Insights into the Mechanism of Copper-Ionophore Mediated Toxicity in Glial Cells

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Copper is an essential enzyme cofactor in oxidative metabolism, iron homeostasis, antioxidant defenses and neurotransmitter synthesis. Copper also has other important biological functions such as modulation of synaptic transmission, involvement in the innate immune response and erythropoiesis. However, due to copper's ability to redox cycle, it can also lead to the formation of reactive species and cell death. Given the growing interest in the use of copper, in the presence of the ionophore elesclomol (CuES) for the treatment of gliomas, we investigated the effect of CuES on the surround parenchyma – namely neurons and astrocytes *in vitro*. We show that astrocytes were highly sensitive to CuES toxicity, while neurons were surprisingly resistant, a vulnerability profile that is opposite of what has been described for zinc. Bolstering these findings, a human astrocytic cell line was similarly sensitive to CuES. Modifications of cellular metabolic pathways implicated in cupropotosis, a form of CuES-regulated cell death, namely inhibition of mitochondrial respiration and knock-down of ferredoxin 1 (FDX1), were insufficient to abrogate CuES toxicity to astrocytes. CuES toxicity was also unaffected by inhibitors of apoptosis, necrosis or ferroptosis. However, we did detect the presence of lipid peroxidation products in CuES-treated astrocytes, indicating the generation of reactive oxygen species following metal exposure. Indeed, treatment with antioxidants mitigated CuES induced cell death in astrocytes, demostrating that oxidative stress is a principal mediator of CuES-induced glial toxicity. Lastly, prior induction of metallothioneins 1 and 2 in astrocytes using zinc plus pyrithione

was strikingly protective against CuES toxicity. These results demonstrate a unique toxic response to copper in glial cells, but not neurons, which differs in cell selectivity profile of other biologically relevant metals such as zinc.

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Preface

I would like to thank all the people whose support has made this thesis possible. I would first like to thank my thesis advisor, Dr. Elias Aizenman, for his guidance, patience, and encouragement during my time in his lab. His support has been instrumental in helping me to grow as a scientist and scientific thinker. I'd also like to thank my committee members, Drs. Dandan Sun, Sarah Berman, Christopher Cunningham, Teresa Hastings, and Amantha Thatiah for their advice, feedback, and support, as well my outside examiner Dr. Paul Rosenberg for his time and insightful comments. I'd especially like to thank Dr. Sarah Berman who has not only served as my committee member, but who is my career advisor in the Medical Scientist Training. Her endless encouragement, kindness, and advice have been invaluable as I've navigated the ups and downs of graduate school. I'd also like to thank the Aizenman lab for their friendship and their assistance over the years. In particular, I'd like to thank Karen Hartnett-Scott who not only makes the lab run smoothly but who has played an instrumental role in key experiments presented in this thesis. I would also like to thank the Medical Scientist Training Program, and Dr. Richard Steinman, who not only care about the scientific and career progress of their students but have put an emphasis on their personal well-being as well.

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1.0 Introduction

1.1 Introduction to Copper Biology

Copper is an essential micronutrient and redox active metal that cycles between its cupric (Cu(II)) and cuprous (Cu(I)) state. In biological systems, its ability to easily release and absorb one electron allows it to participate in redox reactions, making it useful as a cofactor for numerous enzymes (Kaim and Rall, 1996). Indeed, copper can be found at the active site of many critical enzymes, including those essential for neurological function, such as the predominantly cytosolically localized antioxidant Cu, Zn superoxide dismutase (SOD1), the mitochondrial electron transport chain protein cytochrome C oxidase, and the secretory pathway enzymes dopamine β hydroxylase (DBH), and peptidylglycine α-amidating monooxygenase (PAM) (Scheiber et al., 2014), amongst others. Copper is also indispensable for the growth and development of eukaryotes. In mammals, the importance of copper can be appreciated by disorders of copper metabolism that lead to hypocupremia as well as those leading to copper deficiency. The consequences of hypocupremia can include anemia, neutropenia, cardiac hypertrophy, defects in growth and development as well as neurological symptoms such as seizures, myeloneuropathy, and brain atrophy (Lee et al., 2001; Nose et al., 2006; Prasad et al., 2011; Lazarchick, 2012; Zlatic et al., 2015).

Dietary copper is absorbed through specialized intestinal cells called enterocytes expressing the high affinity copper transporter CTR1 on their apical membrane (Nose et al., 2010), which not only import copper but reduces it from Cu(II) to the more bioavailable Cu(I) (Kar et al., 2022). Mice with intestinal epithelial cell specific knockout of CTR1 show profound early

postnatal defects in growth, hypopigmentation, skin laxity, and brittle whiskers, along with decreased activity of cuproenzymes (Nose et al., 2006) – a phenotype reminiscent of patients with Menkes disease, a genetic disease that results in hypocupremia (Ramani and Parayil Sankaran, 2023). Copper accumulation in peripheral tissues in mice with enterocyte specific loss of CTR1 was also significantly reduced underscoring the importance of intestinal absorption of the metal for proper distribution throughout the body (Nose et al., 2006). Indeed, once copper is absorbed by enterocytes, the P-type ATPAse copper-transporter ATP7A translocates from the *trans*-Golgi network (TGN) to the basolateral membrane of the cell where it effluxes copper into the circulation (Lutsenko et al., 2007; Kaler, 2011). Once in the circulation, copper, bound to serum proteins, is delivered primarily to the liver – the main organ for the maintenance of copper homeostasis in the body. Copper is then stored in hepatocytes, secreted in bile, or incorporated into ceruloplasmin in an ATP7B-dependent manner, which contributes to the majority of copper in the plasma (Lutsenko et al., 2007; Lutsenko, 2021). Copper is thought to be transported to the brain by epithelial cells of the choroid plexus, which comprise the blood-brain barrier, and, furthermore, express ATP7A (Niciu et al., 2006; Kaler, 2011)

Of note, the localization of both CTR1 and ATP7A are regulated by copper levels. When extracellular levels of copper are increased, such as under conditions of high dietary copper intake, CTR1 is rapidly internalized via clathrin-mediated endocytosis and the copper transporter does not return to the plasma membrane until extracellular copper levels are decreased (Molloy and Kaplan, 2009; Clifford et al., 2016). Endocytosis of CTR1 may be facilitated by a conserved methionine-rich cluster in the transmembrane domain of the protein, allowing it to act as both a copper sensor and transporter (Guo et al., 2004). As noted previously, ATP7A's localization from the TGN to the membrane of cells is stimulated by increased intracellular copper (Petris et al., 1996). This

copper-dependent localization of CTR1 and ATP7A allows for the regulation of intracellular copper levels, which are tightly regulated as excess copper can lead to toxicity, in part due to its ability to redox cycle and produce reactive species. This tight regulation of cellular copper levels and copper transport is highlighted by the fact that the majority of cytoplasmic copper is buffered by thiol-containing proteins to keep the concentration of labile, or "free" copper in the cytoplasm in the attomolar range (Morgan et al., 2019). Given the low levels of labile copper in the cytoplasm, cuproenzymes require copper chaperones to transport copper from the plasma membrane to their intracellular locations. For example, SOD1 is metalated by the copper chaperone for superoxide dismutase (CCS) (Culotta et al., 1997; Rae et al., 1999; Wong et al., 2000), and the interaction between these two proteins is critical for both the maturation and activation of SOD1 (Banci et al., 2012; Fetherolf et al., 2017; Boyd et al., 2019). CCS has been shown to interact with CTR1 at the plasma membrane, suggesting it acquires copper directly from this copper transporter (Pope et al., 2013; Skopp et al., 2019). Similarly, Atox1 is a copper chaperone that metalates and regulates the activity of the secretory pathway proteins ATP7A and ATP7B (Walker et al., 2002; Hamza et al., 2003; Strausak et al., 2003; Walker et al., 2004; Yu et al., 2017b). Like CCS, Atox1 likely acquires copper through a direct interaction with CTR1 (Kahra et al., 2016). Although evidence suggests that, unlike the cytoplasm, the mitochondria contains a pool of labile copper (Cobine et al., 2004, 2021; Yang et al., 2005; Vest et al., 2013), with the copper chaperones Cox17, Sco1, and Sco2 all involved in the metalation of cytochrome C oxidase's two copper-containing catalytic sites (Capaldi, 1990; Leary et al., 2004; Fontanesi et al., 2006; Banci et al., 2008). As the affinity of SOD1, ATP7A, and cytochrome C oxidase for copper are all greater than their respective chaperones, affinity gradients facilitate the transfer of copper ions from copper chaperones to their cognate proteins (Banci et al., 2010).

Given the tight regulation of cellular copper levels, the importance of copper in antioxidant defense, neurotransmitter synthesis, and energy metabolism, and the severe neurological consequences of systemic copper deficiency and overload in animal models (La Fontaine et al., 1999; Wang et al., 2012; Hwang et al., 2014; Hadrian and Przybyłkowski, 2021), as well as the growing number of human disorders with neurological phenotypes associated with defects in copper transporting proteins (Jaksch et al., 2000; Valnot et al., 2000; Kaler, 2011, 2013; Członkowska et al., 2018; Batzios et al., 2022), it is clear that copper homeostasis is critical for proper neuronal function and its dyshomeostasis can promptly lead to neurological disease.

1.2 Physiological roles of copper in the nervous system

Due to the strong neuronal dependance on mitochondrial respiration for proper functioning, it is not altogether surprising that these cells are heavily reliant on copper due to the critical role of the metal as a catalytic cofactor in cytochrome C oxidase (Complex IV), the final electron acceptor in the electron transport chain. However, copper also serves an important role in neuronal differentiation, acts as a neuromodulator, modifies neuronal circuit excitability, and is involved in neurotransmitter and neuropeptide synthesis. This section will explore the many physiological roles of copper in normal neuronal function.

1.2.1 Copper and Neuronal Differentiation and Synaptogenesis

Neurons, in addition to being highly metabolically active, carry out specific functions unique to this cell type, such as neurotransmitter and neuropeptide synthesis, as well as synaptic

signaling. Given that all these functions require copper and/or cuproenzymes, it may follow that these post-mitotic cells have an increased demand for the divalent metal. Indeed, recent evidence suggests that neuronal differentiation is associated with increased copper dependence. *In vitro* data from differentiated PC12 cells and SH-SY5Y cells, and an *in vivo* study of the embryonic chick spinal cord are all concordant in the finding that copper levels are higher in differentiated neurons as compared to non-differentiated cells (Birkaya and Aletta, 2005; Hatori et al., 2016). This increased cellular demand for copper appears to be compartment specific as the expression of proteins localized to the secretory pathway, such as ATP7A, DBH, and PAM is significantly increased in differentiated neurons. The expression of mitochondrial proteins such as the copper chaperone COX17 and subunit 1 of cytochrome C oxidase are also moderately increased in differentiated neurons. In contrast, the expression of cytosolic copper-containing proteins is not different between undifferentiated and differentiated neurons, even when such proteins are directly involved in metalation (e.g., SOD1's copper chaperone CCS) (Hatori et al., 2016). These findings are supported by observed increased copper levels in the microsomal fractions, containing secretory vesicles and the TGN, in differentiated neurons compared to levels in non-differentiated cells. The increased copper flow to the secretory pathway as well as increased expression of secretory pathway proteins in differentiated neurons suggests that Atox1, which transfers copper to ATP7A, should be more active. Intriguingly, previous research demonstrated a functional partnership between the cell's glutathione/glutathione disulfide (GSH/GSSH) balance and Atox1's redox state where GSH is able to reduce Atox1 and restore its ability to bind copper (Hatori et al., 2012). In both developing chick spinal cord and differentiated SH-SY5Y cells, both glutathione and Atox1 are markedly chemically reduced compared to non-differentiated neurons (Hatori et al., 2016), consistent with a model in which redox-regulation of glutathione allows Atox1 to bind

copper and metalate ATP7A, leading to the maturation of cuproenzymes such as PAM and DBH. Moreover, oxidizing glutathione leads to increased DBH retention suggesting that copper flow to the secretory may be regulated by the redox state of the cell (Hatori et al., 2016). Together these results underscore the importance of both copper and redox regulation for neuronal differentiation and function.

In addition to the requirement for increased copper flow to the secretory pathway for neuronal differentiation, increased intracellular copper and the proper trafficking of copper transporters have been found to be essential for neurite extension and synaptogenesis. As previously noted, Birkaya and Aletta (2005) found that differentiation of PC12 cells with nerve growth factor (NGF) increased cellular copper content within three days of treatment, with intracellular copper levels rising to 14-fold when compared to control cells after 14 days. Moreover, in PC12 cells, this increase in copper is essential for neurite outgrowth. Chelating copper with tetraethylenepentamine (TEPA) not only reduces intracellular copper levels, but results in a marked decrease in neurite branching and length (Birkaya and Aletta, 2005).

The copper ATPase ATP7A is an important regulator of axon extension and synaptogenesis. Indeed, multiple investigations have found that ATP7A is expressed in a developmental rather than constitutive manner in numerous neuronal subpopulations, with expression peaking early in neuronal development, prior to synaptogenesis (El Meskini et al., 2005, 2007; Niciu et al., 2006). Additionally, the localization of ATP7A changes markedly during neuronal maturation. Early in neuronal differentiation, the protein is primarily localized to cell bodies but over time its expression is increased in extending axons (El Meskini et al., 2005). Furthermore, following injury-induced neurogenesis in olfactory receptor neurons, the localization of ATP7A follows a similar pattern, increasing its axonal expression prior to synaptogenesis (El

Meskini et al., 2005), suggesting it likely has a functional role in this process. Mice with mutations in ATP7A not only show aberrant targeting of axons and dendrites, but also an accumulation of immature neurons (El Meskini et al., 2007). These findings suggest that ATP7A plays an important role in both in synaptogenesis as well as in terminal neuronal differentiation at the time of axon extension.

Although El Meskini et al. (2007) posit that this could be a copper-independent process due to the finding that ATP7A can traffic to neuronal processes of hippocampal neurons in response to NMDAR stimulation (Schlief et al., 2005), more recent evidence suggests that copper homeostasis may play an important role in ATP7A-dependent synaptogenesis. Using a *Drosophila* model, which allows for extensive genetic manipulation, Hartwig et al. (2021) demonstrated that copper homeostasis is essential for synaptic development. Moreover, they demonstrated that copper homeostasis is regulated by ATP7A and the conserved oligomeric Golgi (COG) complex. The COG complex is an octamer localized to the Golgi that is involved in vesicle tethering, incoming vesicle fusion, the coordination of retrograde vesicle transport, and glycosylation (Ungar et al., 2002; Miller and Ungar, 2012). Previous work established a biochemical interaction between ATP7A and COG and demonstrated that cells deficient in the COG complex showed increased surface expression of ATP7A along with a concomitant decrease in copper content due to increased efflux (Comstra et al., 2017). This study demonstrated a functional link between ATP7A, the COG complex, and copper homeostasis in neurons. Using genetically modified *Drosophila*, Hartwig et al. (2021) demonstrated perturbations in ATP7A disrupt copper homeostasis and lead to aberrant synaptic morphology in the neuromuscular junction of *Drosophila*, both of which could be rescued by alterations in COG expression. These results suggest that copper dyshomeostasis is involved in the altered synaptic morphology seen in with ATP7A mutants and that COG may be an upstream

modulator of copper homeostasis in neurons. Indeed, further experiments showed that the COG complex controls the function of ATP7A and CTR1 in developing motor neurons (Hartwig et al., 2021), demonstrating the importance of Golgi integrity in neuronal function. These results not only provide insight into copper regulation in developing neurons but also demonstrate a direct link between copper homeostasis and synaptic development. Additionally, this investigation demonstrated that ATP7A expression and copper homeostasis are critical for proper mitochondrial localization to the synapse as well as its functional integrity (Hartwig et al., 2021). Given the high metabolic demands of developing neurites and the importance of axonally localized mitochondria for neuronal development (Spillane et al., 2013; Son and Han, 2018; Rangaraju et al., 2019), these findings may offer an additional clue to the why the Golgi apparatus and ATP7A are critical in synaptogenesis.

