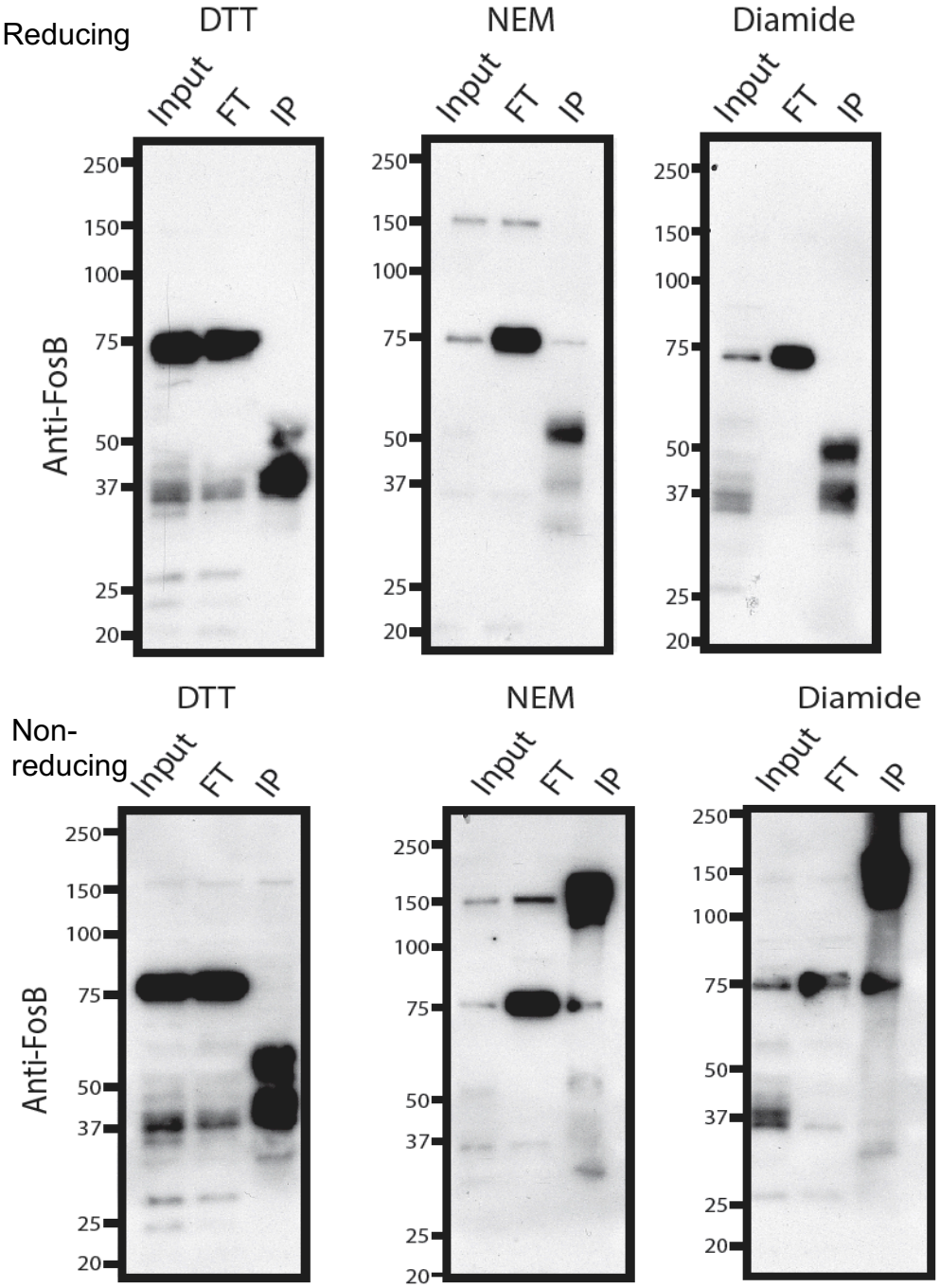

Figure 22 | Delta FosB's redox sensitivity in mouse brain is mediated via disulfide bonds. Mouse dorsal hippocampus processed under reducing (DTT and/or reducing Western blot) conditions maintains bands at Delta FosB's predicted molecular weight (37kDa); whereas conditions that preserve or promote oxidation, NEM or diamide, respectively, lead to a shift in weight due to complex formation through disulfide bonds. Samples wre collected for the input, flowthrough (FT), and immunoprecipitation (IP)

Delta FosB and its partner protein (Figure 22). This higher molecular weight could be

the result of Delta FosB binding to another Delta FosB protein or another AP-1 protein like JunD. Additionally, the number of proteins creating this band could reflect a combination of multimers not just limited to dimers, but tetrameric complexes as well. Nevertheless, these data indicate that Delta FosB forms redox-dependent SDS-resistant complexes in the brain, most likely the result of intra-subunit disulfide bonds.

Discussion

In this chapter, it was determined that redox state of N2a cells and mouse brain alters Delta FosB protein-protein interactions and oligomerization. We used two models to mimic oxidative stress in the brain, one being N2a cells treated with an oxidant (H_2O_2 or diamide) and the other being mouse hippocampal brain tissue treated with NEM that maintained the native state of the protein, including any oxidized cysteines, or diamide which promoted a state of oxidative stress during processing of the tissue. We also utilized Delta FosB mutants in the N2a cells to determine the contributions of individual residues to disulfide bond formation. We chose these two models because the N2a cells are a neuroblastoma cell line that allow functional experiments to be done at a cellular level using a culture that would behave similarly to neurons in the brain. We used mice to perform experiments at a systemic level using a model with translational and preclinical relevance. Additionally, using a system for which there is already an established AD model will allow future experiments to emerge directly from the baseline experiments presented in this work (see future directions).

Producing a state of oxidative stress in N2a cells was approached from two different angles, one being altering the cells after they have been homogenized so that

it is paralleled to what was done with the mouse brain tissue (extracellular redox manipulation) and the other being directly altering the oxidative state of growing, live cells (intracellular redox manipulation). Additionally, cells were either transfected solely with WT Delta FosB cDNA or WT Delta FosB and JunD cDNA to reflect the homomer and heteromer binding, respectively. N2a cells revealed that under reducing conditions, conditions that did not receive an oxidant, Delta FosB remains at its predicted molecular weight of 37kDa, despite if Delta FosB or Delta FosB and JunD were transfected. Under oxidizing conditions, however, the bands shift to a higher molecular weight indicative of complex formation for both conditions, the only difference appears at where the higher molecular weight band shifts to. With the homomer condition, the band shifts to approximately 74kDa, which most likely reflects the combined molecular weight of two Delta FosB proteins. The heteromer condition shows a band at approximately 87kDa. We know this complex is most likely Delta FosB's established binding partner, JunD seeing as the band sits right around where a Delta FosB/JunD complex would reside. Additional support comes from Western blot results using the same samples but probing for JunD instead of Delta FosB. Under native conditions, it is possible that other proteins are part of this complex other than JunD, but the scope of this chapter focuses on how disulfide bonds connecting Delta FosB and the partner protein(s) are altered and not which partner proteins are involved. Additionally, since we are overexpressing JunD and no other potential binding partner, it is likely that other Delta FosB-contained complexes would be limited by the lack of binding partner and the abundance of Delta FosB and JunD. Furthermore, we know that these are disulfide bridges that are mediating the connection between Delta FosB and its partners because despite how the samples are

processed, the denaturing conditions of the sodium dodecyl sulfate (SDS) will remove any non-covalent bonds so that only covalent bonds, like disulfide bridges, will remain.

Next, similar results were seen when H_2O_2 or diamide were added directly to the media the living cells were growing in. This technique provides more translational relevance because it better mimics what happens in the neurons of the brain, making it more similar to what happens in AD. Similar results were found to what was seen extracellularly; reducing conditions revealed bands at the predicted molecular weight of Delta FosB for both heteromer and homomer conditions, whereas, oxidizing conditions showed a shift in the Delta FosB band to higher weights, both of which resembled the same weights as those seen with the extracellular redox manipulations. Additionally, under oxidizing conditions, increasing concentrations of either oxidant resulted in increased ratio of the higher molecular weight band to the lower molecular weight band, indicating there is increased SDS-stable oligomerization occurring. We know that any differences in ratio of oligomerized Delta FosB to single Delta FosB proteins were not due to cells being killed by the oxidant because reducing Western blots using the lysates show similarly expressed bands across all concentrations of oxidant used.

So far WT Delta FosB has been addressed, but an additional level of complexity was added to the cell culture experiments when redox mutants of Delta FosB were introduced. PCR mutagenesis was used to induce a point mutation in cysteine 172, cysteine 222, or both to convert the cysteines at these residues to serines. This rendered the mutated residues “redox insensitive” seeing that serines cannot be oxidized so despite how the samples were processed, they would always remain in a reduced state. As with WT, reducing conditions kept the molecular weight band at the

predicted molecular weight of Delta FosB, despite if either mutant(s) was expressed alone or with JunD. Under oxidizing conditions, when C172S was solely overexpressed, it gave the same results as overexpressing WT, there was a shift to a higher molecular weight. However, for the heteromer, there was a decrease in oligomerization. Although this residue is near the DNA binding region mutating it also affects oligomerization. Surprisingly C222S did not show this attenuation of oligomerization. This was a surprise because it was originally thought that preventing oxidation here destabilizes the complex. Since this is near the leucine zipper region and has previously been thought to stabilize leucine zipper interactions between Delta FosB and its binding partner in a "zip lock" fashion[8], the lack of disulfide bridging capability initially was thought to be detrimental towards complex formation; however, this was not the case. This could potentially be because there are other cysteine residues in Delta FosB and their binding could compensate for the lacking at C222S. Interestingly, however, mutating both C222 with C172S exacerbated the decreased complex formation that occurred with just C172S. It was no surprise that the mutations in the residues did not change oligomerization of the homomers compared to WT because C172 and C222 have only been tested *in vitro* in the heteromer and their significance in homomer complex formation has not been investigated, but our new data suggest that additional disulfide bonds may form in the homomers. Due to the noncanonical arrangements of Delta FosB homomers relative to heteromers[8], it is possible that C44 and C15 could play a significant role in homomer oligomerization.

The evidence for *in vivo* redox sensitivity found in cell culture also fits in with what we found in mouse brain. We used an HA-tagged Delta FosB mouse line because

it allowed us to pull down Delta FosB with an HA antibody made in one animal and probe for Delta FosB using an antibody collected from a different animal. This helped decrease the number of non-specific bands that appeared on the Western blots. Collecting hippocampal punches and treating them with reducing agent, DTT, removed all disulfide bridges and resulted in Western bands at the predicted molecular weight of Delta FosB, like what was seen with the reducing cell culture conditions. Treating hippocampal samples with NEM preserved the native state of the protein, thus preserving any oxidized cysteines while preventing any further oxidation from occurring. This resulted in bands not only at the higher molecular weight band ~74kDa seen in oxidized N2a cells, but there was an additional higher molecular weight band that occurred even higher, around 150kDa, which could reflect a tetrameric complex of four Delta FosB proteins or a combination of Delta FosB and JunD proteins. It is possible that this higher band did not show up in the N2a cells because these cells are grown under extremely stable conditions and the amount of oxidative stress is limited to what I induced, whereas in the brain there are an abundance of sources of oxidative stress. Overall, this provides support for the ability of Delta FosB to not only form dimers in the brain, but other multimeric complexes as well.

Although these findings are critical in supporting Delta FosB's important role in diseases associated with oxidative stress, not just AD, there are a few caveats. One caveat is that we artificially induced levels of oxidative stress in cells and brain with unknown parallels to the levels in an actual patient with AD. Additionally, we artificially overexpressed levels of Delta FosB and JunD in the cells and we do not know how this overexpression compares to that in the brain of AD found previously[5].

The experiments I performed in this chapter suggest that the *in vitro* redox sensitivity of Delta FosB found previously[227] is preserved *in vivo* as well, specifically in the context of Delta FosB's ability to bind other proteins. They also suggest that C172 is instrumental in this ability to form complexes and is also aided in part by C222, particularly in heteromer formation with JunD. However, there is another key step in Delta FosB's ability to regulate gene expression once it forms a homomer or heteromer: DNA binding and transcriptional regulation. The next chapter will assess if the redox sensitivity of Delta FosB found here also extends to Delta FosB's ability to bind DNA and regulate target gene expression.

CHAPTER 4: ALTERATIONS OF DELTA FOSB'S DNA BINDING AND TRANSCRIPTIONAL ACTIVITY IN RESPONSE TO REDOX STATE *IN VIVO*

Author contributions:

Conceived and designed the experiments: Lynch, Aglyamova, Rudenko, Nestler, and Robison

Performed the experiments: Lynch and Aglyamova

Analyzed the data: Lynch, Aglyamova, Rudenko, and Robison

Contributed reagents/materials/analysis tools: Robison, Nestler, Rudenko

**Lynch and Aglyamova contributed equally to this work, Aglyamova on the in vitro purified protein and computational studies and Lynch on all cell culture and mouse work*

Introduction

As discussed in the previous section, there is support for Delta FosB's redox sensitivity through its ability to form disulfide bridges and complexes with other proteins in the test tube, in cells, and in the mouse brain; however, Delta FosB must bind DNA at AP-1 consensus sites and have a functional transactivation domain to regulate gene expression. As discussed, Delta FosB's cysteine residues play a key role in the protein's redox sensitivity, however, how this relates to Delta FosB's DNA binding activity, especially at the C172 "redox switch" found *in vitro*, is unknown.

Previous studies have shown that the "redox switch" plays an important role in the AP-1 complex's ability to bind to DNA. Under reducing conditions, no disulfide bridge forms at C172, allowing for the complex to bind DNA. However, under oxidizing conditions, C172 forms a disulfide bridge with a cysteine residue on its partner protein

(i.e., JunD C279). This creates a kink in the Delta FosB protein which hinders the ability for the complex to bind to DNA. In other words, it becomes conformationally unfavorable to fit into the DNA binding site on target genes of interest. As a result, genes that are normally repressed or activated by Delta FosB no longer have Delta FosB-induced changes in expression, which could lead to the altered gene expression seen in AD.

As mentioned in chapter 3, whereas C172's role as a redox switch has been supported *in vitro* there in no evidence to suggest that this is conserved *in vivo*. Thus, N2a cells were processed under different redox conditions and AP-1 luciferase assays were conducted to test the hypothesis that oxidizing conditions prevent DNA binding and transactivation in cells, whereas basal, reducing conditions lead to increased levels of DNA binding relative to control cells. Additionally, the Delta FosB mutants described previously were used to create redox state-independent cysteine residues at C172, C222, or both to test their relevance to this phenomenon.

Results

Purified Delta FosB protein shows changes in DNA binding under different redox conditions

Although, I did not deal directly with the purified protein *in vitro* studies, our collaborators did, and it was a key component to completing this thesis. Using purified Delta FosB protein, the ability of heteromers versus homomers to bind to the AP-1 consensus sequence derived from cyclin-dependent kinase 5 (cdk5) DNA was investigated under different redox conditions. Cdk5 oligos were chosen because they contain an AP-1 binding site that is regulated by Delta FosB in the brain[318]. Purified

Delta FosB was treated with tris(2-carboxyethyl)phosphine (TCEP), a reducing agent, to induce a reduced state of Delta FosB, whereas diamide was once again the chosen oxidizing agent. Florescence polarization assays were conducted to determine the

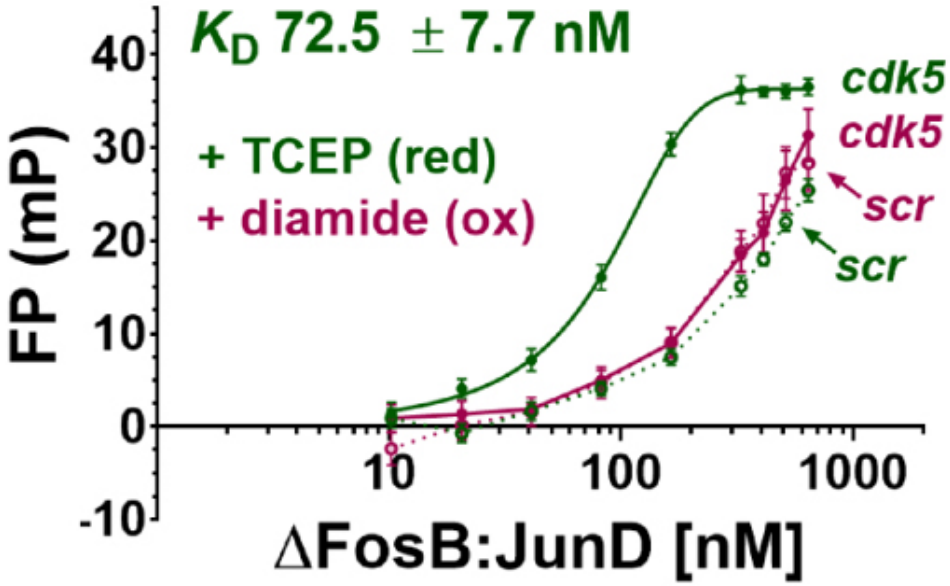

Figure 23 | *In vitro* Delta FosB heteromer loses ability to bind to AP-1 DNA under oxidizing conditions. Fluorescence polarization (FP) assays reveal the ability of the Delta FosB/JunD heteromer to bind to cdk5 oligos (solid lines) under TCEP-induced reducing (green lines) conditions but not diamide-induced oxidizing (pink lines) conditions, compared to control scrambled DNA (dotted lines).

ability of the heteromer or homomer to bind to cdk5. Under reducing conditions, the Delta FosB heteromer can bind specifically to cdk5 oligos at relatively low concentrations and binds poorly to a control scrambled oligo (Figure 23). Scrambled DNA serves as the control and indicates non-specific binding. Under oxidizing conditions, the heteromer is unable to bind the DNA until much higher concentrations and overlaps with the scrambled DNA binding. Thus, the higher concentration needed for the heteromer to bind under oxidizing conditions reflects it being high enough for the heteromer to lose its affinity to bind specifically to cdk5 and sticks non-specifically to any DNA (unpublished, Figure 23). Thus, the heteromer loses its affinity for cdk5 binding

under oxidizing conditions, which could lead to lack of gene expression or too much gene expression of target genes that are induced or inhibited by Delta FosB binding, leading to pathologic states.

We next examined the homomer, and under reducing conditions purified Delta

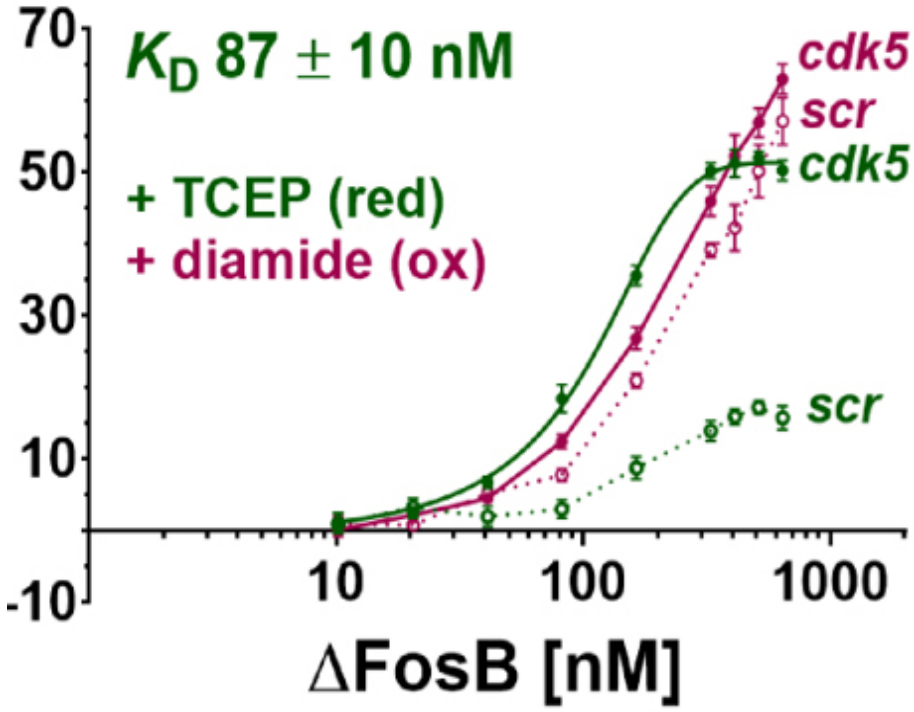

Figure 24 | *In vitro* Delta FosB homomer binds nonspecifically to DNA under oxidizing conditions. Fluorescence polarization (FP) assays reveal the ability of the Delta FosB homomer to bind to cdk5 oligos (solid lines) under TCEP-induced reducing (green lines) conditions or diamide-induced oxidizing (pink lines) conditions relative to scrambled DNA (dotted lines). Ability to bind is relative to the concentration of the heteromer needed to bind DNA.

FosB can bind specifically to AP-1 containing DNA and does not bind strongly to the scrambled DNA (Figure 24), like the heteromer (Figure 23). However, the differences between the heteromer and homomer DNA binding become evident under oxidizing conditions. Under oxidizing conditions, Delta FosB binds at the same affinity to the cdk5 DNA as under oxidizing conditions, but it also readily binds to scrambled DNA (Figure

24). Thus, under oxidizing conditions, the homomer loses its specificity for AP-1 consensus sequence DNA binding. This suggests that the buildup of Delta FosB in diseases like AD where it's more likely for homomers to form may result in aberrant gene expression that could contribute to neuronal pathophysiology.