1.2.2 Copper in the Secretory Pathway.

1.2.2.1 Atox1

Several cuproenzymes, such as ATP7A and ATP7B, needed for neurotransmitter and neuropeptide synthesis, along with vesicular copper release, reside in the secretory pathway. Given the negligible concentration of labile copper in the cytosol, such enzymes require metalation by a copper chaperone that can bind copper in the cytosolic compartment and then deliver it to the secretory pathway, where these proteins are localized. Early studies in yeast suggested that Atx1, a small cytosolically localized protein served as this putative copper chaperone as it functions upstream of the yeast ATP7A and ATP7B homolog Ccc2 in the delivery of copper from CTR1 to the secretory pathway (Lin et al., 1997; Pufahl et al., 1997; Rosenzweig et al., 1999; Huffman and O'Halloran, 2000). One important finding in the elucidation of this pathway was that cells deficient

in Atx1 fail to incorporate copper into Fet3p (Klomp et al., 1997), the yeast homolog of ceruloplasmin and a multi-copper oxidase that functions in high affinity iron transport (Taylor et al., 2005). More importantly, yeast lacking Ccc2 are iron deficient, a sign of Fet3p dysfunction, and this phenotype that cannot be rescued by overexpressing Atx1. Overexpressing Ccc2 in cells deficient in Atx1 however, can rescue iron deficiency, supporting a role for Atx1 in copper delivery to Ccc2 (Lin et al., 1997). Of note, in mammals, ATP7B is the enzyme responsible for the metalation of ceruloplasmin (Terada et al., 1998; Telianidis et al., 2013). Indeed, later studies confirmed that Atx1 is a metallochaperone that transfers Cu(I) to Ccc2 in a direct and reversible manner (Portnoy et al., 1999; Huffman and O'Halloran, 2000), and structural characterization of the Atx1 showed that it coordinates copper through a highly conserved metal binding site (CXXC) located near the surface of the protein (Rosenzweig et al., 1999). Structural and functional characterization of mammalian Atox1 confirmed that there is a specific, copper dependent interaction between this metallochaperone and the metal binding domains of ATP7A and ATP7B that is dependent on the CXXC sequence motifs present in both the Atox1 and ATP7A and ATP7B (Larin et al., 1999; Wernimont et al., 2000). Consistent with these data, cells that are deficient in Atox1 have increased intracellular copper (Hamza et al., 2001, 2003), likely reflecting a lack of copper efflux, due to the inability of Atox1 to transfer copper to ATP7A, which translocates to the plasma membrane under conditions of excess copper to transport copper out of the cell (Petris et al., 1996; Petris and Mercer, 1999). Furthermore, the C-terminus of human Ctr1 has been shown to bind Atox1 with high affinity ($K_d \sim 10^{-14}$ M) and transfer copper to this cytosolic protein (Kahra et al., 2016), supporting a model in which Atox1 serves as a metallochaperone for this divalent metal and delivers it from the plasma membrane to the secretory compartment while keeping the cytosolic concentration of labile copper necessarily low.

Investigations utilizing both yeast and mammalian systems have demonstrated the necessity of cysteine residues in the Atx1/Atox1 metal binding domain in coordinating copper binding (Rosenzweig et al., 1999; Wernimont et al., 2000; Strausak et al., 2003). Mutagenesis experiments of yeast Atx1 demonstrated that its interaction with Cccp is dependent on the two cysteines present in its metal binding domain (Portnoy et al., 1999) and experiments in mammalian cells confirmed that that the two cysteines in Atox1's CXXC motif are required for its interaction with both ATP7A and ATP7B (Larin et al., 1999; Strausak et al., 2003). While this finding is likely a result of the structure of the protein, as the two cysteines are exposed on the surface making them accessible for docking of copper ions (Rosenzweig et al., 1999; Wernimont et al., 2000), it also highlights the potential redox sensitivity of the interaction between Atox1 and its targets. Indeed, recent studies have demonstrated that Atox1 is sensitive to the cytosolic redox environment (Hatori et al., 2012, 2016; Katsuyama et al., 2021). In proliferating cells, Atox1 is mostly reduced. However, GSH can reduce the copper-coordinating cysteines of Atox1 and restore its ability to bind copper. Alternately, when GSSG levels are high and the GSH/GSSG balance is shifted, the copper-coordinating cysteines become oxidized and Atox1 is unable to bind copper (Hatori et al., 2012). As the interaction between Atox1 and ATP7A and ATP7B is dependent on copper binding, the GSH/GSSG ratio bidirectionally modulates the flow of copper through the secretory pathway by regulating the redox state of Atox1. This coupling between Atox1 and the redox state of the cell has significant functional implications in neurons, which will be discussed below.

1.2.2.2 Neurotransmitter and Neuropeptide Synthesis

1.2.2.3 Dopamine-β-hydroxylase

Dopamine-β-hydroxylase (DBH) is a cuproenzyme critical for the synthesis of the neurotransmitter norepinephrine (NE) in noradrenergic neurons of the locus coeruleus and sympathetic nerve terminals. Of note, the locus coeruleus is highly enriched in copper (Naeve et al., 1999; Xiao et al., 2018). In catecholaminergic cells, DBH is responsible for the conversion of dopamine (DA) to NE, which is essential for arousal, attention, and wakefulness (Berridge and Waterhouse, 2003). Human DBH is a tetrameric glycoprotein (Wallace et al., 1973) comprised of four domains – one dopamine-β-monooxygenase N-terminal (DOMON) domain, which belongs to a class of DOMON-like domains, two copper catalytic cores, and a C-terminus dimerization domain (Vendelboe et al., 2016). The catalytic cores are referred to as Cu_H or Cu_M based on whether they are coordinated by three histidines (Cu_H) or two histidines and one methionine (Cu_M). Each copper domain is thought to have a distinct function, with Cu_H transferring electrons from its co-substrate ascorbic acid and Cu_M hydroxylating substrates (Brenner and Klinman, 1989; Klinman, 2006). Underscoring the importance of copper for NE synthesis, DBH thus requires two bound copper for activity (Ash et al., 1984).

Current evidence suggests that DBH is synthesized in the endoplasmic reticulum (Sabban et al., 1983) and is then trafficked to the TGN where it acquires copper from ATP7A (Xiao et al., 2018) before being packaged into secretory granules, converting their DA to NE. DBH exists in both a membrane-bound and soluble form (Sabban et al., 1983) and studies utilizing rat PC12 cells and human SH-SY5Y cells have demonstrated that once packaged into secretory granules, DBH-containing vesicles are released not only in response to stimulation but are also constitutively

released into the extracellular space under resting conditions (Sabban et al., 1983; McHugh et al., 1985; Oyarce and Fleming, 1991). Importantly, extracellular DBH is enzymatically active suggesting it is exported in a copper-bound state (Schmidt et al., 2018). It is thought that this constitutive secretion of DBH also maintains the stable levels of DBH observed in the CSF, which are independent of neuronal activity, though it must be noted that the function of this CSF localized DBH is still unclear (O'Connor et al., 1994). Intriguingly, copper may also regulate DBH secretion as reduced copper delivery to the secretory pathway has been shown to result in increased intracellular levels of the enzyme (Hatori et al., 2016). This finding is supported by recent evidence showing that in SH-SY5Y cells, copper is necessary for the constitutive secretion of DBH (Schmidt et al., 2018). Furthermore, Schmidt et al. (2018) demonstrated that neuronal ATP7B may negatively regulate the export of soluble DBH by sequestering copper in vesicles and inhibiting ATP7A from transferring this cofactor to DBH in the TGN. Thus, not only is copper necessary for noradrenergic signaling by acting as cofactor for DBH, but copper and known copper transporters affect the storage and release of this enzyme, which is likely to have important implications for diseases such as Menke's disease and Wilson disease, in which the function of ATP7A and ATP7B are disrupted.

1.2.2.4 Peptidylglycine α-amidating monooxygenase

Peptidylglycine α-amidating monooxygenase (PAM) is an enzyme that catalyzes C-terminal amidation, an important post-translational modification of many signaling peptides. Over half of all known peptides, including neuropeptides and peptide hormones such as oxytocin, vasopressin, and neuropeptide Y, are produced as glycine-extended precursors with little to no bioactivity (Russo, 2017). PAM catalyzes the cleavage of this glycine, resulting in a bioactive C-

terminal amidated peptide and glycoxylate ((Bradbury and Smyth, 1991; Merkler, 1994; Kumar et al., 2016). Underscoring the importance of this enzyme is the fact that deletion of the PAM gene results in lethality in an early larval stage in *Drosophila* (Jiang et al., 2000) and embryonic lethality in mice (Czyzyk et al., 2005). PAM contains two enzymatic domains – the peptidylglycine α-hydoxylating monooxygenase (PHM) domain and the peptidyl-α-hydroxyglycine α-amidating lyase (PAL) domain that act sequentially to produce an amidated peptide. Of note, the PHM domain contains ~32% homology to DBH (Solomon et al., 2014) including a catalytic core containing Cu_H and Cu_M domains, each requiring two coppers for maximal activity (Kulathila et al., 1994; Eipper et al., 1995; Prigge et al., 1999), which they likely acquire from ATP7A (Otoikhian et al., 2012). Additionally, like DBH, PHM requires ascorbic acid and molecular oxygen as substrates (Murthy et al., 1986).

In addition to copper's role in PHM catalysis, there is evidence to suggest that this metal may also affect endocytic trafficking of PAM. Like DBH, PAM exists in membrane-bound and soluble forms. Soluble PAM is targeted to secretory granules and is released along with bioactive peptides upon stimulation. After exocytosis, membrane PAM appears on the plasma membrane, where it is rapidly internalized and is either packaged into secretory granules or undergoes degradation (Milgram et al., 1994; Steveson et al., 1999). In AtT-20 cells, a pituitary corticotrope tumor cell line, depletion of intracellular copper increases the transit of membrane PAM through the endocytic pathway and into secretory granules. In contrast, increasing intracellular copper decreases cleavage of PAM in secretory granules, which is needed for the release of the soluble enzyme. Additionally, increased copper decreases the targeting of endocytosed PAM to secretory granules and increases its degradation (De et al., 2007). Importantly, De et al. (2007 demonstrated

these effects were mediated through PAM's cytosolic domain, suggesting it may act as a copper sensor, allowing the enzyme to respond dynamically to copper availability.

1.2.3 Synaptic Copper

Although copper is mainly thought of as a cofactor for enzymes with its actions primarily taking place within the intracellular environment, evidence suggests that copper is released from presynaptic neurons and concentrations within the synaptic cleft can rise to micromolar levels, allowing this metal to act on synaptic receptors. It is important to note that due to the reducing environment of the cell, intracellular copper exists primarily as Cu^{+}, however when it is released into the extracellular space, it comes into contact with oxygen and is oxidized to Cu^{2+} (Maryon et al., 2007). Early evidence for the release of copper into the synaptic cleft came from the rat hypothalamus (Hartter and Barnea, 1988), as well as isolated synaptosomal preparations from rat cortical neurons (Kardos et al., 1989). Depolarization with high K^{+} led to the release of copper into the extracellular space and, from these experiments, it was estimated that the concentration of copper within the synaptic cleft was as high as 200-250 µM (Kardos et al., 1989). However, it should be noted that this concentration may not take into account protein-bound copper as later experiments using a fluorescent probe along with ICP-MS found that following depolarization, the concentration of free copper in the synaptic cleft was likely to be closer to 2-3 µM (Hopt et al., 2003). This concentration would be consistent with evidence that the normal extracellular copper concentration in the brain is approximately 0.2-1.7 µM (Tarohda et al., 2004). However, the possibility that higher concentration of this metal can exist in microdomains, as has been found with zinc (Krall et al., 2020, 2021), cannot be ruled out.

Although many of these initial studies used *in vitro* preparations of isolated synaptosomes to study copper release, treatment of primary cultured neurons with glutamate/glycine to stimulate NMDARs also produce an increase in copper levels in the bath solution (Schlief et al., 2005). Moreover, glutamate receptor activation leads to the rapid and reversible trafficking of ATP7A from the Golgi compartment to neuronal processes, suggesting that this P-type ATPAse may play a critical role in copper efflux (Schlief et al., 2005). The direct role of ATP7A in copper efflux is supported by studies using primary neurons from mice with mutant ATP7A receptors. These neurons fail to efflux copper in response to glutamate/glycine treatment suggesting that proper function of ATP7A is an essential component to copper release into the extracellular space (Schlief et al., 2005). Given the rapid efflux of copper following NMDAR activation, Schlief et al. (2005) proposed that ATP7A may accumulate copper in a vesicular compartment that is readily available for exocytosis following activation of the glutamate receptor. Intriguingly, studies using differentiated PC12 cells demonstrated that increasing intracellular copper upregulates proteins implicated in secretory vesicle trafficking such as synaptophysin and SNAP-25, supporting a role for vesicular sequestration and storage of this metal (Duncan et al., 2013).

1.2.4 Targets of Synaptic Copper

1.2.4.1 Ionotropic Glutamate Receptors

Glutamate is a major excitatory neurotransmitter, and its receptors mediate fast excitatory synaptic transmission in the CNS. Fast synaptic action by glutamate is mediated via ionotropic receptors, which include a heterogenous family of tetrameric ligand-gated channels classified based on their structural homology and response to pharmacological agonists. N-methyl-d-aspartate (NMDA) receptors are comprised of an obligatory GluN1 subunit as well as two

GluN2(A-D)/3(A or B) type subunits (Traynelis et al., 2010), with the majority of receptors expressing two GluN1 subunits and two GluN2 subunits (Köhr, 2006). Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors can be comprised of homo- or heteromers of GluA1-4 subunits while kainate receptors are formed from GluK1-GluK5 subunits (Traynelis et al., 2010).

A growing body of evidence suggests that copper can modulate ionotropic glutamate receptors, with early work demonstrating that in cultured rat cortical neurons AMPA/kainate receptors are highly sensitive to inhibition by copper (IC_{50}=4.3 μM) and that application of micromolar concentrations of copper reduced the potency of kainate in activating the AMPA receptor, shifting the EC_{50} of kainate from 100 μM to >300 μM. Intriguingly, application of the reducing agent dithiothreitol (DTT) blocked copper mediated inhibition of kainate-induced currents, suggesting an oxidative mechanism, rather than direct binding, may be partly responsible for copper's inhibition of AMPA/kainate receptors. This supposition is supported by the fact that co-application of kainate with histidine, a high affinity copper chelator, did not affect recovery of receptors from copper block, suggesting that direct copper binding may not be needed to exert its inhibitory effects (Weiser and Wienrich, 1996). However, it is important to note that DTT was not washed out prior to application of copper and thus could have bound the metal (Krężel et al., 2001), which would also explain a lack of copper-mediated inhibition.. A more recent study utilizing primary rat hippocampal neurons found that copper's effect on AMPA receptors is biphasic. Acute application of the metal decreased AMPA evoked currents (IC_{50}=22.9 μM) compared to neurons treated with AMPA alone, in addition to decreasing synaptic activity. In contrast, longer incubation with copper (3 hours) produced potentiation of AMPA evoked currents, shifted the concentration-response curve to AMPA (EC_{50}=8.3 for control neurons and EC_{50}=2.89 for copper

treated neurons), and increased the frequency and amplitude of miniature synaptic currents. This three-hour incubation period also increased intracellular calcium transient frequency supporting a role for increased neurotransmission. However, the increase in AMPAergic transmission disappeared after 24 hours of copper treatment indicating a homeostatic response to increased levels of exogenous copper on a longer timescale (Peters et al., 2011). One question that remains unanswered from this study is whether chelation or wash-out of copper after this 24-hour incubation followed by acute application of the metal would still produce inhibition of AMPA currents or whether the homeostatic response desensitizes receptors to this stimulus. This information would be relevant to neurological disease that result in copper dyshomeostasis and increased brain copper, as desensitization of AMPA receptors may lead to decreased excitation and disturbance of the excitatory-inhibitory balance.