Oxidation prevents Delta FosB from driving AP-1 gene expression

Published findings provide support that the ability of Delta FosB/JunD complex to bind to DNA is regulated by a disulfide bridge at C172 of Delta FosB, which is affected by redox state of the Delta FosB protein[227]. First, we wanted to determine whether Delta

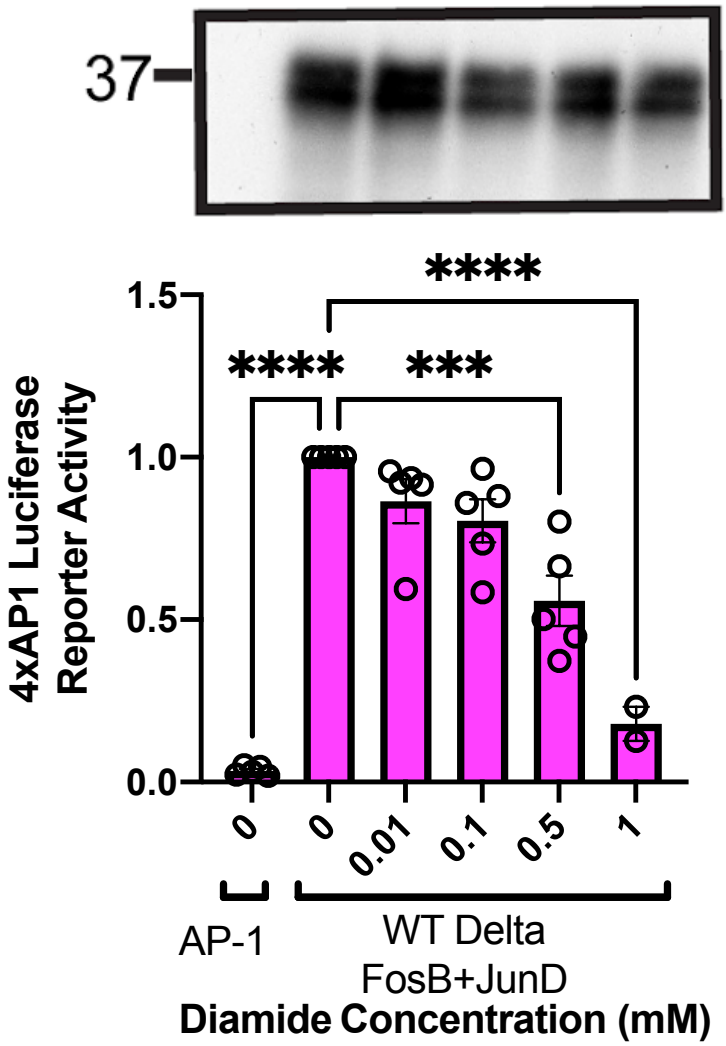

Figure 25 | WT Delta FosB heteromer binding decreases with increased oxidation. Compared to control AP-1 only transfected cells, WT DNA shows increased luciferase reporter activity, that is prevented with increased diamide concentration. The Western blot above reveals this is not due to cell death since unattached, dead cells were rinsed from the plate before scraping the attached, alive cells fro the plate and into a tube for processing.

FosB transactivation of an AP-1 consensus sequence was also dependent on redox

state in cells. To accomplish this, we transfected N2A cells with an artificial 4x AP-1 consensus sequence driving the reporter construct encoding luciferase, and we co-transfected the cells with WT Delta FosB and JunD. Then we added increasing concentrations of diamide to the cell medium, which we previously showed increased disulfide bond formation (see Figure 21 above). We found that increasing

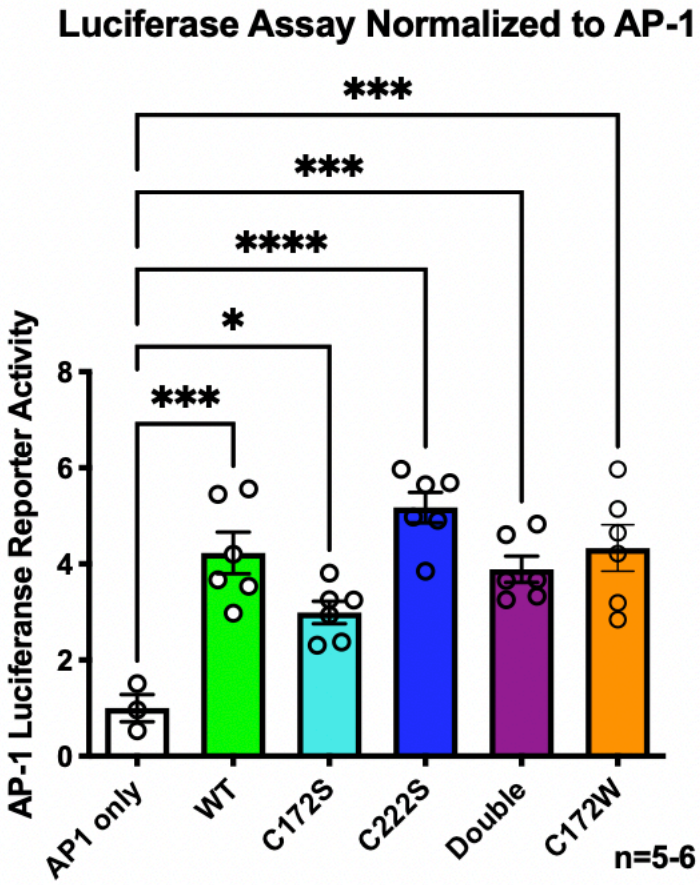

Figure 26 | Delta FosB homomer shows increased DNA binding under reducing conditions. Compared to control AP-1 only transfected cells, WT DNA shows increased luciferase reporter activity. This increase is maintained with the serine and tryptophan mutants, which are insensitive to redox state.

concentrations of diamide attenuated expression of the luciferase reporter construct without killing the cells or decreasing Delta FosB expression (Figure 25). We know that this was not due to cell death because prior to cells being scraped from the plate, they were rinsed with 1 X PBS. Since dead cells become unattached from the plate, this

wash would remove all loose,dead cells so that the only ones remaining for processing were alive. These data clearly indicate that, like regulation of DNA binding *in vitro,* Delta FosB heteromer transactivation of a reporter construct is redox sensitive in cells.

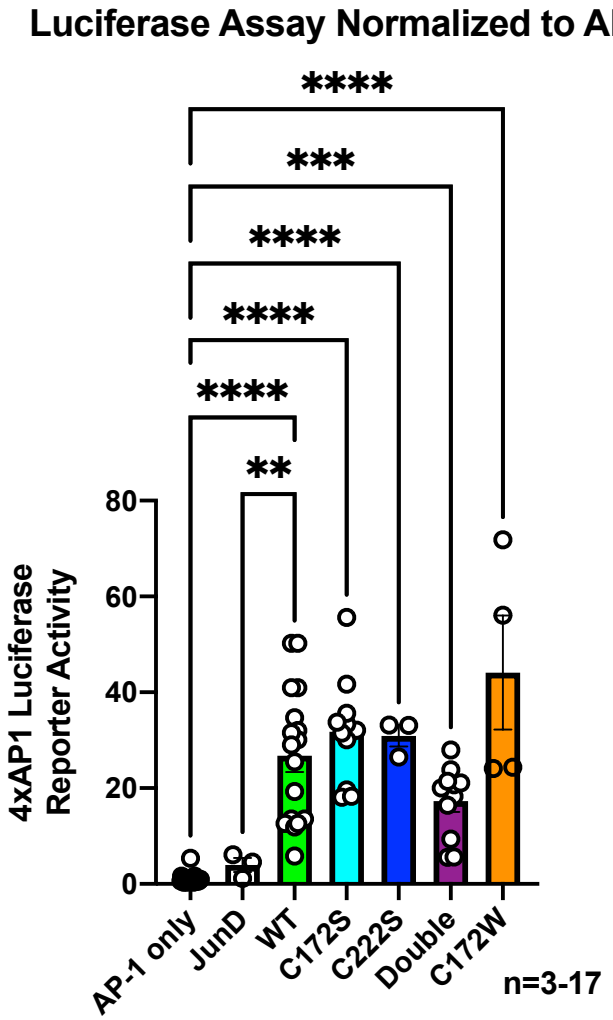

Figure 27 | Delta FosB heteromer shows increased DNA binding under reducing conditions. Compared to control AP-1 only transfected cells, WT DNA co-transfected with JunD shows increased luciferase reporter activity. This increase is maintained with the serine and tryptophan mutants, which are insensitive to redox state.

To investigate the roles of individual amino acids in Delta FosB's redox sensitivity in cells, we used the same mutants described in the previous chapter: C172S, C222S, and C172S/C222S; in addition to a C172S tryptophan (W) mutant which prevents

oxidation at C172 just like the serine. Looking at homomers luciferase activity, we demonstrated that, under basal conditions, without causing redox stress in the cells, all

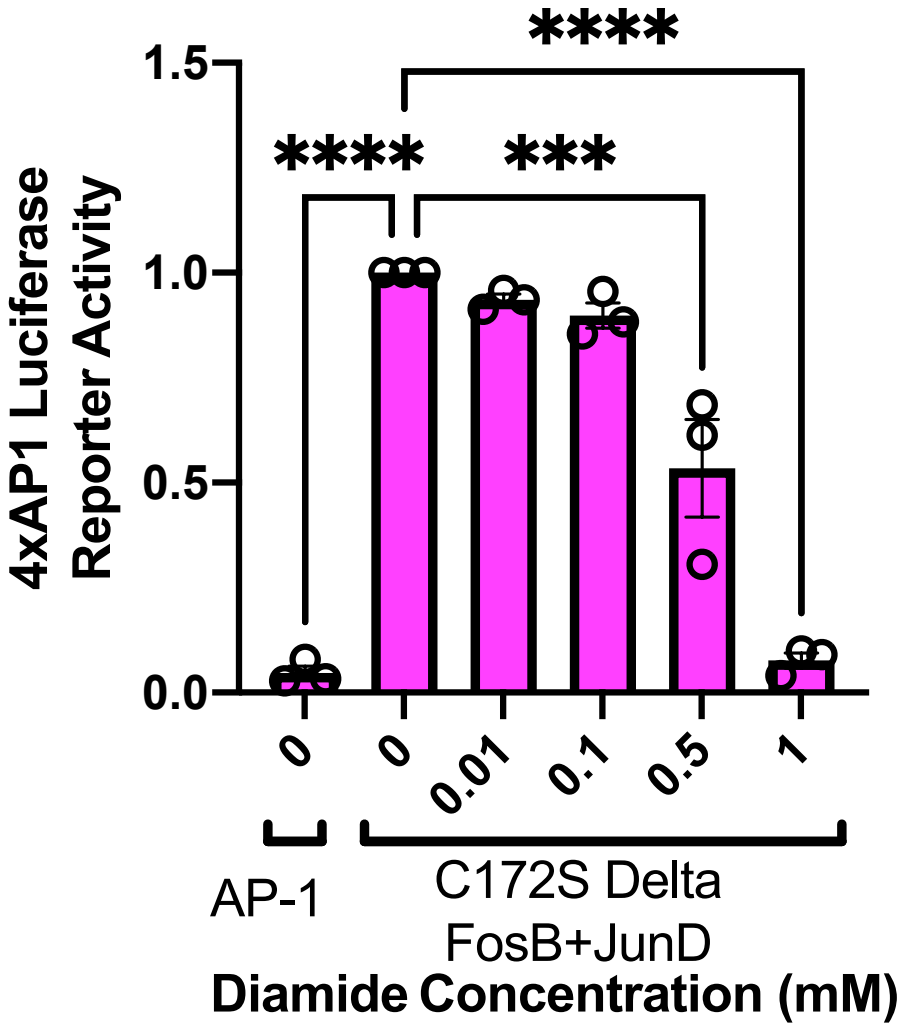

Figure 28 | C172S Delta FosB DNA binding is sensitive to oxidative stress. Compared to control AP-1 only transfected cells, C172S heteromers show increased luciferase reporter activity, that is prevented with increased diamide concentration.

these mutant constructs can drive AP-1 target gene expression just as well as WT Delta FosB (Figure 26). Next, we repeated this experiment looking at the heteromers effect on transcriptional activity without oxidant treatment by transfecting the cells with AP-1, WT, or mutant Delta FosB, and JunD. Just as with the homomers, we found that WT and

mutant Delta FosB co-transfected with JunD shows increased transcriptional activity

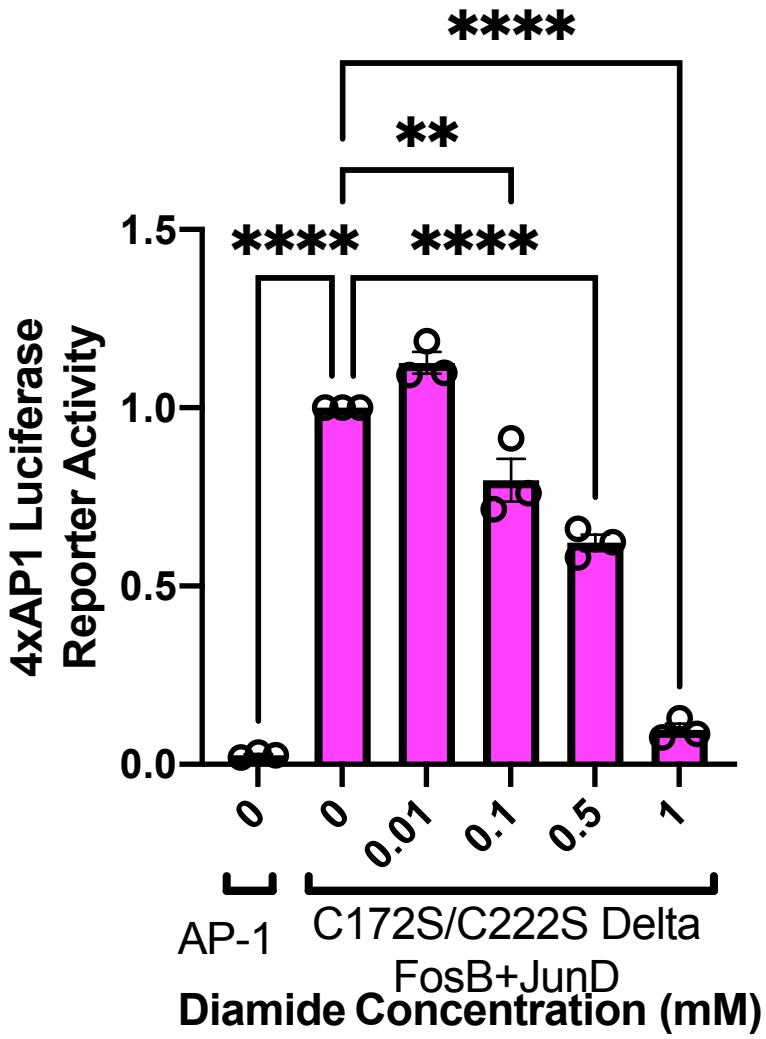

Figure 29 | C172S/C222S Delta FosB DNA binding is sensitive to oxidative stress. Compared to control AP-1 only transfected cells, C172S/C222S heteromers show increased luciferase reporter activity, that is prevented with increased diamide concentration.

under reducing conditions, relative to the AP-1 only control (Figure 27). As an added control, cells transfected with AP-1 and JunD only were analyzed. The data revealed that there is no significant increase in reporter activity relative to AP-1 only , and it is significantly decreased compared to cells transfected with Delta FosB. (Figure 27). This indicates that JunD is not driving the effects seen with Delta FosB.

We then tested the ability of the mutations to prevent the effects of redox state from reducing Delta FosB-driven AP-1 gene expression. We were surprised to find that neither the C172S nor the double C172S/C222S mutant prevented the ability of diamide to reduce Delta FosB driven AP-1 reporter activity (Figures 28 and 29). These data suggest that additional mechanisms beyond the C172 redox switch govern the ability of oxidizing conditions to prevent Delta FosB driven gene transactivation in cells.

Discussion

As was alluded to at the end of the previous chapter, the effect of redox state extends beyond that of just Delta FosB being able to form a complex with other proteins; it also affects the ability of the Delta FosB complex to bind to DNA. Just because the complex has a completed DNA binding domain does not ensure that it will successfully bind to the promoter regions of target genes. This chapter sought to investigate this phenomenon and to determine what, if any, effect redox state has on Delta FosB's ability to bind to DNA and transactivate AP-1 gene expression.

Extensive research has been conducted by our collaborators in Dr. Gabrielle Rudenko's lab, delving into the structural properties of Delta FosB using purified protein from insect cells[8,227]. This has led to the investigation of cysteine residue 172 as being a key cysteine mediating Delta FosB's ability to bind DNA. As discussed in the introduction, under reducing conditions, the lack of a disulfide bond at C172 allows the Delta FosB complex to bind to DNA and continue to regulate transcription; however, under oxidizing conditions, a kink forms at C172 which blocks the complex's DNA binding region from binding target DNA. The evidence for Delta FosB being sensitive to

oxidative state coincides with what was found in the N2a cells and the luciferase assays. Under reducing conditions, WT Delta FosB overexpressed with JunD and the AP-1 promoter shows increased AP-1 expression under reducing conditions, potentially because C172 is not oxidized. Oxidizing conditions prevented WT Delta FosB transactivation of AP-1, potentially due to a kink at C172, which was expected based on *in vitro* data. As mentioned, it was unexpected that the mutants did not rescue the transactivation activity of Delta FosB, specifically C172S, because it was created to prevent any kink from forming that could interfere with DNA binding. Perhaps there are other cysteine residues that come into play that prevent DNA binding. Or it is possible that the kink that forms *in vitro* at oxidized C172 is not the only factor that prevents DNA binding under oxidizing conditions. C222S was not looked at alone due to it being further from the DNA binding region and closer to the leucine zipper regions. Since this residue is thought to mediate the leucine zipper interaction between Delta FosB protein and its binding partner in a “zip lock” fashion[8], it is unlikely to have a role in mediating DNA binding. In addition to the luciferase assay data, we also know that any decreases in DNA binding are not due to cells being killed by the oxidant because any unattached, dead cells were rinsed from the plate prior to processing of the attached, live cells. The processed live cells used for Delta FosB Western blots using the luciferase assay lysates showed similar expression of bands across all concentrations of oxidant used, indicating cell health and Delta FosB expression is preserved.

As with the previous chapter, homomers can also form and bind to DNA *in vitro*[8]; however, little is known about which cysteines are important in this interaction as well as how the complex binds to DNA *in vitro* let alone *in vivo*. There has yet to be evidence

that a kink forms under oxidizing conditions or that homomers are even redox-sensitive at all (see future directions). Thus, we chose to focus this chapter of my dissertation on exploration of the heteromer.

Per usual, there are some limitations to these experiments that must be considered when concluding that Delta FosB DNA binding is sensitive to *in vivo* redox state. As mentioned in the previous chapter, it is difficult to compare the levels of oxidative stress that occur in the brain of a person with AD to the levels of oxidant used to induce stress in N2a cells. However, it is possible to induce oxidative stress in the brains of mice, and mouse models of AD are widely available. Future studies will take advantage of these tools to explore how oxidative stress and AD pathophysiology alter Delta FosB target gene binding and expression in the mouse brain.

CHAPTER 5: SUMMARY, FUTURE DIRECTIONS, AND FINAL SUMMARY

Summary

This dissertation investigated how oxidative stress can drive changes in gene expression seen in disease states like AD, specifically through alterations in the structure-function relationship of transcription factor Delta FosB and its *in vivo* redox sensitivity. Although this research can be applied to many different Delta FosB-dependent diseases such as depression, epilepsy, or addiction, we specifically focused on Delta FosB in the context of AD. Overall, we found that redox sensitivity of Delta FosB found previously *in vitro* also applies to Delta FosB in *in vivo.*

We first delved into how Delta FosB's structure is influenced by the level of oxidative stress, specifically how the ability of it to form disulfide bridges mediates its ability to bind to and form complexes with other proteins. This is a necessary step in Delta FosB's ability to drive changes in gene expression because Delta FosB alone lacks a complete DNA binding domain, thus it must bind to a partner protein, like JunD, so that a complete DNA binding domain can form and fit into DNA binding regions on genes of interest. Dr. Rudenko's lab has already investigated the *in vitro* structure of Delta FosB and how it binds to its partner protein JunD[227] (heteromers) as well as other Delta FosB proteins[8] (homomers). Previous studies have provided evidence that cysteine residues play a major role in this complex and aid in its stability depending on reducing or oxidizing conditions. Specifically, studies from this lab have suggested that C222 of Delta FosB helps to stabilize the leucine zipper interaction between Delta FosB and its partner in a "zip lock" fashion[8].

Using cellular and mouse brain studies, we found that the Delta FosB heteromer can form covalent complexes with other proteins under oxidizing conditions via oxidation of cysteine residues and resulting disulfide bridges holding the complex together. However, under reducing conditions, these bridges no longer form, and SDS-stable complex formation is attenuated. This was demonstrated in both N2a cells and mouse hippocampus through chemical manipulation of redox state. Using the redox-insensitive Delta FosB mutants, we found that two of the four cysteines found in Delta FosB help to mediate Delta FosB interaction under oxidizing conditions. Preventing oxidation of C172 by mutating it to a serine results in decreased complex formation and this decrease is exacerbated when C222 is mutated to a serine as well. C222 alone does not significantly decrease complex formation. Looking at the homomer in this context, we found evidence to support that there are other residues aside from C172 and C222 that drive SDS-impervious complex formation, consistent with the homomer's different structure and altered interacting regions (see Figure 16 above). Nevertheless, **the key advance stemming from these experiments is the demonstration that Delta FosB does indeed form disulfide bridges in cells and in the mouse brain in a manner dependent on redox stress**, implying that the structural data obtained from X-ray crystallography of purified proteins is physiologically relevant and can be used to advance our understanding of the role of Delta FosB in disease states.