NMDA receptors are also intricately involved in the effects of synaptic copper. Not only does NMDAR stimulation allow for the release of free copper into the synaptic cleft, allowing it to act on post-synaptic receptors (Schlief et al., 2005), but studies have demonstrated that copper inhibits NMDAR-induced currents themselves (Trombley and Shepherd, 1996; Vlachová et al., 1996; Weiser and Wienrich, 1996) further indicating that this metal negatively modulates excitatory NMDAR-mediated neurotransmission. The estimated potency of copper in blocking NMDAR receptor currents in these studies ranged from micromolar (IC_{50}=15 µM-22 µM) (Trombley and Shepherd, 1996; Weiser and Wienrich, 1996) to nanomolar concentrations (IC_{50}=270 nM) (Vlachová et al., 1996). One potential explanation for the difference in the IC_{50} values between those reported by Weiser & Wierich (1996) and those reported by Vlachova et al. (1996) is the timing of their experiments. While both studies utilized primary rat cortical neurons in their investigations, Weiser & Wierich (1996) used their cultures between 11-21 days *in vitro*

while Vlachova et al. (1996) used cultures at 5-9 days *in vitro*. As NMDARs undergo a developmental switch, with GluN2B being the predominant subunit expressed until the second week of development at which time GluN2A expression strongly increases (Sanz-Clemente et al., 2013), it is possible that differences in NMDAR subunit identities may underlie these differences. Despite the wide range of IC_{50} values, all studies were in agreement regarding the voltage-independence of this inhibition, although the mechanism by which copper inhibits the NMDAR remains unknown.

While the effect of copper on NMDARs may occur via direct binding, work by Marchetti et al. (2014) and Schlief et al (2006) suggests the possibility of a redox modulatory component. Importantly, it has long been known that NMDARs are subject to redox modulation mediated by key cysteine residues on GluN1 and GluN2A subunits (Aizenman et al., 1989, 2020). In contrast to Vlachova et al. (1996) which found that nanomolar concentrations of copper inhibited NMDAR-induced currents, Marchetti et al. (2014) found that in cerebellar neurons and transiently transfected HEK293 cells, low micromolar concentration of the metal potentiated the current (EC_{50}=4.6 µM for GluN1/GluN2B; IC_{50}= 2 µM for GluN1/GluN2A) and increased intracellular calcium. As cerebellar neurons express GluN1/GluN2C NMDARs, as well as triheterometric GluN1/GluN2A/GluN2C NMDARs (Bhattacharya et al., 2018; Sibarov et al., 2018), differences in subunit composition could contribute to the potentiation in cerebellar neurons. This potentiation was absent when neurons were pre-treated with the reducing agent DTT, although it should be noted that DTT itself caused potentiation of NMDAR currents. This finding is consistent with previous research showing that reducing agents potentiate NMDAR currents while oxidizing agents inhibit them (Aizenman et al., 1989). Intriguingly, these experiments also demonstrated a moderate decrease in NMDAR currents when copper was applied following DTT treatment raising

the possibility that copper may oxidize redox modulatory sites when they are in a reduced state. However, given that copper facilitates NMDAR currents in the absence of a reducing agent and is more likely to act as an oxidizing agent in its Cu^{2+} state, it is likely that if copper-mediated facilitation occurs through redox modulation of the NMDAR, it is through an indirect, rather than direct mechanism. One possibility could involve copper-induced release of an endogenous reducing substance, such as dihydrolipoic acid, which has been found to reduce the NMDAR redox modulatory site (Tang and Aizenman, 1993) and thus indirectly contribute to the potentiation of NMDAR currents.

Consistent with Trombley & Shepherd's (1996) and Weiser & Wienrich's (1996) investigations, concentrations above 30 µM copper inhibited NDMAR-mediated currents (IC_{50}=24 µM for GluN1/GluN2B, IC_{50}=26 µM for GluN1/GluN2A) in transiently transfected HEK293 cells and cerebellar neurons (Marchetti et al., 2014). One potential explanation for this inhibition, posited by Schlief et al (2006), may involve S-nitrosylation of the receptor, a post-translational modification involving the attachment of a •NO group to the thiol side chain of cysteine. S-nitrosylation has been suggested to result in blockade of the receptor (Lei et al., 1992), consistent with inhibition of NMDAR currents by copper. Schlief et al. (2006) showed that in primary hippocampal neurons, treatment with 200 µM copper blocked NMDAR activation and resulted in endogenous nitric oxide production. Furthermore, treatment with a nitric oxide synthase inhibitor eliminated the copper induced blockade of NMDAR activation while the effect of copper was restored after treatment with an exogenous nitric oxide donor (Schlief et al., 2006). These results suggest that copper indirectly modulates NMDARs function through its effect on nitric oxide production. However, it is worth noting that the concentrations of copper used in this study are likely supraphysiological and may result in oxidative stress triggering the production of reactive

oxygen and reactive nitrogen species. Furthermore, whether nitric oxide actually modifies redox modulatory sites on the NMDAR has been called into question (Aizenman and Potthoff, 1999; Hopper et al., 2004; Aizenman et al., 2020). Thus, the mechanism by which copper inhibits NMDAR currents, and whether it is a result of redox modulation remains to be determined.

Although the mechanism by which copper inhibits NMDARs has yet to be fully elucidated, it appears to have homeostatic and neuroprotective functions. When 200 μM copper, a concentration that did not cause overt neuronal toxicity, was applied to primary hippocampal neurons exposed to excitotoxic levels of glutamate/glycine, it completely abrogated cell death. Conversely, when the copper chelator bathocuproine disulfate (BCS) was applied to cultures to reduce intracellular copper prior to exposure glutamate/glycine, neuronal death was increased (Schlief et al., 2006). Importantly, all experiments were performed in the presence of the AMPA/kainate antagonist CNQX to isolate NMDAR stimulation by glutamate/glycine. These results suggest that the vesicular copper released from neurons following NMDAR stimulation (Schlief et al., 2005) may act on NMDARs themselves to rein in excessive excitation, and that additional copper release can protect neurons from excitotoxic cell death. Co-application of copper with glutamate/glycine also inhibited the previously observed elevation in intracellular calcium upon NMDAR hyperactivation, indicating that copper likely protects against excitotoxic cell death by preventing downstream signaling events following receptor stimulation (Schlief et al., 2006). These findings may have implications for diseases in which neuronal copper is increased and thus, more is available to be released from the cell upon NMDAR stimulation. In support of the homeostatic and neuroprotective nature of copper release is the finding that primary neurons derived from mice with loss of function of *Atp7a*, which is critical for neuronal copper release (Schlief et al., 2005), were shown to be more sensitive to excitotoxic injury induced by exposure

to high K^+ to induce synaptic glutamate release, as compared to control neurons. However, application of exogenous copper before and during high K^+ stimulation abrogated this increased sensitivity to excitotoxic injury in ATP7A mutant neurons (Schlief et al., 2006). These findings strongly indicate a protective role for AT7A-dependent copper release in neurons and may partly account for the neurodegeneration seen in ATP7A-linked disorders such as Menke's disease, which shows decreased function of this protein.

1.2.4.2 $GABA_A$ Receptors

$GABA_A$ receptors ($GABA_ARs$) are ionotropic, chloride-gated channels and serve as the major inhibitory receptors in the CNS. $GABA_ARs$ are pentameric assemblies that are usually comprised of three different proteins selected from 19 different genetically encoded subunits (α1-6, β1-3, γ1-3, δ, ε,θ, π, and ρ1-3). Evidence suggests that most $GABA_AR$ are comprised of two copies of a single α subunit, two copies of a single β subunit, and one copy of another subunit such as γ or δ, although $GABA_ARs$ can be heteromers of α and β subunits alone (Olsen and Sieghart, 2008). The subunit composition of these receptors not only affects their physiological and pharmacological properties but their localization within neurons as well. It is generally accepted that α1-3βγ subunits comprise synaptic $GABA_ARs$ whilst those that contain α4-6 subunits as well as αβ heteromers are extrasynaptically localized (Nusser et al., 1998; Hannan et al., 2020). The localization of $GABA_AR$ is a large determinant of the type of inhibition these receptors exert. Synaptic $GABA_ARs$ are thought to modulate rapid phasic inhibition while extrasynaptic receptors are typically thought to influence tonic inhibition, which is important for proper regulation of the excitatory-inhibitory balance.

A large body of work has investigated the effect of zinc on $GABA_A$Rs and it is now established that these receptors are allosterically inhibited by this metal (Krall et al., 2021). Importantly, this inhibition is subunit dependent with isoforms containing only αβ subunits being most sensitive to zinc block. Inclusion of a δ, ε, or π subunit reduces the affinity of zinc for the $GABA_A$R but strikingly, the inclusion of a γ subunit renders these receptors almost insensitive to zinc block (Draguhn et al., 1990; Nagaya and Macdonald, 2001). This loss of zinc sensitivity is thought to be a result of a conformational change to the α and β subunits when the γ subunit is inserted, disrupting the putative zinc binding site(s) (Hosie et al., 2003). Thus, tonic GABAergic signaling is most likely to be affected by zinc binding. In contrast to zinc, there has been comparatively little research into the effect of copper on GABAergic signaling. However, early experiments utilizing rat dorsal ganglion neurons demonstrated that micromolar concentrations of copper reversibly inhibited $GABA_A$Rs with a similar potency to zinc (IC_{50}=16 μM and 19 μM, respectively) (Ma and Narahashi, 1993; Narahashi et al., 1994). These concentrations are similar to what was found for zinc- and copper-mediated inhibition of $GABA_A$ receptors in rat olfactory bulb neurons (IC_{50}=17 μM) (Trombley and Shepherd, 1996). Moreover, in dorsal root ganglion copper antagonized the blocking actions of zinc on $GABA_A$Rs in a concentration-dependent manner suggesting these divalent metals share a binding site (Ma and Narahashi, 1993). These findings were supported by work in Purkinje cells which demonstrated that copper attenuates zinc induced $GABA_A$R block and vice versa. However, these studies found that copper inhibited $GABA_A$Rs at nanomolar (IC_{50}=35 nM) rather than micromolar concentrations (Sharonova et al., 1998). Furthermore, these authors posited that copper and zinc bind the $GABA_A$ receptor at distinct, but conformationally linked sites (Sharonova et al., 2000).

Further investigation into copper binding to and its inhibition of $GABA_A$ receptors has revealed that in contrast to zinc, γ containing $GABA_ARs$ are sensitive to inhibition by copper and that those comprised of α1β3γ2 subunits are more sensitive to block by this metal than those containing α6β3γ2 (Kim and Macdonald, 2003). These studies also revealed that an N-terminal motif in the α1 and α2 subtypes is a major determinant of high copper sensitivity and that Val^{134}, Arg^{135} (Gln for α2), and His141 within this motif are the most important determinants for copper binding (Kim and Macdonald, 2003). Additionally, His^{267} within the M2 domain of the β3 subunit and His^{273} within the M2-M3 loop of the α6 subunit, which are major determinants of $GABA_AR$ sensitivity to zinc (Fisher and Macdonald, 1998; Hosie et al., 2003), were found to contribute to copper inhibition (Kim and Macdonald, 2003). Together, these data suggest that zinc and copper bind to overlapping but non-identical sites on the $GABA_AR$.

While the finding that copper potently blocks α1β3γ2 $GABA_ARs$ suggests that this metal inhibits fast synaptic, or phasic GABAergic signaling in contrast to zinc, which can only inhibit tonic GABAergic signaling, it is important to note extrasynaptic receptors are sensitive to copper block and, therefore, tonic GABAergic signaling as well. In cerebellar granule cells, the α6 subunit is obligatory for δ expression (Jones et al., 1997). Despite the lower affinity of copper for α6 receptors, application of micromolar concentrations of copper to α6β3δ expressing cerebellar granule cells reduces GABA evoked responses (McGee et al., 2013). Moreover, chelating copper from these cells enhances tonic conductance, further suggesting that copper binds to and inhibits extrasynaptic $GABA_ARs$. In addition to blocking tonic conductance in cerebellar granule cells, application of copper to medium spiny neurons from the striatum significantly inhibits their GABAergic tonic conductance (McGee et al., 2013), demonstrating the ability of copper to block tonic GABAergic signaling is not restricted to one brain region. It is important to note that like

cerebellar granule cells, medium spiny neurons are also known to express extrasynaptic δ-expressing $GABA_A$ receptors (Santhakumar et al., 2010). Given the importance of tonic GABAergic signaling in regulating neuronal excitability, it is intriguing to consider the implications of pathological conditions, such as Wilson disease, in which brain copper is elevated and may disrupt tonic GABA signaling.

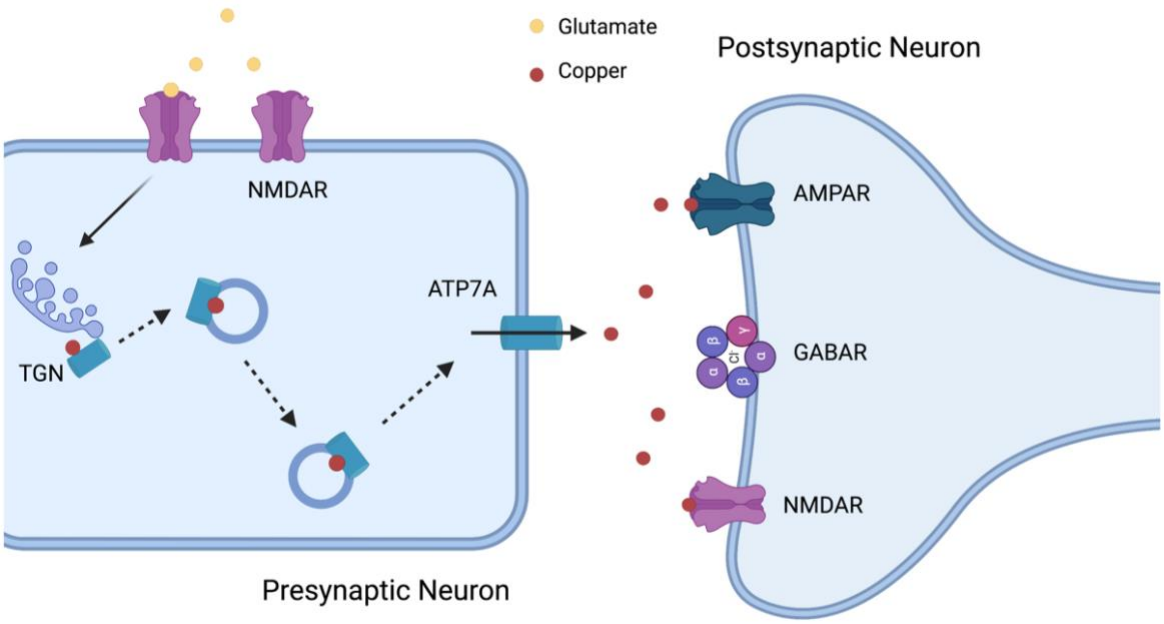

Figure 1. Synaptic Copper. Glutamate binding to NMDA receptors stimulates ATP7A to traffic out of the trans Golgi network (TGN) to the pre-synaptic membrane to induce copper efflux into the synaptic cleft. Copper can then bind to post-synaptic NMDA receptors, $GABA_A$ receptors, and AMPA receptors, subsequently modifying their activity.

1.2.5 Copper and Network Excitability

Given copper's actions on both excitatory and inhibitory ligand-gated receptors, one would expect that that application of copper might affect neuronal circuit function. Indeed, using rat hippocampal slices as a neuronal network model, Maureira et al. (2015) found that broadly, copper fine enhances network excitability. Application of 10 nM nanomolar copper increased the spontaneous action potential (AP) firing rate in the CA1 region. More intriguingly, chelation of endogenous of copper with bathocuproine decreases spontaneous spiking activity, suggesting that endogenous copper regulates spiking activity in active hippocampal networks (Maureira et al., 2015). Additionally, consistent with previous research demonstrating that nanomolar concentraions of copper inhibit $GABA_A$ receptors (Trombley and Shepherd, 1996; Sharonova et al., 1998), Maureira et al. (2015) found that application of 50-100 nM copper affected the excitatory/inhibitory balance in the hippocamus. Stimulation of Schaffer collaterals in the CA3 resulted in a transient silence of spiking activity in the CA1 of the hippocampus. However, application of 10 and 50 nM copper decreased the silent period (or time to first spike), consisent with a decrease in $GABA_A$ mediated inhibition. Supporting this point, application of copper did not change basal field excitatory post-synaptic currents (fEPSCs) suggesting the decreased time to first spike was a result of decreased inhibition (Maureira et al., 2015). Based on these results, the authors posit that copper can overcome CA1 feedforward inhibition as the inhibition overcome by copper application is mediated by feedforward inhibitory neurons.

Intriguingly, despite evidence that copper increases spontaneous activity and network excitability, Dodani et al. (2014) found that copper chelation rather than copper application increased hippocampal network excitability. Confocal and two-photo calcium imaging of dissociated hippocampal cultures revealed temporally uncorrelated calcium transients. However,

treatment with the copper chelator BCS resulted in an increase in the correlation between calcium transients, which reflect synchronized action potentials produced by neurons in the circuit (Mao et al., 2001). Similar results were obtained when using isolated retinal tissue (Dodani et al., 2014), which is a well-understood model for studying correlated spontaneous activity in developing circuits (Blankenship and Feller, 2010). Although BCS is a cell-impermeable chelator, it is worth noting that imaging with a fluorescent Cu(I) probe showed that BSC application reduced labile copper pools in both dissociated hippocampal neurons and isolated retinal tissue suggesting that application of the chelator affects intracellular copper pools (Dodani et al., 2014). Taken together, these data are consistent with a model in which disrupting copper stores via acute chelation increases network excitability as evidenced by an increase in correlated activity. Further emphasizing the importance of copper in regulating spontaneous activity, $CTR1^{+/-}$ mice, which have half the amount of copper in their nervous system compared to WT mice, had more spontaneous calcium transients in their retinas than those isolated from their WT littermates, paralleling previous work using a copper chelator to disrupt cellular copper (Dodani et al., 2014). Together results from this study suggest that endogenous copper alters spontaneous activity in developing neuronal circuits in a CTR1-dependent manner.

1.2.6 Copper and Circadian Rhythms

The suprachiasmatic nucleus (SCN) in the hypothalamus is the principal circadian clock in the brain and is entrained to light/dark cycles via retino-hypothalamic innervation from a subset of retinal ganglion cells that release glutamate in response to light. The release of glutamate and subsequent activation of NMDARs within the SCN is the dominant cue for photic entrainment

(Ding et al., 1994; Ebling, 1996). Depending on what phase the circadian clock is in, release of glutamate can also cause delays or advances in the circadian rhythm through NMDAR activation.

Intriguingly, copper may play an important role in the regulation of circadian rhythms. The copper chaperone and transporter Atox1 (Lee et al., 2013) is expressed rhythmically in the SCN, as is ATP7A (Yamada and Prosser, 2020). Additionally, electrophysiology studies in acute SCN slices from mice demonstrated that modulation of copper availability can shift circadian clock phase. Reducing copper availability via chelation with tetrathiomolybdate (TTM) or BSC at early subjective night induces a significant delay in peak neuronal activity (~3 hours) (Yamada and Prosser, 2014). This phase delay is consistent with the delay induced by application of glutamate to SCN slices, suggesting that copper chelation may increase NMDAR activity, consistent with studies demonstrating copper-induced inhibition of NMDAR currents (Trombley and Shepherd, 1996; Vlachová et al., 1996; Weiser and Wienrich, 1996). Furthermore, co-application of TTM with the selective NMDAR antagonist 2-amino-5-phosphonopentanoate (AP5) inhibited the TTM-induced phase-delay supporting the supposition that this effect is NMDAR mediated. In addition to causing phase delays at early subjective night, copper chelation, like glutamate application, results in phase advances at late subjective night (Yamada and Prosser, 2014). Although previous evidence suggests that different molecular mechanisms underlie glutamate-induced phase shifts at early and late subjective night (Golombek and Rosenstein, 2010), the NDMAR appears to be involved in both copper chelation-induced phase shifts at both time points (Yamada and Prosser, 2014). Taken together, these findings indicate that basal copper release in the SCN may rein in excessive activation of NMDARs and serve as a homeostatic role in the regulation of circadian rhythms. Surprisingly, application of copper itself also results in similar phase shifts as copper chelation at early and late subjective night. Unlike copper chelation however, these circadian phase

shifts were independent of NMDAR activation (Yamada and Prosser, 2014). Yamada and Prosser (2020) suggest that these results may best be explained by biphasic effects of copper in the SCN where too little or too much copper can phase-shift the SCN clock and activate or inhibit distinct signaling pathways.

In support of the role of copper in the regulation of circadian rhythms, a distinct splice variant of the copper transporter ATP7B (PINA) was identified in the pineal gland (Borjigin et al., 1999). This splice variant occurs via an alternative promoter and results in an open reading frame that is identical to the C-terminal half of the full ATP7B protein. PINA is expressed in pinealocytes and a subset of photoreceptors in adult rats where its expression is 100-fold greater at night than during the day. The nocturnal expression of PINA is under control of the SCN clock, which transmits its signals through innervation of the pineal gland by the superior cervical ganglion (SCG) (Borjigin et al., 1999). Of note, the SCG provides sympathetic innervation via the release of norepinephrine, which, as noted earlier, is itself copper-dependent due the requirement of copper for the conversion of dopamine to norepinephrine by DBH. Indeed, Dbh $^{-/-}$ mice have altered circadian rhythms (Swoap et al., 2004). Intriguingly, while PINA lacks the N-terminus of the ATP7B protein containing metal binding sites, when the protein is expressed in *S. cerevisiae* lacking the ATP7A/ATP7B homolog ccc2, mutant *S. cerevisiae* present copper transport abilities, suggesting PINA is a putative copper transporter (Borjigin et al., 1999). These findings suggest that ATP7B may contain additional copper binding sites in its C-terminus and that the function of PINA in the pineal may involve copper transport.

1.2.7 Copper, Sleep, and Arousal

The locus coeruleus (LC) is a brain structure located in the upper brainstem that has been shown to an important structure for the regulation of a wide range of behaviors including sleep, arousal, attention, learning, and memory (Aston-Jones and Bloom, 1981; Berridge and Waterhouse, 2003; Sara, 2009). Notably, it is also the brain region that has the highest concentration of copper (Que et al., 2008), which is not altogether surprising considering that the LC is the main source of central NE (Lutsenko et al., 2019), the synthesis of which requires the cuproenzyme DBH. As many behaviors linked to the LC, including sleep and arousal, are associated with NE, disruption of copper homeostasis may also disrupt these processes.

Evidence for the importance of copper in the regulation of sleep and arousal comes from a recent study examining the effect of brain copper depletion in the LC of zebrafish. *Atp7a* mutants were generated to investigate the effect of copper dysregulation in the LC. Intriguingly, *Atp7a* mutant larval zebrafish were nearly identical to their wildtype siblings in regard to their gross morphology, growth, and fertility, but had significantly reduced copper levels in the brain (Xiao et al., 2018). The phenotype allowed the authors to study the behavior of these zebrafish in the context of copper dyshomeostasis, without the confounds of growth delay and neurological abnormalities seen in animal models of Menkes disease, which also occurs due to loss of ATP7A function (Kim and Petris, 2007; Guthrie et al., 2020). Supporting the hypothesis that altered copper homeostasis in the LC would lead to alterations in sleep and arousal due to impaired NE synthesis, *Atp7a* mutant larvae showed differences in rest-activity cycles manifested as increased spontaneous swimming activity during the night and decreased activity during the day compared to wildtype zebrafish. Additionally, they had altered arousal behavior as measured by the acoustic startle response. Unlike wildtype larvae which have a lower maximal escape response during the

day compared to the night, indicating increased startle and decreased arousal during the night, the acoustic startle response did not differ between day and night in *Atp7a* mutant larvae and resembled the wildtype larvae's night startle response, suggesting overall increased arousal (Xiao et al., 2018). Importantly, larvae treated with the copper chelator BCS to reduce brain copper recapitulated the behavioral deficits observed in *Atp7a* mutant, zebrafish confirming that these effects were a result of reduced brain copper rather than an unspecified effect of the mutation. Furthermore, larvae that had LC neurons ablated also showed similar behavioral changes to those deficient in brain copper, establishing that copper deficiency within the LC drives alterations in rest-activity cycles and arousal. Finally, increasing synaptic NE within the LC by treating mutant larvae with NE reuptake inhibitors partially rescued the rest-activity phenotype (Xiao et al., 2018). Together, these data demonstrate that copper homeostasis is important for modulation of rest-activity and arousal by the LC through a mechanism requiring metalation of DBH by ATP7A for NE synthesis. These data are supported by *in vivo* work in mice that utilized an Cre-inducible CRISPR/Cas9 system to knockdown (KD) *dbh* in LC neurons (Yamaguchi et al., 2018). Consistent with DBH's known role in the conversion of DA to NE, mice with *dbh* KD had decreased NE concentrations in the LC as well as its projection areas. Functionally, this disruption resulted in increased latency of rapid eye movement (REM) and non-REM sleep-to-wake transitions in response to optogenetic stimulation of neurons within the LC (Yamaguchi et al., 2018). These results support a role for NE in arousal, consistent with the above-described work in zebrafish larvae. In addition to altered sleep-to-wake transitions, LC-specific *dbh* gene KD decreased the total wake time and increased the non-REM time during the dark phase of the light-dark cycle suggesting that NE release is important for the maintenance of wakefulness (Yamaguchi et al., 2018). These findings are consistent with previous work showing that spontaneous discharge of

LC-NE neurons co-varied with the sleep-wake cycle in rats with the highest activity occurring during waking and virtually no activity occurring during REM sleep (Aston-Jones and Bloom, 1981). Yamaguchi et al.'s (2018) findings also support previous results from the group showing a causal relationship between LC-NE firing, sleep-to-wake transitions, and maintenance of wakefulness in mice (Carter et al., 2010) but expand them to implicate NE synthesis by DBH, and thus indirectly copper, in these processes.

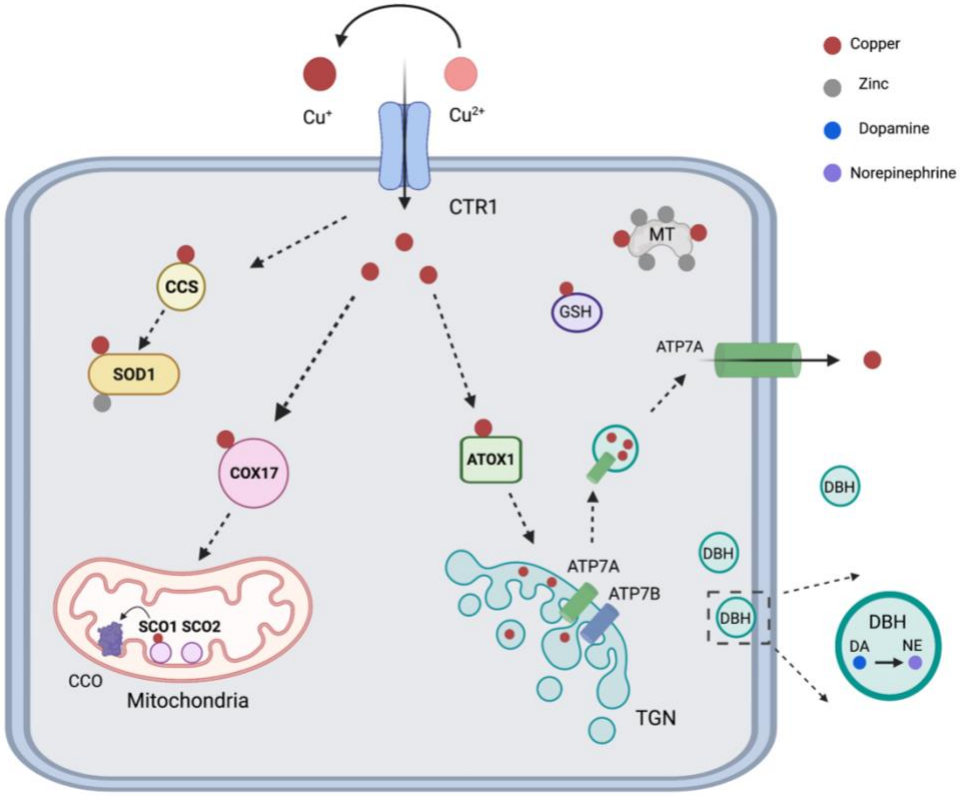

Figure 2. Schematic of copper handling in neurons. Copper is reduced from Cu(II) to Cu(I) by the high affinity copper transporter CTR1 before being transported into neurons. The copper chaperone CCS transports copper to the primarily cytosolically localized cuproenzyme, Cu/Zn superoxide dismutase 1 (SOD1), while the copper chaperone Cox17 binds copper and delivers it to the mitochondria, where it is subsequently transferred to SCO1 before being delivered to cytochrome C oxidase (Cco), the final electron

acceptor in the electron transport chain. SCO2 acts as a copper thiol oxidoreductase for SCO1, altering the ratio of oxidized/reduced cysteines in the protein depending on mitochondrial copper levels. Atox1, a copper chaperone for the secretory compartment, transports copper from the plasma membrane to the trans-Golgi network (TGN), where it transfers copper to ATP7A and ATP7B, allowing these ATPAse copper transporters to metalate secretory proteins such as dopamine-β-hydoxylase (DBH), which is released in vesicles and converts dopamine (DA) to norepinephrine (NE). ATP7A also translocates to the plasma membrane to mediate copper export, thereby regulating cytosolic levels of copper. Metallothioneins (MTs) and glutathione (GSH) bind copper in order keep labile levels of the metal necessarily low and avoid copper-mediated toxicity.

1.3 Pathophysiology of copper in the nervous system

Copper is indispensable for neurological function as is homeostasis of this metal. Both excess copper and copper deficiency lead to severe neurological consequences as exemplified by Wilson disease and Menkes disease, respectively. Disorders of copper homeostasis or copper transporters can lead to a wide range of neurological symptoms including parkinsonism, sleep disturbances, seizures, muscle weakness, and ultimately result in neuronal and glial death. This section will describe important pathologies associate with copper dyshomeostasis.

1.3.1 Wilson disease

Wilson disease (WD) is an autosomal recessive disorder of copper metabolism that leads to pathological copper accumulation, primarily in the liver and the brain. In 1993, two studies identified *Atp7b*, which maps to a region on chromosome 13, as the disease-causing gene (Bull et

al., 1993; Tanzi et al., 1993). Since then, over 500 disease-causing mutations have been identified in ATP7B (Bandmann et al., 2015). ATP7B is highly expressed in the liver where it mediates excretion of copper into bile and delivers copper for incorporation into ceruloplasmin, which is the main copper transporting protein in the blood (Lutsenko et al., 2007). As the liver is the main site for metabolism of dietary copper, dysfunctional ATP7B leads to copper overload in hepatocytes, resulting in liver pathology, and excess copper, not bound to ceruloplasmin, is released into the circulation where it can pathologically accumulate in other tissues, including the brain (Członkowska et al., 2018). Indeed, brain copper in WD patients has been found to be 10-15 higher than in brains of control individuals (Poujois et al., 2017).