After the Delta FosB complex has formed, it must then bind to the DNA and transactivate gene expression to have physiological effects. We wanted to know how redox state affects this process. Again, Dr. Rudenko's lab provided *in vitro* evidence with purified proteins that DNA binding is indeed redox-sensitive and that oxidizing

conditions limit DNA binding whereas reducing conditions allow it. This was done using FP assays, which provide information on the DNA binding capability of the purified protein. These assays showed that under oxidizing conditions, the Delta FosB heteromer is unable to bind to target genes, whereas the homomer binds DNA just as well but loses its specificity for binding to AP-1 consensus sequences. This would suggest that in diseases of oxidative stress, there could be aberrant gene expression not only due to proper Delta FosB target genes not being enhanced/repressed by Delta FosB binding but also because there may be many random genes being enhanced or repressed by non-specific Delta FosB binding that normally are not targets.

Furthermore, Dr. Gabrielle Rudenko's lab was able to delineate why, at least in part, Delta FosB/JunD heteromers are sensitive to redox state. This is because they interact in a way that causes a kink in Delta FosB's structure at C172 under oxidizing conditions, thereby preventing it from binding to DNA[227]. Conversely under reducing conditions, C172 does not get oxidized, and thus the kink is not formed, and the complex is freely able to bind to DNA. This parallels nicely with the results I obtained from cell culture *in vivo* because the luciferase assays revealed that oxidative stress decreases AP-1 transcriptional activity of the overexpressed heteromer. Taking this a step further, we investigated the importance of Delta FosB cysteine residues C172 and C222 by looking at how mutating one or both impacts this redox regulation. Under reducing conditions, mutant Delta FosB had normal AP-1 transactivation comparable to the WT which was expected as neither mutation was expected to directly prevent DNA binding or transactivation. Surprisingly, C172 and the double mutant (C172S/C222S) did not prevent the decrease in Delta FosB heteromer DNA binding under oxidizing

conditions. This could be due to other residues contributing to redox sensitivity *in vivo*, or because AP-1 target expression requires the function of other proteins (polymerase or other transactivators) that are also redox sensitive. Further experiments will be needed to understand these results.

Overall this research provided two major contributions: 1) Delta FosB forms disulfide bridges in cells and in the mouse brain; and 2) Delta FosB DNA binding and target gene activation are redox-sensitive in cells. It provided further evidence for the *in vitro* and new evidence for the *in vivo* redox sensitivity of the Delta FosB heteromer and touched on the redox insensitivity of the Delta FosB homomer. It also shed light on the roles of C222 and C172 as important residues involved in Delta FosB's response to redox state. Evidence presented in this dissertation and past literature support Delta FosB as a redox-sensitive protein whose structure and function can be altered in diseases of oxidative stress, like AD, thus potentially leading to aberrant and pathological gene expression.

Collaborative Experiments in Progress

A critical next step in this project is to determine the target genes of Delta FosB under oxidizing vs reducing conditions in both cells and in mouse brain. To this end, I prepared N2A cells transfected with Delta FosB and JunD (or non-transfected controls) and treated the cells with 1.0mM diamide (or vehicle) for 2 hrs, conditions I have shown reduce Delta FosB-dependent gene expression without killing cells (Figure 25). I then harvested these cells and sent them to the Mount Sinai School of Medicine, where our collaborator Dr. Eric Nestler is performing Cleavage Under Targets and Release Using

Nuclease (CUT&RUN) using a Delta FosB antibody. CUT&RUN is a chromatin profiling strategy in which antibody-targeted controlled cleavage by micrococcal nuclease releases specific protein-DNA complexes into the supernatant for paired-end DNA sequencing[319]. Unlike Chromatin Immunoprecipitation (ChIP), which fragments and

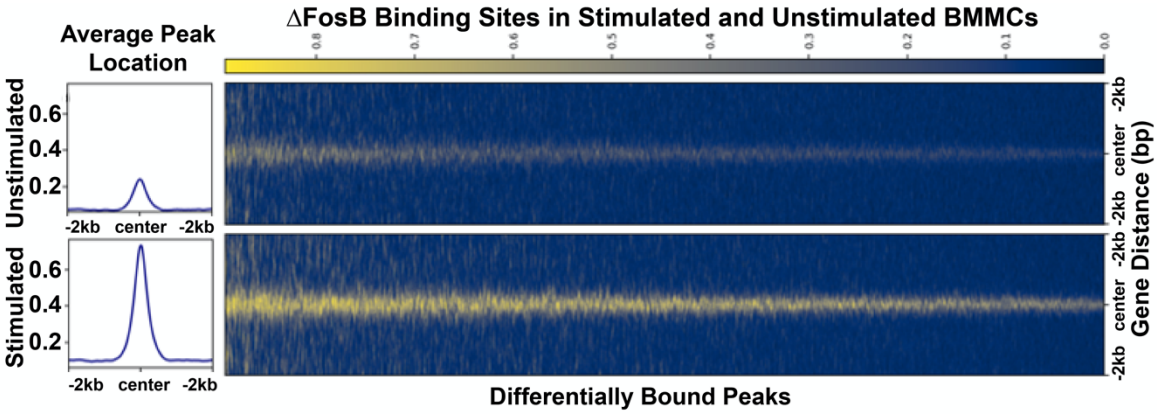

Figure 30 | CUT&RUN reveals Delta FosB gene targets in BMMCs. BMMCs were stimulated using IgE-DNP, and cell pellets were subjected to CUT&RUN using a Delta FosB antibody. Data demonstrate increased Delta FosB binding to gene promoters following stimulation.

solubilizes total chromatin, CUT&RUN is performed *in situ*, allowing for both quantitative high-resolution chromatin mapping and probing of the local chromatin environment. We expect the results of this assay to show us Delta FosB binding regions in the DNA of N2A cells, and we expect that under basal conditions, these will include many gene promoters with AP-1 consensus sequences. Indeed, that is exactly what we see when applying this technique to cultured mast cells in a related project (Figure 30). However, we expect that the CUT&RUN performed on N2A cells treated with diamide will reveal binding to more random chromatin locations, with significantly less enrichment for AP-1 consensus sites.

To determine whether oxidative stress alters Delta FosB gene target binding in the mouse brain, we are taking a similar approach. We treated WT adult male C57 mice with 0.11mg/ml potassium dichromate in their drinking water (or untreated water as a control) for seven days, a paradigm known to cause oxidative stress in the mouse

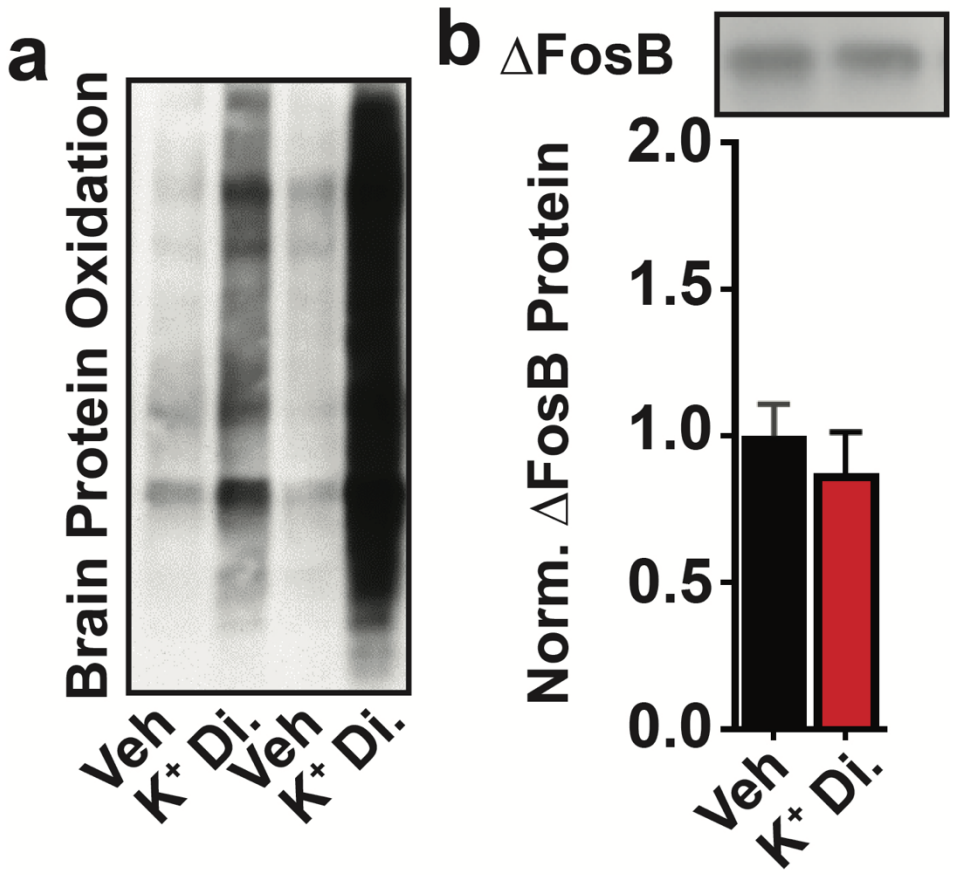

Figure 31 | Increased oxidative stress in mouse brain. a) Adult male mice exhibit protein oxidation in hippocampus (Protein Carbonyl Assay Kit, abcam ab178020) after treatment with $K_2Cr_2O_7$ (systemic oxidizing agent, 25 mg/kg/day oral). b) Delta FosB expression is unaffected by treatment with $K_2Cr_2O_7$ (right).

brain[320]. This paradigm did indeed cause protein oxidation in the brains of mice without changing the overall levels of Delta FosB expressed in the mouse hippocampus (Figure 31). We have sent hippocampus and nucleus accumbens punches from these mice to Dr. Nestler's lab for CUT&RUN analyses, and we again expect that oxidative stress will reduce the binding of Delta FosB to chromatin sites containing AP-1 consensus

sequences and increase binding to non-specific sites. We will correlate the results of these assays with Delta FosB ChIP target gene data from J20 AD model mice published by our collaborator Dr. Jeannie Chin[5,321], and we expect that oxidative stress caused by potassium dichromate treatment may cause a similar pattern of Delta FosB gene binding to that reported in AD model mice.

Future Directions

The data presented in this dissertation and in the literature suggest that Delta FosB's role in disease is complex. Thus, our results beg for further research to be done in four main areas: 1) Continued biochemical dissection of Delta FosB's role in diseases associated with oxidative stress; 2) Manipulation of Delta FosB redox state in preclinical models; 3) Identification of Delta FosB redox switch-targeting compounds; 4) Investigation of Delta FosB in other redox-sensitive diseases.