Although the presentation of WD patients is highly variable, patients typically present between the ages of 5-35 with clinical features that can include hepatomegaly and hepatitis, Kayser-Fleischer rings in the eyes, neurological manifestations including but not limited to dystonia, dysarthria, and parkinsonism (Svetel et al., 2001; Machado et al., 2006; Lorincz, 2010), as well as psychiatric symptoms such as depression, personality changes, and psychosis (Bandmann et al., 2015; Członkowska et al., 2018). Although not typically described as a symptom of WD, a number of studies have shown sleep disturbances in WD patients (Nevsimalova et al., 2011; Cochen De Cock et al., 2018, 2021) and a meta-analysis found that 54% of patients suffered sleep disorders (Xu et al., 2020). Although the cause of sleep disturbances is not known and may be related to neurological damage, it is interesting to consider the previously described link between copper and the maintenance of circadian rhythms in this context, as copper dyshomeostasis may disrupt sleep cycles.

Given the variable clinical presentation, a diagnosis is usually made in combination with biochemical tests (Ryan et al., 2019; Salman et al., 2022). Additionally, given that treatment is

most effective if given early in the disease course or before patients become overtly symptomatic (Dzieżyc et al., 2014), there is an increasing use of molecular diagnostics in first degree relatives of patients with WD to identify known homozygous or compound heterozygous mutations in such individuals (Ala et al., 2007; European Association for Study of Liver, 2012), although it should be noted that disease-causing mutations have been found to have variable penetrance (Bandmann et al., 2015). Treatment for WD consists of the use of copper chelating agents such as D-penicillamine, trientine, and tetrathiomolybdate, which directly bind copper in blood and tissue and facilitate its excretion, and/or the administration of zinc salts that induce metallothioneins in intestinal cells, which, in turn, bind excess copper and thus interfere with copper absorption (Członkowska et al., 2018; Stremmel and Weiskirchen, 2021; Avan et al., 2022). It should also be noted that the zinc transporter ZIP4 is expressed in intestinal cells where it functions in zinc absorption (Cousins, 2010). Recent work has shown that human ZIP4 is able to transport Cu(II) with micromolar affinity when expressed in oocytes (Antala and Dempski, 2012). Therefore it is possible that zinc treatment leads to decreased enterocyte copper uptake through competition for uptake through ZIP4, although CTR1 is still likely to be the main copper transporter in this cell type based on knockout studies (Nose et al., 2006). While copper chelating agents are highly effective in improving hepatic function, the response rate for patients with neurological symptoms is less favorable (Weiss et al., 2013; Stanković et al., 2023), which may be reflective of irreversible brain damage. Additionally, the initiation of copper chelating agents can paradoxically worsen neurological symptoms during initial treatment (Weiss et al., 2013; Weiss and Stremmel, 2014; Bandmann et al., 2015), although the mechanism by which this occurs is still unknown.

Different brain regions show differential susceptibility to WD-induced copper toxicity. Pathological changes including astrogliosis, demyelination, and necrosis are most often reported

in the basal ganglia, thalamus, cerebellum, and upper brainstem (Członkowska et al., 2018). It is of note that that many of these regions have been implicated in dystonia (Bhatia and Marsden, 1994) and patients with the neurodegenerative disease X-linked dystonia-parkinsonism show both basal ganglia and cerebellar pathology (Hanssen et al., 2018). Furthermore, both neuroimaging and neuropathological studies consistently show lesions in deep brain structures (Mikol et al., 2005; da Costa et al., 2009; Shribman et al., 2022). Thus, damage to brain regions that are vulnerable to copper-induced pathology correspond to neurological symptoms frequently experienced by patients with WD. In addition to the aforementioned lesions, patients with WD show widespread brain atrophy, which has been correlated with both neurological disease as well as increased levels of serum levels of non-ceruloplasmin bound copper (Smolinski et al., 2019, 2022; Shribman et al., 2022). In addition to neuronal pathology, a histopathological feature or WD is the presence of Opalski cells – large glial cells with a foamy cytoplasm and periodic acid-Schiff staining granules that are derived from degenerating astrocytes (Poujois et al., 2017). Two additional abnormal astrocytic cell are observed in WD patient brains – Alzheimers's type I and type II astrocytes, both of which are misshapen and characterized as reactive astrocytes (Mikol et al., 2005; Meenakshi-Sundaram et al., 2008; Poujois et al., 2017; Sosunov et al., 2020; Goldman, 2022). Although Alzheimer's type II astrocytes are associated with hepatic encephalopathy (Poujois et al., 2017), the cause of Alzheimer's type I astrocytes, which are present in a number of neurodegenerative disorders, remains a mystery (Goldman, 2022). Unfortunately, although several animal models for WD exist (Reed et al., 2018; Hadrian and Przybyłkowski, 2021), most poorly recapitulate the neurological manifestations of disease and are better suited for investigation of hepatic pathology, making it difficult to investigate the causes of neuronal and glial cell death and injury in this disease. However, of the animals that do show neurological abnormalities, such as

Atp7b $^{-/-}$ mice, evidence suggests that oxidative stress, respiratory chain defects, and copper mislocalization may all contribute to the observed neuropathology (Sauer et al., 2011; Dong et al., 2015; Reed et al., 2018).

Due to the number of identified mutations in ATP7B, there has been growing interest in finding a genotype-phenotype correlation for pathogenic variants. However, patients with the same mutation can have vastly different clinical presentations. For example, in a Japanese cohort of patients, of the two identified patients with the same c.2871delC mutation, one presented with fulminant hepatitis while the other only showed mild neurological findings at diagnosis (Okada et al., 2000). Additionally on a molecular level, even when pathogenic amino acid substitutions are in the same functional domain, the properties of these ATP7B variants vary widely in regards to stability, localization, and catalytic and transport activity (Huster et al., 2012), making detailed molecular characterizations necessary to understand the consequences of each mutation on protein function. Moreover, the majority of patients with WD are compound heterozygous and recent evidence suggests that the properties of some ATP7B mutants, when expressed *in vitro*, differ when they are expressed alone versus when they are co-expressed with other mutants, suggesting that a functional interaction between pathogenic variants could exist (Roy et al., 2020). However, characterizing the molecular mechanisms underlying defective ATP7B function is still an important step in developing new treatment strategies – especially those that may better improve neurological outcomes. The ATPB-H1069Q mutation is the most frequent disease-linked mutation in European and North American populations. Over 50% of patients from these populations harbor the mutation in homozygosity or compound heterozygosity (Gomes and Dedoussis, 2016). Characterization of this aberrant protein in hepatocyte-like cells derived from WD patient fibroblasts demonstrated that while a significant portion of ATP7B-H1069Q is retained in the ER

where it is rapidly degraded, some protein correctly traffics to the TGN. Furthermore, exposing cells to copper increases trafficking of ATP7B-H1069Q from the TGN to endolysosomal compartments, suggesting it retains some functional properties of the wild-type protein (Parisi et al., 2018). Using proteomics, a recent study identified that this mutation promoted ATP7B interaction with heat shock protein 70 (HSP70), accelerating its degradation in the ER. Importantly, suppression of HSP70 reduced ER retention and degradation of the mutant protein and promoted transport to the Golgi and cell surface, suggesting that HSP70 could be a viable pharmacological target to increase proper localization of ATP7B-H1069Q. These results prompted a three-dimensional similarity search of FDA-approved drugs that resembled the allosteric HSP70 inhibitor used in previous experiments, identifying domperidone, an anti-emetic agent, as a possible drug that could be repurposed for WD. Indeed, domperidone increased ATP7B-H1069Q levels and promoted its export from the ER to the Golgi in HepG2 cells (Concilli et al., 2020). Although these results only show the potential utility of domperidone for the treatment of WD *in vitro*, this study demonstrates the importance of functional characterization of mutants, even when a clear genotype-phenotype correlation cannot be established.

1.3.2 ATP7A-linked Neurodegenerative Disorders

Unlike AT7B, where mutations in the gene result in a single known clinical phenotype (Wilson disease), mutations in ATP7A cause three X-linked neurodegenerative diseases – Menkes disease, occipital horn syndrome (OHS), and X-linked distal hereditary neuropathy (dHMN) (Kaler, 2011). Menkes disease is the most severe of the three and is caused by mutations in the gene that result in its loss of function. OHS is considered to be a milder version of Menkes and is typically caused by a “leaky” splice junction in ATP7A, resulting in only a portion of transcripts

from this allele containing the mutation, or mutations that result in a hypofunctional protein (Kaler et al., 1994; Møller et al., 2000; Dagenais et al., 2001; Kaler, 2011). dHMN is the least severe of the three conditions and has a distinct clinical phenotype from Menkes and OHS, which is thought to be due to mutations in ATP7A that impair but do not abolish correct mRNA splicing. Of note, in dHMN these mutations occur almost exclusively in the carboxyl half of the protein (Kennerson et al., 2010; Kaler, 2013).

Menkes disease and OHN reside on the same clinical spectrum with overlapping symptoms including lax skin and joints, reduced bone density, and vascular tortuosity, although Menkes disease patients typically present within the first 2-3 months of life while OHS patients can present during mid-childhood or later (Menkes, 1988; Kaler, 2011, 2013). Additionally, Menkes patients have coarse, hypopigmented hair (Ramani and Parayil Sankaran, 2023). Biochemical signs of both diseases include low serum copper and ceruloplasmin (Kaler, 2013). This copper deficiency is a result of a failure of ATP7A in enterocytes to efflux copper into the circulation, leading to enterocyte copper accumulation (Kaler, 2011; Ramani and Parayil Sankaran, 2023). However, Menkes disease and OHN diverge in the severity of their neurological manifestations. Neurological features of Menkes disease can include seizures, hypotonia, severe developmental delay, and brain atrophy, which unfortunately contribute to death by 3 years of age (Kaler, 2013; Zlatic et al., 2015). Neuropathological examination of Menkes patient brains shows widespread atrophy of both white and gray matter and neuronal loss that is most pronounced in the cerebral cortex but also affects the hippocampus, striatum, hypothalamus, and thalamus to variable degrees (Zlatic et al., 2015). In contrast, the primary neurological symptom associated with OHS is dysautonomia (Kaler, 2013). The difference in severity of neurological symptoms is thought to be a result of residual ATP7A activity in OHS patients, estimated to be approximately 20-30% due

to a mixture of properly and aberrantly spliced mRNA (Kaler et al., 1994; Gu et al., 2001; Tang et al., 2006). However, it has been reported that levels as low as 2-5% of correctly spliced mRNA permits this milder phenotype (Møller et al., 2000). OHS patients also have a distinctive, and pathognomonic radiological feature – occipital exostoses (excess bone growths) (Kaler et al., 1994). Many symptoms of Menkes and OHS can be attributed to lack of metalation of cuproenzymes. For example, tyrosinase, which catalyzes the production of melanin, is a copper-containing enzyme and lack of catalytic activity contributes to the hypopigmented hair seen in classical Menkes. Similarly, skin and joint laxity in Menkes and OHS patients can be attributed to lack of metalation of lysl oxidase – a cuproenzyme that is important for the production of extracellular matrix proteins like collagen and elastin (Ramani and Parayil Sankaran, 2023).

While it has been posited that the neurodegeneration observed in Menkes disease is a result of the lack of metalation of cytochrome C oxidase (Sparaco et al., 1993; Prasad et al., 2011; Zlatic et al., 2015), in part due to ultrastructural alterations observed in the mitochondria of neurons in Menkes patient post-mortem brain tissue (Hirano et al., 1977; Yoshimura and Kudo, 1983), this doesn't fully explain why OHS patients suffer from symptoms associated with loss of cuproenzyme activity but are spared the severe neurological consequences seen in Menkes – especially considering evidence that copper flux is greater in differentiating neurons than proliferating cells and that ATP7A levels are higher in differentiated neurons compared to undifferentiated cells (Hatori et al., 2016; Lutsenko, 2021). However, recent evidence from a murine model of Menkes disease supports the hypothesis that pathogenic symptoms may be a result of lack of mitochondrial copper (Guthrie et al., 2020). ATP7A-mutant mice treated with the drug elesclomol (ES) – a lipophilic molecule that binds copper with high affinity (Wu et al., 2011; Yadav et al., 2013)– in a complex with Cu(II) (CuES) survived significantly longer than vehicle-

treated animals or those treated with copper histidine (CuHIS) – a hydrophilic copper complex (median age of survival for vehicle treated mice – 14 days; median age of survival for CuHIS treated mice – 18 days; median age of survival for CuES treated mice – 203 days). These survival benefits have been recapitulated in a *C. elegans* model of Menkes disease (Yuan et al., 2022). Neurologically, CuES treated mice had significantly improved better on neuromotor assessments and did not suffer from seizures while on a neuropathological level CuES-treated animals brains showed preservation cortical and hippocampal neurons as well as a preserved Purkinje layer in the cerebellum (Guthrie et al., 2020). Importantly, mitochondrial COX1 levels, one of the two catalytic subunits of cytochrome C oxidase that contains a copper-binding site (Capaldi, 1990; Timón-Gómez et al., 2018), showed a 14% increase suggesting that delivery of copper to the mitochondria may underlie the improvements in Menkes pathology, consistent with *in vitro* studies showing that ES escorts copper to the mitochondria and rescues cytochrome C oxidase levels (Soma et al., 2018; Zulkifli et al., 2023). It must be noted, however, that CuES metalates cuproenzymes in other cellular compartments such as the Golgi (Guthrie et al., 2020). While these results are exciting and suggest that CuES treatment may be a viable treatment strategy for disorders of copper metabolism, whether CuES will be useful for the treatment of Menkes disease likely will rely on early detection as neocortical and cerebellar ATP7A levels peak early in development (Niciu et al., 2006) and it has been suggested that treatment of Menkes disease patients should commence within 10 days of birth due to the early onset of disease (Kaler, 2013).

X-linked distal motor neuropathy, as its name suggests, is an X-linked disorder that results in progressive atrophy and weakness of distal muscles as well as abnormal nerve conduction velocities, but unlike Menkes and OHS, patients with dHMN do not present with any CNS manifestation or other clinical abnormalities. Moreover, there does not appear to be any

derangement of copper metabolism as reflected by normal serum copper and ceruloplasmin. Additionally, in contrast to Menkes and OHS, dHMN is often an adult-onset disease, although it can present in late childhood. These differences in dHMN and the two other ATP7A-linked diseases reflect its milder disease course likely since mutations linked to dHMN do not cause as severe a loss of normal ATP7A function. Indeed, the first mutations linked to this disease were missense mutations (p.P1386S and p.T994I) occurring in the conserved C-terminal portion of ATP7A (Kennerson et al., 2010) – a region that does not directly involve the transporter's known functional domains (Yu et al., 2017a). Additionally, *in vitro* characterization of these mutations revealed no change in ATP7A splicing, or a reduction in mRNA or protein levels, although both ATP7A mutants showed defects in trafficking from the TGN to the plasma membrane in response to copper supplementation in patient-derived fibroblasts (Kennerson et al., 2010). However, when expressed in HEK293 and differentiated NSC-34 cells, a motor-neuron like cell line (Cashman et al., 1992), both mutant ATP7A proteins show increased plasma membrane localization under basal copper conditions compared to WT ATP7A (Yi et al., 2012). Together, these results suggest that trafficking defects are a characteristic of dHMN-linked mutations in ATP7A. It should be noted that fibroblasts derived from patients with the P1386 mutation showed an intermediate copper-retention phenotype (Kennerson et al., 2010), suggesting their ability to appropriately efflux copper is compromised by the mutation.

Although the C-terminus does not contain metal binding domains, which reside in its N-terminus, and is not known to participate in metalation of cuproenzymes (Yu et al., 2017a), more recent research has demonstrated that the carboxyl half of the protein is critical for anterograde and retrograde trafficking of ATP7A through its interaction with adaptor protein (AP) complexes 1 and 2 (Yi and Kaler, 2015) – which play important roles in the regulation of intracellular vesicle

trafficking (Robinson, 2004). Of note, the ATP7A missense mutation P1386S partially disturbs interactions with both AP1 and AP2 and when the mutant protein is expressed in differentiated NSC-34 cells it leads to abnormal axonal localization of ATP7A. Whereas WT ATP7A is normally expressed in the somatodendritic compartment and traffics to axons under conditions of elevated copper, ATP7A^{P1386S} is axonally localized under basal copper conditions (Yi and Kaler, 2015). The authors speculate these finding may reflect a role for AP1 in tethering ATP7A to the TGN until copper triggers its release. Intriguingly, further investigation of the ATP7A T994I missense mutation, also linked to dHMN, revealed an abnormal interaction with valosin-containing protein 97 (p97/VCP), which, like AP1 and AP2, functions in endolysosomal trafficking (Bug and Meyer, 2012). While WT ATP7A and ATP7A^{P1386S} do not strongly interact with this protein, immunoprecipitation experiments demonstrated a selective interaction between p97/VCP and ATP7A^{T994I}. Moreover, siRNA knockdown of p97/VCP in HEK293 cells significantly increased the amount of ATP7A^{T994I} retained in the TGN, directly implicating the aberrant interaction between these two proteins in the mislocalization of ATP7A^{T994I} at the plasma membrane (Yi et al., 2012). Thus, altered interactions between endolysosomal proteins appears to be a common mechanism underlying trafficking defects observed in dHMN-linked ATP7A mutants. Furthermore, *in* vitro characterization revealed the importance of the C-terminus of ATP7A in proper recycling between the TGN and the plasma membrane in response to changing intracellular copper levels. Although it is unclear why these missense mutations in ATP7A cause selective degeneration of motor neurons, it is interesting to note that a missense mutation in carboxyl half of ATP7A (p.M1311V) was recently linked to a patient with ALS and characterization of patient-derived induced pluripotent stem cell motor neurons showed trafficking defects reminiscent of

those described for dHMN-linked ATP7A mutants (Bakkar et al., 2021). Therefore, motor neurons may be a cell type that is selectively vulnerable to copper dyshomeostasis.

1.3.3 Amyotrophic Lateral Sclerosis

Amylotrophic lateral sclerosis (ALS) is the most common adult motor neuron disease with an incidence of approximately 2 per 100,000 individuals per year (Hardiman et al., 2017; Masrori and Van Damme, 2020). ALS is characterized by selective death of upper and lower motor neurons causing progressive muscle atrophy, weakness, and spasticity. Paralysis eventually results and death is often a result of respiratory failure, typically within 2-5 years of ALS onset (Rothstein, 2009; Masrori and Van Damme, 2020). Approximately 10% of ALS is inherited (familial ALS) while close to 90% of cases are sporadic. Although over 30 mutations have been identified, mutations in four genes – *C9orf72*, *TARDP*, *FUS*, and *SOD1* – comprise nearly 70% of familial ALS (fALS) cases (Hardiman et al., 2017). Of these four genes, mutations in the Cu/Zn superoxide dismutase *SOD1*, a copper-dependent enzyme that is important in detoxification of superoxide radicals, has been studied the longest as it was first identified as an ALS-associated mutation in the early 1990s (Siddique et al., 1991; Rosen et al., 1993). As such, the number of identified molecular mechanisms underlying *SOD1*-associated neuropathology/neurotoxicity including, but not limited to, protein misfolding, proteasome impairment, excitotoxicity, oxidative stress, ER stress, impaired axonal transport, axonapathy, inflammation, and mitochondrial dysfunction, are vast (Ferraiuolo et al., 2011). Unfortunately, there does not yet exist effective treatment strategies for this disease. Despite initial excitement over antisense oligonucleotide (ASO) therapy for *SOD1* ALS (Smith et al., 2006; McCampbell et al., 2018), a strategy that has been successfully used to treat conditions such as spinal motor atrophy (Stein and Castanotto, 2017; Scoles et al., 2019), a

phase III study of an ASO targeting this mutant gene failed to improve clinical end points and was associated with adverse events (Miller et al., 2022). Thus, there is still a great need to understand underlying molecular mechanisms of this disease to develop targeted treatment strategies.

There have been several animal models developed to model *SOD1* ALS, with overexpression of the mutant protein being sufficient to cause disease in mice. One common feature to both animal models of *SOD1* ALS and human disease is the deposition of aggregate SOD1 protein in neuronal and glial inclusions (Stieber et al., 2000; Watanabe et al., 2001; Philips and Rothstein, 2015). One of the most widely used rodent models of *SOD1* ALS is the SOD^{G93A} model, which harbors a mutant form of human SOD1 in which an alanine is substituted for glycine at codon 93 (Rosen et al., 1993). This model recapitulates important features of human ALS including the degeneration of spinal motor neurons, muscle weakness, and paralysis, as well as histological features such as pathological inclusions in neurons and glia, some of which contain mutant SOD1 (Stieber et al., 2000; Watanabe et al., 2001). Intriguingly, recent studies examining mutant SOD1 suggest that the aggregation of this protein may be due to a lack of proper metalation. The WT SOD1 is a homodimer with each subunit binding a one copper ion and one zinc ion. While the copper ion is necessary for catalytic activity of the enzyme, the zinc ion is thought to stabilize the structure of the SOD1 (Sirangelo and Iannuzzi, 2017). Multiple studies have demonstrated that metal deficiency increases the aggregation propensity of both WT and mutant SOD1 (Banci et al., 2007; Oztug Durer et al., 2009; Tiwari et al., 2009). In support of the theory that SOD1 aggregation may be linked to problems with metalation, a study utilizing NMR spectroscopy to investigate the structure of fully metalated WT SOD1 and the G93A SOD1 variant found this mutation resulted in local destabilization of protein structure at the site of the mutation, but more importantly selectively destabilized the metal binding region of SOD1 (Museth et al., 2009). Destabilization

of the metal binding site may reduce the metal affinity or binding ability of G93A SOD1 resulting in the pathological aggregation observed in mouse models and humans with this mutation. Additionally, evidence suggests that metal deficiency destabilizes the SOD1 protein and structural distortion of apo-SOD1 results in its aberrant oligomerization (Strange et al., 2007; Teilum et al., 2009).

Based on the findings described above, it would be expected that mutations in the SOD1 copper chaperone CCS would also lead to SOD1 aggregation due to lack of copper metalation. However, surprisingly, an investigation of CCS deficiency showed that while motor neurons in *CCS*$^{-/-}$ mice showed significantly diminished incorporation of copper into WT and mutant SOD1, the absence of CCS did not affect the onset and progression of motor neuron disease in fALS-linked SOD1-mutant mice. This finding suggests that motor neuron disease in *SOD1* ALS is independent of CCS-mediated copper loading (Subramaniam et al., 2002). This result is especially surprising given that finding that this SOD1 copper chaperone is necessary for the activation of the enzyme (Wong et al., 2000) and that mice deficient CCS$^{-/-}$ show increased vulnerability to the oxidative stressor paraquat (Subramaniam et al., 2002) , which exerts its toxicity, in part, through the production of superoxide anions (Alizadeh et al., 2022). Thus, it is possible that, *in vivo,* protein misfolding independent of SOD1 metalation leads to a toxic gain-of-function independent of copper loading. It should be noted, however, that mice with neuronal specific deletion of SOD1 show increased denervated neuromuscular junctions, loss of axonal integrity, and accelerated age-related loss of skeletal muscle (Pollock et al., 2023) suggesting that loss of SOD1 function likely does contribute to the ALS phenotype.

Supporting this possibility, multiple studies investigating the effect of oral administration of the metallo-complex Cu(II)atsm, a low-molecular weight, lipophilic compound that is able to

cross biological membranes, including the blood brain barrier (Dearling and Blower, 1998; Nikseresht et al., 2023), on SOD1^{G93A} mice found that it improved the neurological phenotype of the animals, as assessed by motor performance tests, decreased spinal cord pathology, delayed the time to symptom onset, and improved survival in a dose-dependent manner (Soon et al., 2011; Hilton et al., 2017). Transdermal administration of Cu(II)atsm also significantly extended survival and decreased the percentage of copper-deficient mutant SOD1 in the spinal cord (Williams et al., 2016), suggesting that the improved neurological phenotype may be related to increased SOD1 function. Indeed, supplementation with Cu(II)atsm increased the catalytic activity of mutant SOD1 in the spinal cord of transgenic animals (Hilton et al., 2017), which likely contributed to the reduction in oxidative stressors markers observed in the spinal cords of SOD1^{G93A} mice (Soon et al., 2011; Hilton et al., 2017). Importantly, when Cu(II)atsm was administered to non-transgenic animals, no increase in SOD1 activity and no change in cytochrome C activity was observed, suggesting that Cu(II)atsm does not alter the activity of copper-replete cuproenzymes (Hilton et al., 2017). Intriguingly, in addition to its therapeutic effect on rodent motor neurons, Cu(II)atsm treatment may also improve astrocyte function in patients with SOD1 mutations (Dennys et al., 2023), which is significant as glia reprogrammed from fibroblasts of patients with *SOD1* mutations have been shown to induce hyperexcitability and cell death in healthy control motor neurons (Di Giorgio et al., 2008; Marchetto et al., 2008; Ferraiuolo et al., 2016). A recent *in vitro* study demonstrated that when astrocytes from SOD1-linked ALS patient-derived fibroblasts were pre-treated with 1 µM Cu(II)atsm before co-culture with healthy murine embryonic stem cell derived motor neurons, motor neuron survival was increased compared to vehicle-treated or untreated reprogrammed astrocytes. Intriguingly, however, Cu(II)atsm does not improve astrocyte dysfunction by increasing *SOD1* activity but by decreasing mitochondrial respiration, which is

increased in these cells, and increasing glycolysis (Dennys et al., 2023). These finding suggest that the beneficial effects of Cu(II)atsm are multifaceted and involve the improvement of both neuronal and glial function in cells carrying *SOD1* mutations. While these results are promising, it is necessary to acknowledge that treatments that have successfully delayed disease onset and extended lifespan in animal models of ALS, such as the antibiotic minocycline (Kriz et al., 2002; Zhu et al., 2002), have not translated to success in clinical trials (Gordon et al., 2007), and, as such, these findings should therefore be interpreted with caution.

1.3.3.1 Multiple Sclerosis

Multiple sclerosis (MS) is a demyelinating disease of the CNS in which the immune system attacks oligodendrocytes, the myelinating cells of the CNS, causing their cell death, resulting in progressive demyelination in the brain and spinal cord. A number of animal models have been developed to study this neurodegenerative disease, including cuprizone (CPZ) treatment. The CPZ model allows the study of processes underlying primarily non-autoimmune demyelination and remyelination, in contrast the experimental autoimmune encephalomyelitis (EAE) model in which mice are artificially sensitized to autoantigens. CPZ is a copper chelator, and the CPZ model of MS involves the addition of CPZ to mouse chow. Despite the widespread use of this model to study MS, no consensus yet exists as to CPZ's mechanism of action although it is thought that it may exert its toxicity by disrupting mitochondrial enzymes of the respiratory chain through copper chelation, as these require copper as a cofactor for proper function. It is posited that this disruption leads to oxidative stress and eventually results in oligodendrocyte cell death along with activation of microglia and astrocytes (Kipp et al., 2017).

Recently, however, several studies have challenged that demyelination in the CPZ is a result of copper chelation and direct oligodendrocyte death. If CPZ causes demyelination through copper chelation, then copper supplementation may offset some of its damaging effects. However, the addition of copper in stoichiometric excess of CPZ to the chow of C57Bl/6 mice did not reduce CPZ-induced demyelination (Morgan et al., 2022). Furthermore, when equivalent doses of other copper chelators including neocuproine and D-pencillamine were added to mouse chow, they failed to induce CNS demyelination, suggesting that CPZ may be acting through another mechanism. Supporting this notion, the addition of D-pencillamine to the CPZ diet paradoxically prevented demyelination in a dose-dependent manner (Morgan et al., 2022), rather than exacerbating the CPZ-phenotype as one might expect with the addition of another copper chelator. These data suggest that the binding of the CPZ to copper may give rise to a toxic species. Indeed, there is evidence that the binding of CPZ to Cu^{2+} gives rise to an unusually high valent Cu^{3+} state which yields a $Cu^{3+}(CPZ)_2^-$ complex (Messori et al., 2007; Yamamoto and Kuwata, 2009). The high oxidation state of copper could allow it to participate in reactions that lead to peroxidation of lipid-rich structures, such as myelin. Thus, evidence of CPZ-induced lipid peroxidation and subsequent oligodendrocyte death (Jhelum et al., 2020) may be a result of copper toxicity rather than copper deficiency. Furthermore, the finding that CPZ leads to ferroptosis of oligodendrocytes (Jhelum et al., 2020) is consistent with recent work demonstrating that copper can amplify sensitivity of cells to this cell death pathway (Xue et al., 2023).

Supporting the idea that copper toxicity rather than deficiency may lead to or exacerbate demyelination comes from a study that examined MS lesions in human MS patients as well as in EAE mice and CPZ mice. Colombo et al (2021) found that in MS patients, while circulating serum copper levels were unchanged, there was a striking increase in the expression of CTR1, ATP7A, and

ATP7B in GFAP-positive astrocytes during neuroinflammation. These data could indicate that in MS patients, a rise in astrocytic copper uptake from the bloodstream and subsequent redistribution to other cell types such as oligodendrocytes drives cell death and demyelination. This idea is supported by the strong inverse correlation found between CTR1 levels and myelin content in areas of active MS lesions and the direct correlation between CTR1 or ATP7B and astrogliosis, in as well as the upregulation of all copper transporters in chronic inactive lesions, with high levels of CTR1 and AT7B on GFAP-positive astrocytes in human brain samples. Consistent with what the authors observed in the human CNS, both EAE mice and CPZ mice showed intense upregulation of ATP7A, ATP7B, and CTR1 on astrocytes in the spinal cords in comparison to control mice (Colombo et al., 2021). Together these data suggest that astrocyte-mediated copper dyshomeostasis and copper toxicity is a common mechanism underlying demyelination in MS and animal models of MS.

1.3.4 Acquired Copper Deficiency

Although Menkes disease demonstrates the devastating neurological consequences of early copper deficiency, acquired copper deficiency has become well-recognized as a cause of neurological disease (Jaiser and Winston, 2010). Risk factors for acquired copper deficiency include bariatric surgery, prolonged total parental nutrition, malabsorption syndromes such as Celiac disease, and zinc overload (Goodman et al., 2009; Jaiser and Winston, 2010; Btaiche et al., 2011; Doherty et al., 2011). Patients with hypocupremia typically present with both hematological abnormalities such as anemia and neutropenia (Lazarchick, 2012), in addition to neurological abnormalities, which often resemble subacute combined degeneration of the spinal cord, a complication of vitamin B12 deficiency involving sensory deficits, paresthesia, weakness, and ataxia (Qudsiya and De Jesus, 2021), due to the involvement of the dorsal column (Kumar et al.,

2004). Although neurological symptoms due to hypocupremia can be severe and include generalized weakness, impaired sensation of position and vibration, and gait ataxia, they can also be useful in distinguishing between myelodysplastic syndrome and hypocupremia. Indeed, in one case, the presence of neurological symptoms in a patient with suspected myelodysplastic syndrome, who was being evaluated for a bone marrow transplant, prompted her physicians to test her serum copper levels, which were undetectable, with symptoms resolving following copper supplementation (Kumar et al., 2005). However, it is important to identify and treat hypocupremia early as copper supplementation resolves hematological abnormalities, but neurological manifestations are not always reversible (Gabreyes et al., 2013; Rohm et al., 2019).