Continued Biochemistry

This dissertation investigated the redox sensitivity of both Delta FosB heteromers and homomers. Although Delta FosB is traditionally known to favor heteromer formation particularly with JunD and has a higher affinity for forming heteromers *in vitro*[220], under certain concentrations Delta FosB homomers have been formed *in vitro*. Knowledge of Delta FosB homomer formation, DNA binding, and function is limited as data supporting the ability of Delta FosB to form homomers is relatively new. So far, there is evidence to suggest that Delta FosB homomer formation and DNA binding is possible; however, Delta FosB proteins interact with each other to

form a complex in a vastly different arrangement compared to heteromers[8]. Furthermore, this is restricted to data that has been collected from experiments done *in vitro* using purified protein and our more recent cell culture studies. Our cell culture studies looking at DNA binding support that Delta FosB homomers appear to be redox-insensitive. However, aside from what has been laid out in this dissertation, the prevalence of Delta FosB homomers in the brain is unknown. This is an issue that needs addressing, as Delta FosB appears in the brain at much higher levels in both animal models of and patients with AD[5]. Although the exact concentration is unknown, the concentration needed for Delta FosB to form homomers becomes more likely under these conditions, and other binding partners like JunD may become saturated. Thus, better understanding of the structure and function of Delta FosB homomers becomes increasingly important.

The first experiment I would conduct to address this issue would be to determine how much Delta FosB protein is needed for homomer formation to be favored over heteromer formation *in vivo*. This would be conducted by transfecting N2a cells with different concentrations of Delta FosB under oxidizing conditions and performing Western blots to determine the amount of homomer band (~74kDa) to heteromer band (87kDa) that occurs at each condition. I could also do this in mouse brain by injecting different amounts of Delta FosB virus into the hippocampus of HA-tagged FosB mice then performing an immunoprecipitation where I use a HA antibody to pull down the HA-tagged Delta FosB then probe for Delta FosB using another antibody to see if there are any Delta FosB homomer interactions. I would expect that as I increase concentrations of Delta FosB in cell culture, the ratio of homomer band expression compared to

heteromer band would increase. In the mouse brain, I would except increasing concentrations of Delta FosB would also lead to an increased expression of a Western blot band at the homomer molecular weight and less at the heteromer.

Once I have determined the correct concentration of Delta FosB needed to saturate other binding partners and ensure enough homomer formation to overshadow the heteromer effects, I can repeat the same structural and functional experiments I performed in this dissertation but using an adjusted concentration of Delta FosB. For example, I could mutate other residues that previous literature has deemed important for Delta FosB oligomerization in cell culture and/or mice and determine if they play an important role in the ability of the Delta FosB homomer to form based on how much of the homomer Western blot band is detected under non-reducing conditions. Additionally, I could use this Delta FosB concentration transfected in cells and look at DNA binding through luciferase assays to see the amount of AP-1 promoter activity relative to the heteromer. Finally, I could do a CUT&RUN assay with cells transfected with the Delta FosB concentration to determine if the homomer has altered target gene expression relative to the heteromer (which would need to be done as well), perhaps in the context of genes altered in AD.

Finally, although we have collected evidence that the homomer is redox-insensitive, it would be good to re-test this redox sensitivity using the proper concentrations of Delta FosB necessary for homomer formation. It is likely that since the experiments done in this dissertation had a high concentration of Delta FosB expressed that would have saturated any native binding partner amounts already, we would get the same results, but it would be best to redo transfecting the N2a cells with Delta FosB and

using different redox treatments to determine if oligomerization through disulfide bond formation and luciferase assay DNA binding results are redox-sensitive.

Manipulation in preclinical models

The scope of this dissertaion uses N2a cells and mice to assess the response of Delta FosB to oxidative stress. In both cases, under states of oxidative stress SDS-stable oligomerization of Delta FosB with partner proteins occurs, indicating the presence of disulfide bonds, but DNA binding and transactivation of AP-1 gene expression is prevented. Using redox-sensitive mutants of Delta FosB manipulates heteromer response by preventing disulfide bond formation specifically at C172 and/or C222. Although this is important in that is reflects the *in vivo* redox sensitivity of Delta FosB in both N2a cells and mice, these cells and mice are not models of AD. For example the N2a cells did not overexpress amyloid beta protein nor did the mice possess an AD phenotype. Thus, a next step would be applying what we know about the redox sensitivity of Delta FosB in non-pathologic cells and mice to determine if there is a redox-dependent way to reverse the pathological phenotype seen in models of AD. I will also note that the suggested experiments would be focused on Delta FosB heteromers since data presented in the previous chapters support that the homomer may has different interactions with itself and with DNA. More information about the structure and DNA binding properties of the homomer is needed before steps are taken to alter these properties *in vivo*.

Cellular AD models

When considering methods to elucidate the effect of redox state of Delta FosB in models of AD, several methods present themselves. Starting with N2a cells, we could use cells that have been treated with purified amyloid beta, or transfected to express amyloid beta 42, to mimic AD conditions. Another option would be to collect stem cells from patients with AD and reprogram back into a neuronal cell type, however this is a complicated and delicate process, thus using the amyloid beta model would most likely be the most promising option. Once the cellular model is established, we can then transfect the cells with WT Delta FosB and see how the AD model affects Delta FosB's ability to bind DNA using a luciferase assay. It is expected that Delta FosB DNA binding would be reduced or prevented[144] based on the state of oxidative stress that the cells are under shown in previous literature using these models. This likely would reflect the kink at C172 forming and conformationally hindering DNA binding. Next, we could see if we could rescue DNA binding by treating or transfecting the AD-model cells with some type of antioxidant. Since this could prevent oxidation at the "redox switch," we would expect DNA binding to be preserved even under the cellular state of oxidative stress since no kink will form. Additionally, we could perform CUT&RUN experiments on lysates collected from these cells to look at Delta FosB-target gene binding. It is likely that the AD cells transfected with WT Delta FosB will show aberrant target gene expression. This aberrant expression could be reversed if we add some type of antioxidant, and we would expect to see similar results to the N2a cells that were not treated with any oxidant. Perhaps antioxidants can rescue any aberrant effects on DNA binding or Delta FosB-target gene expression that is seen in the cells modeling AD.

Additionally, we could also test some of the compounds that our group is currently producing in collaboration with the Rudenko lab that specifically target the DNA binding region of Delta FosB (see section on targeting compounds below)

AD mouse models

A critical next step would be testing this in a mouse model of AD, for example the J20 model. This model overexpresses human amyloid precursor protein (APP) and contains two mutations linked to familial AD[153]. These mice present with amyloid beta in the hippocampus between six and 36 weeks of age and develop plaques within five to seven months[153]. Additionally, they display impairments in learning and spatial memory by 4 months[322]. Of course, this model shows increased levels of oxidative stress[323]. Not only could we test the biochemical effects of the oxidative state of these mice on Delta FosB structure and function, but we could also test behavioral outcomes. Starting with the biochemical evidence, we could do an immunoprecipitation as in chapter three, to determine if Delta FosB is able to interact with other binding partners. This would require crossing the HA-tagged Delta FosB mice with the J20 mouse line so that Delta FosB would have the HA tag in the AD mouse line, and indeed we have begun these crosses in our lab already. It is likely that we would see protein interactions, likely with JunD and/or with other FosB proteins, that would indicate disulfide bridge formation. The state of oxidative stress present in these mice is hypothesized to be elevated enough to drive redox bridge formation and show similar results to the HA-tagged Delta FosB mice that were treated with diamide and non-reducing Laemmli buffer, but in this case the oxidation state of HA-Delta FosBxAD mice would be inherent.

To examine DNA binding and target gene activation, we could also collect hippocampal punches from the HA-Delta FosBxAD mice and perform a CUT&RUN assay to look at Delta FosB-target genes and compare it to HA-Delta FosB mouse brain sample. We would expect altered Delta FosB-target gene expression in the HA-Delta FosBxAD mice, some of which could be like what might be seen in amyloid beta treated N2A cells as well as AD target genes in the literature[5,321].

Switching gears to looking at the behavioral effects in these mice, we could see if we can rescue the behavioral impairments seen in the AD mice by using the Rudenko lab compounds. A variety of behavioral paradigms could be used to assess the cognitive effects in mice (see introduction for examples), but I would start with the Morris Water Maze (MWM). For this experiment I would compare the control AD mice to AD mice that received the Delta FosB-targeting compound. For the control AD mice, I would expect there to be impairments in learning and memory as shown previously[5], which would be indicated by prolonged time to find the platform during the trial tests of the MWM and decreased time spent in the target quadrant during the probe test. I would expect mice that receive redox switch-targeting compounds to reverse the cognitive impairments seen in the AD mice, which would be shown by decreased time to find the platform during the trials and more time spent in the target quadrant of the pool during the probe test. Other behavioral tests could be done as well including contextual fear conditioning and novel object recognition discussed previously in the introduction. Regardless of the paradigm being used, it is hypothesized that the result would be the control AD mice showing cognitive impairments and the compound-treated mice rescuing the behavioral deficits.

Identification of Delta FosB-targeting compounds

The evidence in this dissertation provides further support that targeting Delta FosB could provide a major therapeutic pathway for diseases like AD, which could completely change how the medical world is currently pursuing AD intervention. Many of today's treatments for AD are aimed at slowing disease progression rather than prevention. Perhaps targeting Delta FosB and preventing downstream changes in gene expression early on could prevent a patient's cognitive condition from deteriorating. As mentioned in the previous section, we, and our collaborators in the Rudenko lab have already started designing and creating compounds that specifically target the region

near the redox switch, C172, of Delta FosB (Figure 32). The idea is like what we did initially with the C172S mutant, in that by preventing oxidation of Delta FosB at C172 we