While many patients with hypocupremia present with hyperzincemia, the source of increased zinc levels is not always identifiable (Hedera et al., 2003; Merza et al., 2015). However, one surprising and intriguing cause of hyperzincemia leading to myeloneuropathy reported in the literature is the use of zinc-containing dental adhesives. Indeed, a number of reports describe the development of hematological abnormalities and hyperzincemia alongside progressive myeloneuropathy that could be traced back to excessive use of dental adhesive (Nations et al., 2008; Tezvergil-Mutluay et al., 2010; Doherty et al., 2011; Carroll et al., 2017; Cathcart and Sofronescu, 2017; Jamal et al., 2021). In some cases, the neurological damage to the dorsal column of the spinal cord was severe enough to be detected on magnetic resonance imaging (Gabreyes et al., 2013; Carroll et al., 2017). The widespread recognition of hyperzincemia-mediated hypercupremia has led to the development of new, zinc-free formulations of dental adhesives; however, some popular dental adhesive brands still contain zinc. Thus, dentists should advise patients on the importance of properly fitting dentures and physicians should be aware of this association when evaluating patients with hyperzincemia and hypocupremia of unknown origin.

1.4 Conclusion

Copper is a critical micronutrient for growth, development, and brain development. Disorders of copper metabolism such as Menkes disease, demonstrates the devastating consequences of early copper deficiency on neurological function (Kaler, 2011) while the neurological symptoms associated with acquired copper deficiency such as myeloneuropathy (Btaiche et al., 2011; Doherty et al., 2011; Rohm et al., 2019) highlight the continual need for copper for proper neuronal function. Indeed, the metal plays critical roles in neuronal metabolism, antioxidant defense, as well as neurotransmitter and neuropeptide synthesis (Hatori and Lutsenko, 2013; Lutsenko et al., 2019; Wen et al., 2021). Moreover, copper can act as a neuromodulator through its effects on NMDAR and GABAR activity (Narahashi et al., 1994; Trombley and Shepherd, 1996; Vlachová et al., 1996; Sharonova et al., 1998; Peters et al., 2011; Marchetti et al., 2014), has been found to alter network excitability (Dodani et al., 2014; Maureira et al., 2015), and can influence behavior through its indispensable role in the synthesis of norepinephrine (Yamada and Prosser, 2014; Xiao et al., 2018). Although only briefly referenced in in this review of the literature, the recent advent of fluorescent probes that are highly selective for copper and sensitive to oxidation states now allows for the investigation of labile Cu(I) in living cells (Chung et al., 2019; Morgan et al., 2019) and will likely lead to new and exciting discoveries regarding the physiological and pathophysiological mechanisms of copper signaling in the brain.

1.5 Dissertation Goals

There is a wealth of research regarding neurons and their copper handling systems. In contrast, there is significantly less literature exploring the ways that astrocytes utilize and buffer copper. However, based on the research that does exist, it is thought that that astrocytes may protect neurons against copper toxicity *in vivo* due to their contact with capillary endothelial cells, thought to be responsible for the transport of copper across the blood-brain-barrier, as well as by the high capacity of astrocytes to store copper and thus act as so-called metal "depots" or "sinks"(Scheiber and Dringen, 2013; Scheiber et al., 2014) At the same time there is a growing interest in the use of copper and the copper-ionophore elesclomol (CuES) to treat cancers, including gliomas, which raises the question of how the surrounding brain parenchyma will respond to this insult. We have observed that the treatment of primary rat mixed cortical cultures with CuES showed that, to our surprise, astrocytes rather than neurons were selectively vulnerable to this treatment. Given this observation, the goals of the dissertation were to: 1) Determine whether a glioma cell line is susceptible to CuES treatment, which may indicate potential therapeutic utility, 2) Establish the selective vulnerability of astrocytes to CuES compared to neurons, 3) Determine the mechanism(s) underlying CuES-induced astrocyte cell death, and 4) Investigate potential glioprotective strategies. These dissertations goals are covered in Chapter 2 while a discussion of the implications of our findings are discussed in Chapter 3.

2.0 Copper-elesclomol induces neuron sparing, FDX1-independent astrocyte toxicity

2.1 Overview

Copper is an essential enzyme cofactor in oxidative metabolism, antioxidant defenses and neurotransmitter synthesis. However, intracellular copper, when improperly buffered, can also lead to cell death. Here, we show that copper, in the presence of the copper ionophore elesclomol (CuES), is highly toxic to rat C6 glioma cells *in vitro*, supporting computational studies that have established glioma as likely to be highly susceptible to copper-mediated cell death. As glioma are generated within the brain parenchyma, we also examined the vulnerability of neurons and astrocytes to CuES in tissue culture. While we observed that astrocytes were similarly highly sensitive to CuES toxicity, neurons were surprisingly resistant, a vulnerability profile that is opposite of what has been described for zinc. A human astrocytic cell line was similarly sensitive to CuES. Modifications of cellular metabolic pathways implicated in CuES-regulated cell death, cuproptosis, namely inhibition of mitochondrial respiration, and knock-down of ferredoxin 1 (FDX1), were insufficient to abrogate CuES toxicity to astrocytes. CuES toxicity was also unaffected by inhibitors of apoptosis, necrosis or ferroptosis. We did detect the presence of lipid peroxidation products in CuES-treated astrocytes, suggesting the presence of reactive oxygen species following metal exposure. Indeed, treatment with antioxidants mitigated CuES induced cell death in both astrocytes and glioma cells. Lastly, prior induction of metallothioneins 1 and 2 in astrocytes with zinc plus pyrithione was strikingly protective against CuES toxicity. These results demonstrate a unique toxic response to copper in glial cells, but not neurons, which differs in cell selectivity profile of zinc.

2.2 Introduction

Copper is a catalytic and structural cofactor for cuproenzymes involved in oxidative phosphorylation, iron homeostasis, antioxidant defense, as well as neurotransmitter synthesis (Scheiber et al., 2014; Ruiz et al., 2021). Copper also serves important biological functions in the brain, including modulation of synaptic transmission (D'Ambrosi and Rossi, 2015). The critical role of copper in key physiological processes is evidenced by the fact that the transition metal is an essential micronutrient in virtually all eukaryotes (Festa and Thiele, 2011). Furthermore, genetic mutations that result in copper deficiency lead to human disorders with profound clinical consequences (Jaksch et al., 2000; Kaler, 2013; Garza et al., 2023). However, copper is able to redox cycle, allowing it to form toxic free radicals, displace other metals from enzymes, and disrupt proper protein folding (Festa and Thiele, 2011). Indeed, the accumulation of intracellular copper is the key driver of pathology in Wilson's disease (Ala et al., 2007). Moreover, copper dyshomeostasis has been linked to neurodegenerative disorders (Gromadzka et al., 2020; Chen et al., 2022). As such, intracellular concentrations of copper are tightly regulated (Morgan et al., 2019).

A copper-dependent regulated cell death pathway, cuproptosis, was recently described (Tsvetkov et al., 2022). Cuproptosis involves the disruption of mitochondrial respiration through increased lipoylation of tricarboxylic acid (TCA) cycle proteins (Tsvetkov et al., 2022). In this cell death pathway, lipoylation, a post-translational modification of lysine, is mediated by ferredoxin 1 (FDX1), an iron-sulfur cluster protein involved in electron transfer for a variety of biological processes (Schulz et al., 2023). The bulk of research on cuproptosis has involved the copper ionophore elesclomol, which has been previously investigated as a possible anti-neoplastic drug (Yadav et al., 2013). Although it has had limited efficacy in clinical trials (O'Day et al., 2013;

Monk et al., 2018), elesclomol is a potent inhibitor of cancer cell proliferation *in vitro* (Blackman et al., 2012; Nagai et al., 2012; Hasinoff et al., 2015) and, as such, the discovery of cuproptosis has reignited interest in copper's potential as a treatment strategy for difficult-to-treat cancers. Indeed, cuproptosis-associated genes have been evaluated as predictors of prognosis and treatment response in glioma (Ye et al., 2022; Zhang et al., 2022, 2023; Zhu et al., 2022). Gliomas are tumors derived from aberrant glial or glial precursor cells and are amongst the most common and most deadly types of primary brain tumor (Ostrom et al., 2019; Nicholson and Fine, 2021). Unfortunately, no novel pharmaceutical treatments that affect patient survival have been successfully developed for this malignancy since temozolomide was introduced over 15 years ago (Stupp et al., 2005; Nicholson and Fine, 2021). As the majority of studies examining the role of cuproptosis in glioma have been computational in nature, we sought to determine if a glioma cell line would be sensitive to copper-elesclomol (CuEs) treatment. Additionally, we aimed to determine how CuEs would affect the tumor microenvironment. We discovered that while glioma cells are indeed vulnerable to CuEs-induced death, astrocytes are also extremely sensitive to CuEs toxicity. To our surprise, neurons were strongly resistant to this insult. Importantly, our results also indicate that copper/elesclomol toxicity to astrocytes is not via cuproptosis, as it lacks dependence on FDX1.

2.3 Materials and Methods

2.3.1 Compounds

Chemicals: elesclomol (#S1052), Z-VAD-FMK (S7023), necrostatin-1s (S8641), deferoxamine mesylate (S5742), and ferrostain-1 (S7243) were purchased from Selleck Chemicals. Cupric chloride dihydrate (C3279), Antimycin A (A8674), Sodium azide (S8032), EUK-134 (SML0743) and pyrroloquinoline quinone (PQQ) (D7783) were purchased from Sigma. Dizocilpine hydrogen maleate (MK801) (M107) was purchased from Milipore. Primary antibodies for immunocytochemistry: chicken anti-MAP2 (Abcam, ab5392, 1:5000), rabbit anti-GFAP (Abcam, ab7260, 1:1000), mouse anti-4-hydroxynonenal (12F7) (Invitrogen, MA5-27570, 1:100). Secondary antibodies for immunocytochemistry: Alexa Fluor 488, Alexa Fluor 546, Alexa Fluor 647 (Thermo Fisher, 1:1000).

2.3.2 Cerebrocortical cultures

All procedures involving the use of animals were reviewed and approved by the University of Pittsburgh IACUC (Protocol # 21039053). Primary cortical cultures were prepared from embryonic day 16-17 Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) as previously described (Hartnett et al., 1997). Briefly, pregnant rats were housed alone in a standard cage in the University central animal facility for a maximum of three days with free access to food and water and were sacrificed humanely and painlessly by CO_2 inhalation and further exsanguinated via severing of the jugular vein to assure completion of euthanasia. The animals showed no sign of distress during this procedure and perished quickly and painlessly. Embryos of

either sex were removed and quickly decapitated, although they likely had also perished before the dissection was performed. Five-10 embryonic cortices from a single littler were pooled and dissociated with trypsin and cells were plated on 12 mm, poly-L-ornithine (PLO) coated glass coverslips in six well plates at a density of 635,000 cells per well. We consider a single dissociation, from one pregnant animal, a biological replicate. Non-neuronal proliferation was inhibited after 14 days *in vitro* (DIV) with 2 µM cytosine arabinsoside. Cultures were utilized at 3-4 weeks *in vitro*. We calculate that approximately 50-70 pregnant rats (approximately 450 embryos) were utilized for these studies as we normally perform one dissociation per week throughout the year. On occasion, a pregnant animal of the wrong gestation age was shipped, and these were excluded from the study (i.e. no cultures were obtained). Otherwise, no cultures were excluded from the study.

2.3.3 Astrocyte cultures

To generate astrocyte cultures, mixed cortical cultures were exposed to 1 mM kainate (KA) in HEPES-buffered minimal essential media with 0.01% BSA (MHB) for 18-24 hours to eliminate the neuronal component, which comprise 10-20% of cultures at 3-5 weeks *in vitro*, while sparing the astrocytes which comprise 80-90% of cells in this preparation (Rosenberg and Aizenman, 1989). Following overnight KA treatment, astrocytes were transferred to fresh MHB solution. For experiments utilizing both mixed cultures and astrocyte cultures, coverslips containing neurons and astrocytes were treated in MHB without KA in parallel to control for manipulation of coverslips.

2.3.4 C6 glioma cells

Rat C6 glioma cells (CCl-107, ATCC) and were cultured in Ham's F12 Nutrient Mixture with GlutaMAX supplemented with 2.5% fetal bovine serum (Thermo Fisher) and 15% horse serum and maintained in an incubator at 37°C with 5% CO_2. Cells were split twice a week. For LDH assays, C6 cells were plated at a 188,000 cells per well PLO-coated 24 well plates and for CellTiter Glo assays, cells were plated at a density of 32,000 cells per well onto PLO-coated 96 well plates. The maximum number of passages utilized for these cells was ~25.

2.3.5 Immortalized Human Astrocytes

Immortalized human fetal astrocytes (hTert) (Applied Biological Materials, T0281) were cultured on poly-D-lysine (PDL) (Sigma) coated flasks in Prigrow IV medium (Applied Biological Materials) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 ng/mL human epidermal growth factor (EGF) (Gibco), and 100 U/mL penicillin, 100 µg/mnL streptomycin (Sigma). Cells were split twice a week and seeded at a density of 40,000 cells per well onto PDL-coated 24 well plates for LDH assays and 14,000 cells per well onto PDL-coated 96-well plates for CellTiter Glo assays. The maximum number of passages utilized for these cells was ~20.

2.3.6 Cell Treatments

All experimental paradigms were performed in MHB unless otherwise stated. Cells were exposed to indicated concentrations of copper elesclomol (CuES) overnight or for a two-hour

pulse. For CuES pulse experiments, cells were exposed to CuES for 2 hours and then rinsed with MHB. Cells were then incubated overnight in fresh MHB before toxicity/viability measurements. All experiments using drugs to modify CuES toxicity, with the exception of zinc pyrithione (ZnPyr), included a one-hour pre-incubation period before the addition of CuES. Drugs remained in their respective treatment groups overnight before toxicity or viability assays were conducted. For zinc pre-conditioning experiments, cortical cultures were simultaneously exposed to 1 mM KA and 20 µM zinc in the presence of 250 nM pyrithione. Following overnight incubation with KA and ZnPyr, coverslips were moved to fresh MHB before treatment with CuES.

2.3.7 siRNA Treatment

Mixed cortical cultures were treated with 1 µM Acell rat *FDX1* SMARTPool siRNA (Horizon) (Horizon) for 48 hours in DMEM-Glutamax supplemented with 2% FBS and 31 mM HEPES. Sister cultures were treated in parallel with 1 µM Acell non-targeting control siRNA (Horizon). For initial confirmation of *FDX1* knockdown, RNA was collected after 48 hours. For CuES toxicity experiments, siRNA treated coverslips were transferred to MHB after 48 hours and exposed to CuEs overnight. RNA was collected from sister cultures treated identically to confirm the persistence of knockdown.

2.3.8 Toxicity Assays

The toxicity of treatment conditions was assessed using a using a commercially available colorimetric lactate dehydrogenase (LDH) activity assay kit (Sigma). Following experimental treatment, medium was collected for LDH assays as this enzyme is rapidly released from cells

following damage to the plasma membrane (Aras et al., 2008). Toxicity is represented by increased OD450 values. A minimum of three experiments from separate culture dates were performed, each in triplicate.