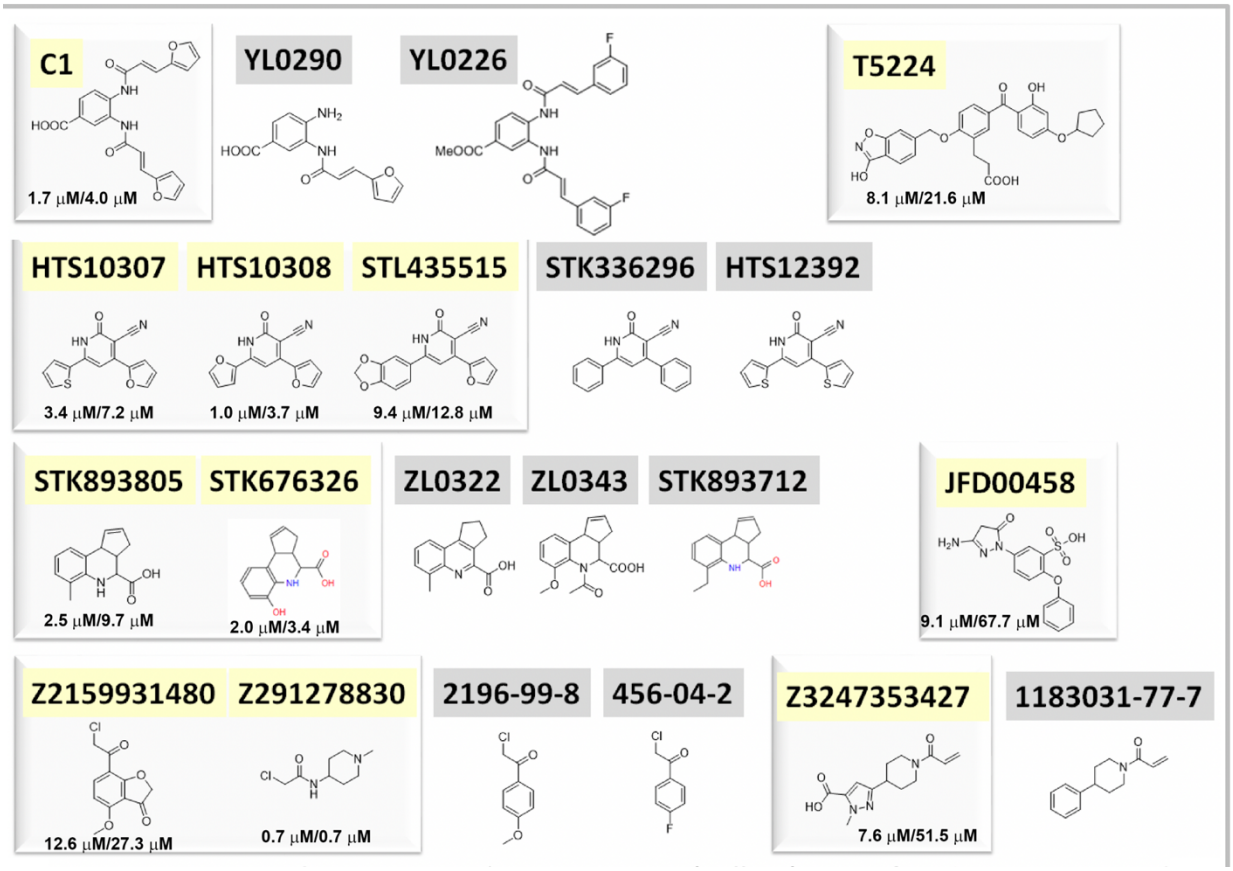

Figure 32 |Compounds targeting Delta FosB. Yellow compounds represent active compounds, IC_{50} for the heteromer Delta FosB/JunD and the homomer Delta FosB/Delta FosB, respectively. The controls are inactive compounds in grey, IC_{50}>>100 uM.

can prevent the kink that hinders Delta FosB from binding to DNA. We hypothesize that this would prevent the changes in gene expression that occur in AD and other states of oxidative stress.

Both our lab and Dr. Eric Nestler's lab, another collaborator, have already started testing these compounds in cell culture and conducting luciferase assays. Dr. Nestler's lab has been treating cells expressing the AP-1 promoter with the drug of choice under basal conditions, meaning with only native Delta FosB, and has determined that some

of these drugs increase or decrease reporter activity (unpublished). These data are limited in that they do not allow us to conclude that it is Delta FosB and not other Fos family proteins that are driving the effect. That's where work in our lab comes into play. We are utilizing our N2a cell line and transfecting cells with AP-1, Delta FosB, and JunD cDNA then treating the cells with the compounds to determine whether these compounds work through inhibition of Delta FosB, and not some off-target effect. In other words, because we are assessing changes the compound is having on Delta FosB ability to drive AP-1 promoter activity specifically, we know any effects are occurring directly through Delta FosB. I have already tested one compound, T5224, and determined that it decreases Delta FosB DNA binding, which agrees with what was found in the Nestler lab when the same compound was tested (Figure 33). As was shown earlier in this dissertation, I could also collect cell lysates treated with the

compound at a concentration that gives the desired effect and send them out for CUT&RUN analysis to determine if there are differences in Delta FosB-target gene expression relative to Delta FosB controls and to Delta FosB under oxidizing conditions.

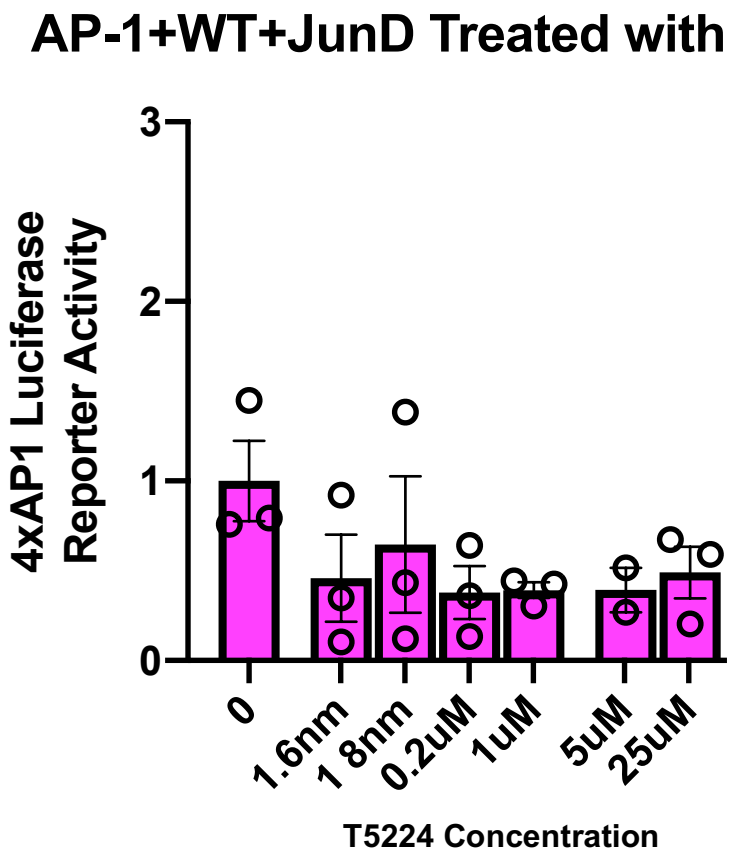

Figure 33 | T5224 decreases Delta FosB transcriptional activity. N2a cells transfected with AP-1, WT Delta FosB, and JunD cDNA were treated with increasing concentrations of compound T5224, which led to decreases in Delta FosB transcriptional activity.

I would expect that compounds that increase or decrease Delta FosB transcriptional activity would lead to alterations in gene expression relative to cells that were not treated with the compound. Determining the effects these compounds have on Delta FosB transcriptional activity supports the next step I would take, which would be utilizing the compounds to correct or prevent cognitive deficits in models of AD.

As mentioned in the previous section, there are two models of AD I would use: a N2A cellular model overexpressing amyloid beta and the J20 mouse line. For the AD cellular model, I would treat cells with my compound of choice, then collect lysates from treated and nontreated cells for cut and run analysis. I would expect there to be changes Delta FosB binding to target genes based on compound treatment, perhaps preventing the reduced target gene binding caused by APP expression and/or oxidizing conditions. Using the mouse models would be especially informative. I would stereotaxically inject the compounds directly into the hippocampus of AD mice. Then I would put these mice through a battery of memory paradigms to assess whether there are improvements or further deficits in the cognitive function of the AD mice receiving the compound versus the mice that did not. Another, less invasive avenue would be to add the drug to the drinking water of the mice or dilute it in saline and inject it into the mice interperitoneally; however, this would then require us to determine whether the drug is palatable (or aversive), bioavailable, and whether it is able to cross the blood brain barrier. Overall, utilizing AD models to assess drug efficacy would be extremely beneficial due to the translational relevance.

Although Delta FosB-targeting compounds could be an additional avenue for therapeutic intervention, they do not come without complications. As discussed in the introduction, both too much or too little Delta FosB can lead to impairments in learning and memory. Thus using a compound that alters Delta FosB function, whether through enhancement or repression of its function, could drive cognitive impairments. One suggestion for this would be to have drugs that target downstream Delta FosB-target genes rather than Delta FosB itself. A very simplified example is the Delta FosB target

calbindin, a protein that plays a role in cognitive function, shows decreased levels in patients suffering from cognitive decline, perhaps increasing expression or function of that protein by other means could help rescue some of the cognitive deficits. However, I say simplified because it is not likely that only one protein will be the “cure-all” for AD. Another suggestion would be to use compounds that are specific for Delta FosB heteromers vs. homomers. We know that in AD, Delta FosB is highly upregulated[5]. It is at high concentrations that Delta FosB starts to form homomers because the amount of other binding partners becomes saturated. Thus, perhaps targeting heteromers or homomers differentially could lead to different therapeutic effects.

Delta FosB in other redox-sensitive diseases

One of the most exciting things about the research conducted in this dissertation is that it not only applies to AD, but other redox-sensitive diseases that Delta FosB plays a role in including epilepsy[**5**], depression[251], and addiction[324]. All these diseases are under a state of oxidative stress and have increased levels of Delta FosB that we now know is redox-sensitive, and thus could be driving the changes in gene expression in these diseases in response to oxidative stress. Since AD was already discussed during this dissertation, I will focus on epilepsy, depression, and addiction.

Epilepsy

Previous studies have shown that Delta FosB is induced by seizure activity in the brain of rats modeling epilepsy, specifically in areas associated with seizure generation and propagation[5,256,318]. This could help explain the increases in Delta FosB in AD,

because patients with AD exhibit subclinical epileptiform activity in the brain early in the disease. Further, work from our lab suggests that Delta FosB is a negative feedback mechanism that is induced by strong neuronal activity but works to reduce excitability of glutamatergic neurons[285,325]. Thus, drugs that could increase the function of Delta FosB could act to reduce neuronal excitability and prevent seizures.

To further assess Delta FosB's pertinence to this disorder, I would treat mice with pilocarpine (a seizure stimulating drug) to mimic epilepsy. Prior to treatment, half of these mice would stereotaxically receive bilateral injections of WT Delta FosB, C172S, C222S, or C172S/C222S, into the hippocampus. I would record seizure activity to assess number of seizures and severity then sacrifice and collect hippocampal brain punches at various time points starting with 24 hours after the pilocarpine injection. I could not collect brain punches any sooner than 24 hours because this gives time for other FosB isoforms induced by the initial stimulation to decrease, and only the stable Delta FosB protein to persist. I would expect that overexpression of any form of Delta FosB would reduce seizure activity. Next, I would conduct Western blot on some of the hippocampal punches to verify that Delta FosB is increased in the mice treated with pilocarpine compared to non-treated mice. I would then prepare the rest of the punches for CUT&RUN analysis to determine if overexpressing different mutants of Delta FosB lead to alterations in gene expression. I would expect the mice treated with WT Delta FosB to have aberrant Delta FosB-target gene expression like that which has previously been published in models of Epilepsy[5]. The C172S and C172S/C222S mutant should show gene expression that more closely resembles that of non-treated mice, because Delta FosB in these instances should be insensitive to the state of oxidative stress

induced by the seizures[291]. I would have another set of mice under the same conditions that would go through behavioral tests to assess cognitive function since diseases associated with epileptiform activity show deficits in cognition[291]. These would include tests previously mentioned, the Morris Water Maze, novel object recognition, and contextual fear conditioning. In all cases I would expect pilocarpine treated mice to have increased cognitive deficits compared to non-treated mice. Furthermore, mice containing redox-insensitive mutants of Delta FosB may have improved cognitive function compared to controls or mice receiving WT Delta FosB.

Depression

Depression is another disease associated with oxidative stress[76-78]. Additionally, it is a disease in which Delta FosB has been found to play a major role. Delta FosB is not only induced in the hippocampus by antidepressants but overexpressing it in mice has also been found to be pro-resilient in response to chronic stress, a key risk factor for depression[251]. To assess whether the redox state of the brain is driving behavioral responses to chronic stress, and thus depression susceptibility, I would use a similar approach to what was discussed in the Epilepsy section. I would inject WT Delta FosB and the cysteine mutants into the hippocampus of mice. Next, I would expose the mice to a chronic stress paradigm, such as chronic social defeat stress. This paradigm involves exposing male C57 mice to retired male CD1 breeders that are generally more territorial and aggressive. Over the course of 10 days, the C57 mice will share a cage with the retired breeders with only a perforated plexiglass wall between the two mice. The divider will then be removed from the cage so that the mice are able to directly

interact for a given period each day. This interaction will be monitored to assure that the C57 mouse does not get hurt. After the 10 days is up the mice will then go through a series of paradigms to assess their level of depression- and anxiety-like behaviors. This would include social interaction for reduced mood and social withdrawal and the elevated plus maze (EPM) for anxiety-like behavior. Since Delta FosB has a pro-resilient effect, I would expect the mice with overexpressed WT Delta FosB to have less depression- and anxiety-like symptoms (i.e. spend more time interacting with the target mouse and more time in the open arms of EPM) and mice that are insensitive to redox state to have pro-depressive effects (i.e. more time spent in the corners and less time spent in the open arms). In other words, if it is the state of oxidative stress that is driving Delta FosB to function in a manner that creates a pro-resilient effect, then altering this ability using the redox-insensitive mutants would prevent resilience.

Addiction

Addiction is the final disease I will touch on and it is different in the other redox-sensitive diseases mentioned previously because in this case the target brain region is the nucleus accumbens (NAc) rather than the hippocampus. However, it is still under a state of oxidative stress which is caused by drugs of abuse[326]. Delta FosB expression is induced by drugs of abuse and actually enhances sensitivity to the behavioral effects of addiction including drug seeking behavior[324]. Again following a similar pattern to the previous diseases mentioned, I would overexpress WT or mutant Delta FosB in the NAc of mice. Next, I would put them through chronic cocaine exposure for a period of 10 days and test the behavioral phenotypes of these mice. Focusing specifically on

cocaine as an example drug of abuse, studies have already shown that when WT Delta FosB is overexpressed, it leads to increased locomotor sensitization with repeated cocaine administration, increased self-administration of low doses of cocaine, increased preference for cocaine in progressive ratio assays, and increased rewarding responses to cocaine in place-conditioning assays[250,324]. Thus if I test these same paradigms, I expect the same results when I overexpress WT Delta FosB. Conversely, overexpressing the mutants that are insensitive to redox state would lead to the opposite effects; decreased sensitivity to cocaine including decreased locomotor sensitization, decreased self-administration of low doses, and decreased preference for cocaine in the progressive ratio assays. Additionally, assessing whether Delta FosB-target gene expression that is altered in addiction can be rescued, I would investigate if overexpressing redox-insensitive mutants of Delta FosB returns the gene expression levels back to basal levels.

Final Summary

This dissertation presents evidence for the *in vitro* and *in vivo* redox sensitivity of Delta FosB regarding covalent complex formation and AP-1 gene transactivation. These findings elucidate the complex nature of Delta FosB's structure and function and provide a possible explanation for how oxidative stress can drive long term changes in gene expression seen in many neurological disorders (like AD) through Delta FosB. It is to be hoped that these studies will set the groundwork for further exploration of Delta FosB's ability to behave as a sustained molecular switch in disease and to be a potential

therapeutic target for AD (and possibly epilepsy, addiction, and/or depression) in the future.

APPENDIX

APPENDIX

A key tool that I helped to produce and will play a huge role in the future directions of the project are viral vectors for expression of mutant Delta FosB constructs

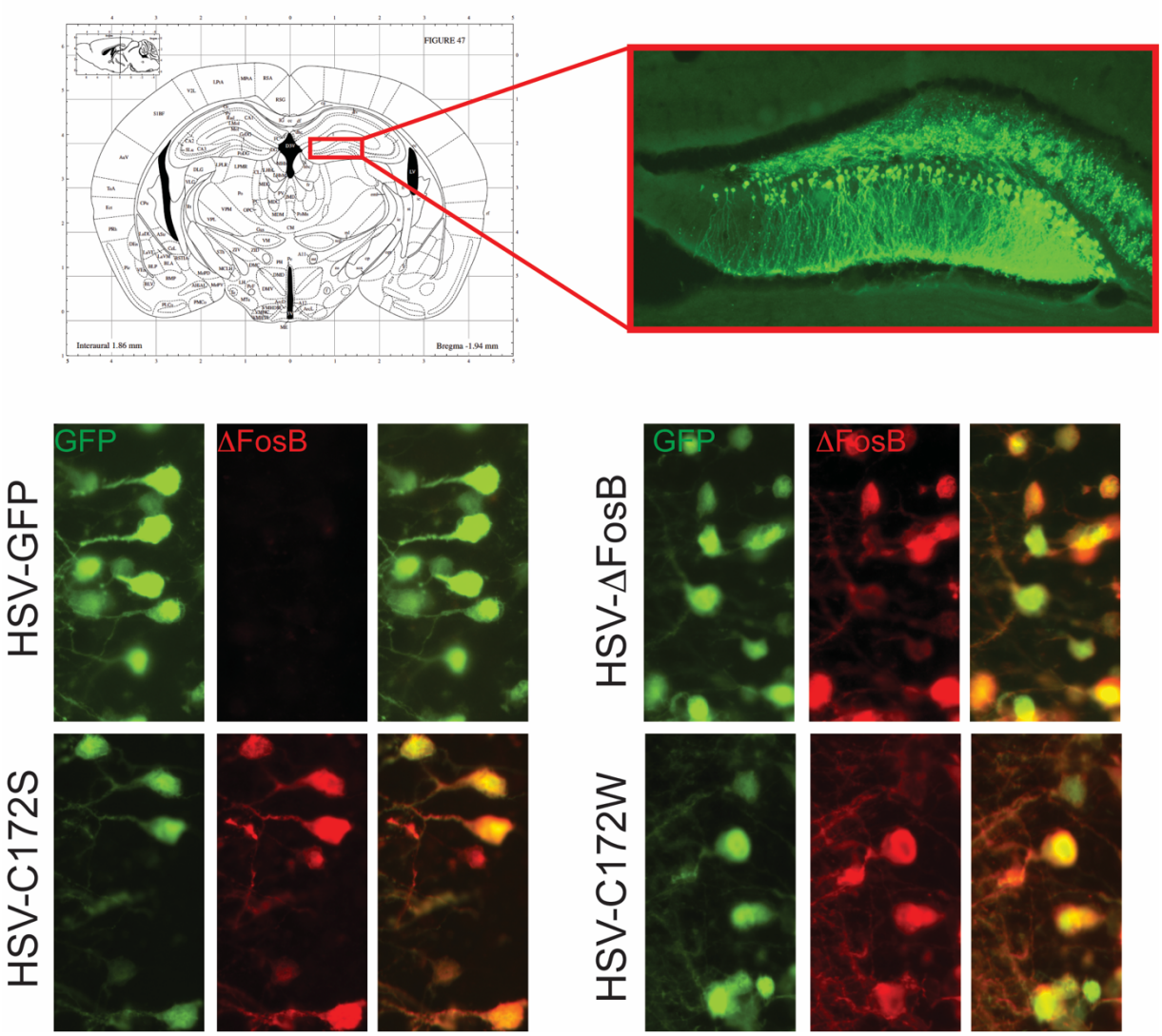

Figure 34 | Viral-mediated gene transfer of WT Delta FosB or mutants in the hippocampus. (Top) Representative fluorescent coronal image of the hippocampus 3 d after injection of HSV-GFP. (Bottom) Immunofluorescence of dentate gyrus neurons close to site of HSV-GFP (green) and HSV-GFP- WT Delta FosB or HSV-GFP-C172S or HSV-GFP-C172W (red).

in mouse brain. Viral tools allow not only for local enhancement or inhibition of native

proteins *in vivo*, but they also allow for circuit-specific and cell-specific manipulations that allow researchers to test the significance of specific circuits of interest or individual cell types. The specific viral type that will be dominant for next steps of looking at the role of redox state and Delta FosB is herpes simplex virus (HSV). These viruses are icosadeltahedral enveloped viruses containing double stranded DNA and are large relative to other viruses[327]. Once infected, or in this case injected into the mouse brain, expression of the transgene begins after approximately 10 hours and lasts for about eight to 10 days[328]. Peak expression occurs between three to five days, providing a relatively short window, so that follow-up experiments can be conducted within a quick timeframe compared to other viruses where the researcher must wait potentially weeks for the virus to reach peak expression. This window has already been shown to be beneficial for testing the roles of transcription factors at specific stages of behavioral expression and acquisition, including Delta FosB[255,313].

Using HSVs to manipulate Delta FosB gene expression in the brain, although is not included in this thesis, will be a key next step for this project. I helped to produce viral tools that contain the Delta FosB cysteine single and double mutations mentioned in the previous chapters. Dr. Rachel Neve and her viral core at Massachusetts General Hospital generated a few of these new vectors for us, and we plan to use them for more *in vivo* work in the future. I produced preliminary evidence that C172S and C172W as well at WT Delta FosB express well *in vivo* in the mouse dorsal hippocampus (Figure 34). Intracranial injections were conducted through stereotaxic surgeries as previously described[255]. Mice were anesthetized with a 0.9/0.1mg/kg mixture of ketamine and xylazine and injected with 0.5uL/hemisphere of virus while in a stereotax via Hamilton

glass syringes (Bregma coordinates are shown in Table 3). As can be seen in the figure, compared to GFP alone control vector, all three Delta FosB expressing vectors cause overexpression of Delta FosB along with GFP in neuronal cells. This is important as we can utilize these vectors to mimic or prevent multiple redox states of Delta FosB to see if certain states enhance or repress Delta FosB dependent behaviors (i.e. learning and memory[255]). Moreover, we will be able to determine whether overexpressing a redox insensitive (C172S) Delta FosB mutant can rescue behaviors seen in mouse models of redox-dependent disease with aberrant Delta FosB expression and/or function.

Table 3 | Surgical Coordinates

Region	Angle	Anterior/Posterior	Medial/Lateral	Dorsal/Ventral
Dorsal Hippocampus	10	-2.2	+2.0	-2.1/-1l9

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