2.3.9 Viability Assays

The CellTiterGlo Luminescent Cell Viability Assay (Promega) was used to assess viability following copper-ionophore treatment in experiments utilizing EUK-134 and PQQ, as these compounds interfered with the enzymatic reaction utilized in the LDH assay. Experiments were performed according to the manufacturer's directions. Viability was calculated as percent of control luminescence values due to variability between experiments in absolute luminescence values. A minimum of three experiments from separate culture dates were performed, each in triplicate, for primary cultures and from three separate split dates for cell lines.

2.3.10 Immunocytochemistry

Coverslips were fixed with 4% paraformaldehyde/4% sucrose for 20 minutes, washed three times with PBS, and were then blocked and permeabilized with 0.05% Triton-X with 10% normal goat serum in PBS for 45 minutes. Coverslips were then incubated with primary antibodies at 4°C overnight. The following day, cells were incubated with secondary antibodies for one hour at room temperature before mounting with Fluoromount-G mounting medium (Thermo Fisher). Three to four random fields of view were imaged from each coverslip (60x) on a Nikon A1R laser scanning confocal.

2.3.11 Quantitative Polymerase Chain Reaction

Total RNA was isolated from cortical cultures using the TRIzol Plus RNA Purification Kit (Invitrogen). RNA samples were eluted in RNAse free H_2O and approximately 500 nanograms of RNA from each sample was reversed transcribed into cDNA using iScript (Bio-Rad). Quantitative PCR (qPCR) was performed with using the following primers: β-actin sense (FW): 5′-ACTCTTCCAGCCTTCCTTC-3′; β-actin antisense (RV): 5′- ATCTCCTTCTGCATCCTGTC-3′ FDX1 sense (FW): 5'-TCGATGGATTTGGTGCGTGT-3'; FDX1 antisense (RV): 5'-CAGGCACACGGACAGTCATA; MT1 sense (FW): 5'-CACCGTTGCTCCAGATTCAC-3'; MT1 antisense (RV): 5'- GCAGCAGCACTGTTCGTCAC-3'; MT2 sense (FW): 5'-GCAGCAGCACTGTTCGTCAC-3'; MT2 antisense (RV): TGCACTTGTCCGAAGCCTCT-3'. qPCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and a CFX96 Touch Real-Time PCR detection system (Bio-Rad). The qPCR reactions for each sample were run in triplicate and all targets were normalized to β-actin.

2.3.12 Statistics

Data are presented as means ± SEM. The number of experiments noted for each experiment represents a biological replicate. All statistical analyses were performed using GraphPad Prism 9 (GraphPad). Normality was assessed by the Shapiro-Wilk test. For comparison of two sample means, a two-tailed *t*-test was used. A one sample t-test was used for analysis of normalized values (control matched to 1). For comparison of more than two sample means, a one-way analysis of variance (ANOVA) with Sidak's or Dunnet's test for multiple comparisons was used (specified in figure legends). For analysis of LDH values in experiments comparing cultures with neuron and

astrocytes to astrocytes alone, a two-way ANOVA with Sidak's test for multiple comparisons was used to compare the effect of drug treatments between sample groups. Although no formal power analysis was performed, samples sizes were determined *a priori* based on a large number of previous studies utilizing our cell culture models to examine cell death (Hartnett et al., 1997; Aizenman et al., 2000; Aras et al., 2008).

2.4 Results

2.4.1 Copper ionophore treatment induces cell death in C6 glioma cells

To determine whether copper induces cell death in glioma cells *in vitro*, we used rat C6 glioma as this cell line has been reported to express similar genetic changes to those seen in human brain tumors (Sibenaller et al., 2005). C6 cells were exposed overnight to increasing concentrations of copper (0.1-10 µM) in the presence of the highly selective copper ionophore, elesclomol (ES; 100 nM). Although elesclomol was not toxic to C6 cells on its own, 2 µM copper in the presence of 100 nM elesclomol (CuES) induced significant cytotoxicity as evidenced by increased LDH release, an indicator of cell death and damage (Fig. 1A). Additionally, even at sublethal concentrations of CuES, we observed a striking change in morphology with cells losing their spindle-like appearance and becoming more circular in shape (Fig 1B).

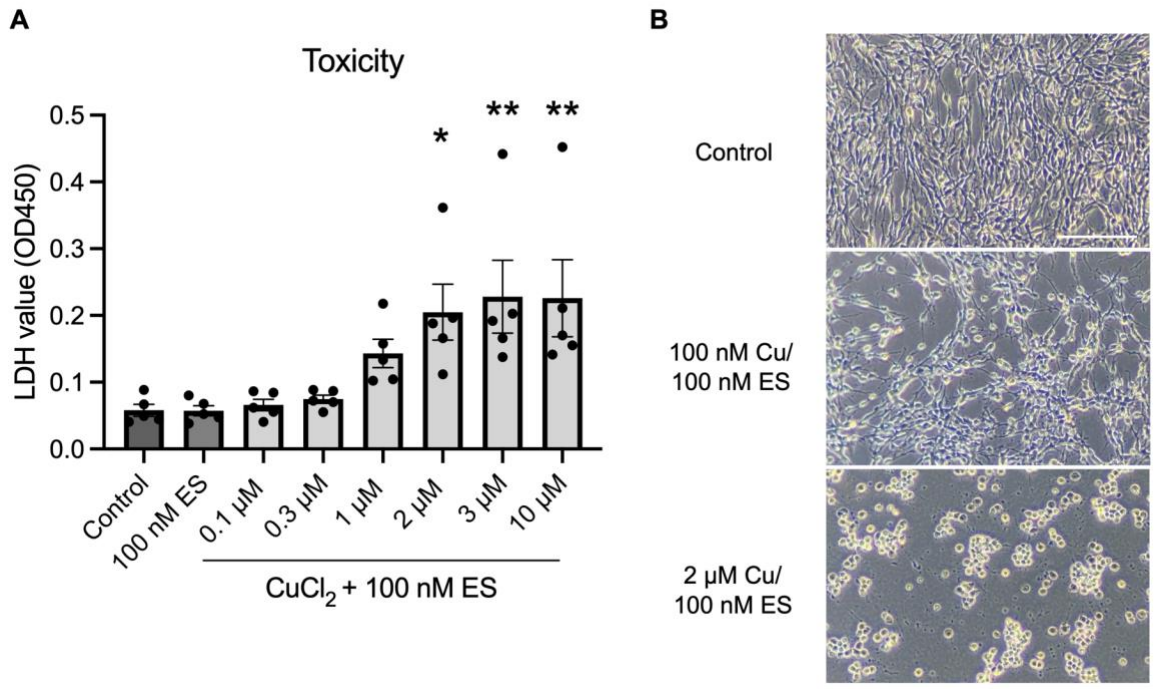

Figure 3. C6 glioma cells are susceptible to copper ionophore induced cell death. (A) LDH assay showing concentration-dependent copper toxicity in the presence of 100 nM elesclomol (*p<0.05, **p<0.01, one-way ANOVA, Dunnet post-hoc, n=5 biological replicates). **(B)** Phase contrast images of C6 glioma cells after overnight treatment with CuES demonstrating concentration-dependent alterations in cell morphology. Data are expressed as mean ± SEM. Scale bar is 50 µM.

2.4.2 Primary rodent neurons and astrocytes are differentially vulnerable to copper-elesclomol induced toxicity

Because gliomas are generated within the brain parenchyma, an optimal treatment would selectively target glioma cells while leaving the surrounding tissue uninjured. To assess the effect of CuES on the glioma microenvironment, we used mixed cerebrocortical cultures containing

neurons and astrocytes to evaluate the susceptibility of these cell types to CuES treatment. We found that overnight treatment with concentrations at or above 2 μM copper in the presence of 1 μM elesclomol resulted in significant cytotoxicity as evidenced by increased LDH release (Fig. 2A). Strikingly, phase contrast microscopy revealed the presence of healthy-appearing neurons surrounded by swollen astrocytes in CuES-treated cultures (Fig. 2B). This finding was surprising in light of the significant body of evidence demonstrating that neurons are exquisitely sensitive to toxicity induced by transition metals, particularly zinc, while astrocytes are generally resilient against this cell stress (Choi et al., 1988; Dineley et al., 2000). Indeed, exposing the cerebrocortical cultures to overnight treatment with 10-30 μM zinc along with 250 nM of the zinc ionophore pyrithione (ZnPyr) resulted in a significant elevation in LDH release (Fig. 2C) and phase contrast microscopy confirmed that neurons were primarily affected by this treatment, while astrocyte morphology remained intact. (Fig. 2D).

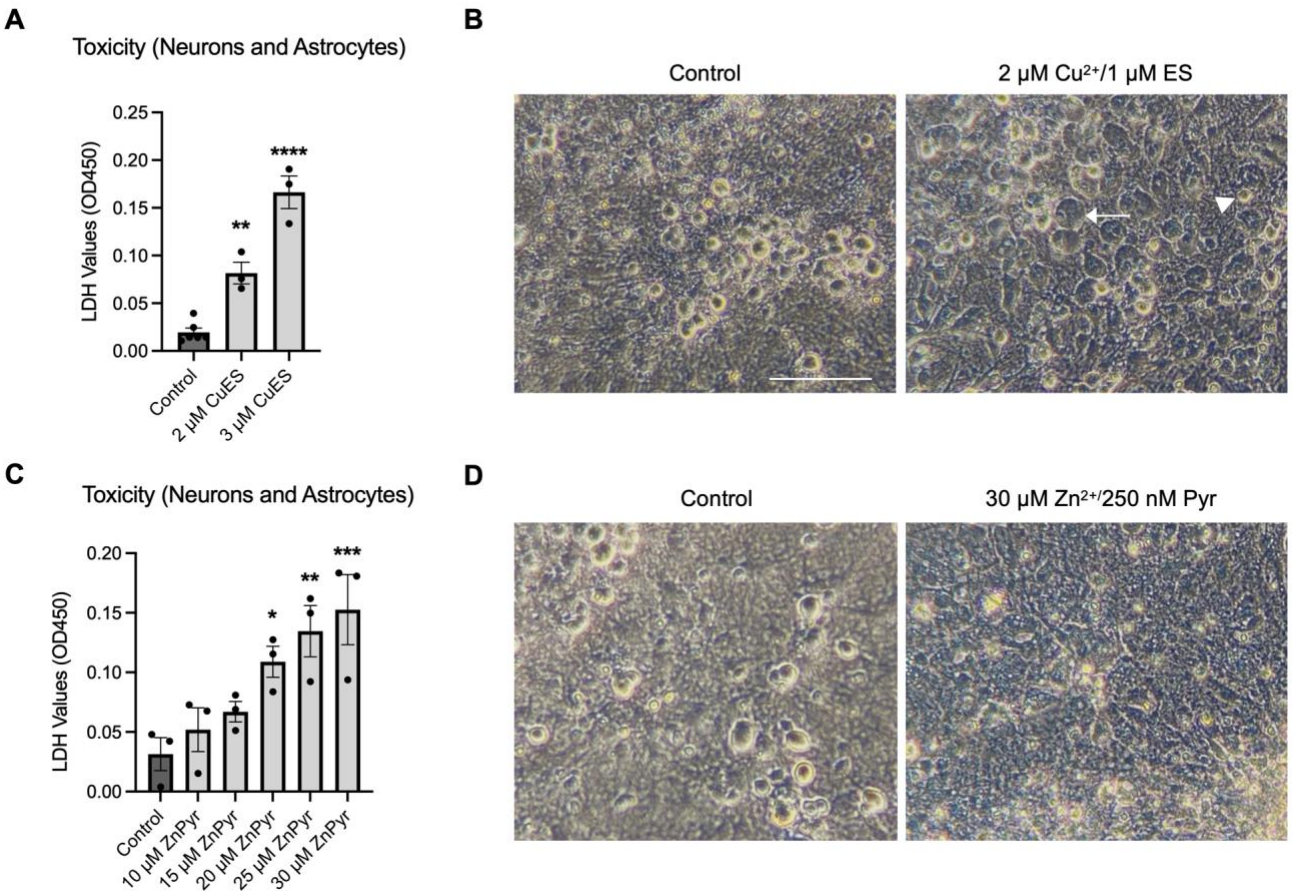

Figure 4. Copper and zinc toxicity appear to target different cell populations in mixed neuronal glial cultures. (A) LDH assay showing concentration-dependent copper toxicity in the presence of 1 μM elesclomol in cerebrocortical cultures (**p<0.01, ***p<0.001, one-way ANOVA, Dunnet post-hoc, n=3 biological replicates). **(B)** Phase contrast images of cerebrocortical cultures following overnight treatment with CuES, demonstrating swollen glia (arrow), but seemingly healthy-appearing, phase-bright neurons (arrowhead). **(C)**. LDH assay showing concentration-dependent zinc toxicity in the presence of 250 nM of the zinc ionophore, pyrithione (*p<0.05, **p<0.01, ***p<0.001, one-way ANOVA, Dunnet post-hoc, n=3 biological replicates). **(D)**. Phase contrast images of cerebrocortical cultures following overnight treatment with ZnPyr showing a lack of phase bright neurons, but seemingly healthy glia. Data are expressed as mean ± SEM. Scale bar is 100 μM.

As LDH values represent the total LDH released by both neurons and astrocytes in our cultures, we next assessed the relative contributions of each cell type to CuES- and ZnPyr-induced LDH release by comparing cultures containing both neurons and astrocytes to those containing astrocytes alone. To achieve astrocyte cultures lacking neurons (astrocyte cultures), mixed cerebrocortical cultures were treated overnight in media containing 1 mM kainate, as previously described (Aizenman et al., 2000), which causes widespread neuronal death without affecting the viability of astrocytes (Prieto and Alonso, 1999; Aizenman et al., 2000; Qu et al., 2003). Following kainate or vehicle treatment, cultures were treated with increasing concentrations of CuES or ZnPyr overnight. As suggested by our observations noted above, astrocytes in the mixed cultures contributed the majority of the LDH signal following CuES treatment. In cultures containing neurons and astrocytes, significant toxicity was seen at concentrations at or above 3 μM CuES while in cultures containing astrocytes alone, significant toxicity was observed at concentrations at or above 2 μM CuES (Fig. 3A). These data demonstrate that astrocytes are the primary cell type affected by CuES in our mixed cultures. To confirm the differential sensitivity of neurons and astrocytes to zinc and copper toxicity, we used the same experimental paradigm described above to assess the relative contributions of neurons and astrocytes to zinc-ionophore induced LDH release. These experiments confirmed that astrocytes are relatively resistant to zinc-induced toxicity while neurons remain highly sensitive (Fig. 3B). Indeed, while mixed cultures showed significant toxicity at or above concentrations of 20 μM Zn^{2+} in the presence of 250 nM pyrithione, no concentration of ZnPyr tested elicited significant LDH release in astrocyte cultures lacking neurons, underscoring the differential vulnerability of neurons and astrocytes to zinc ionophore and copper ionophore induced toxicity.

Intriguingly, our results suggested that not only are neurons more resistant to CuES toxicity but that they may protect astrocytes against CuES mediated injury. Elesclomol concentration response experiments demonstrated that in cultures containing both neurons and astrocytes in the presence of 2 μM Cu^{2+}, 200-300 nM ES was necessary to induce significant elevations in LDH signal (Fig. 3C.). However, as little as 50 nM elesclomol was sufficient to induce significant cytotoxicity in astrocyte cultures (Fig. 3D), demonstrating increased sensitivity in the absence of neurons. Moreover, in our initial experiment comparing the vulnerability of neurons and astrocytes to CuES toxicity, described above, (Fig. 3A), a two-way repeated measures ANOVA revealed an interaction between the kainate treatment and CuES ($F(4,16)$, $p=0.0086$), reflecting the possibility that astrocyte cultures may be more sensitive to CuES toxicity in the absence of neurons.

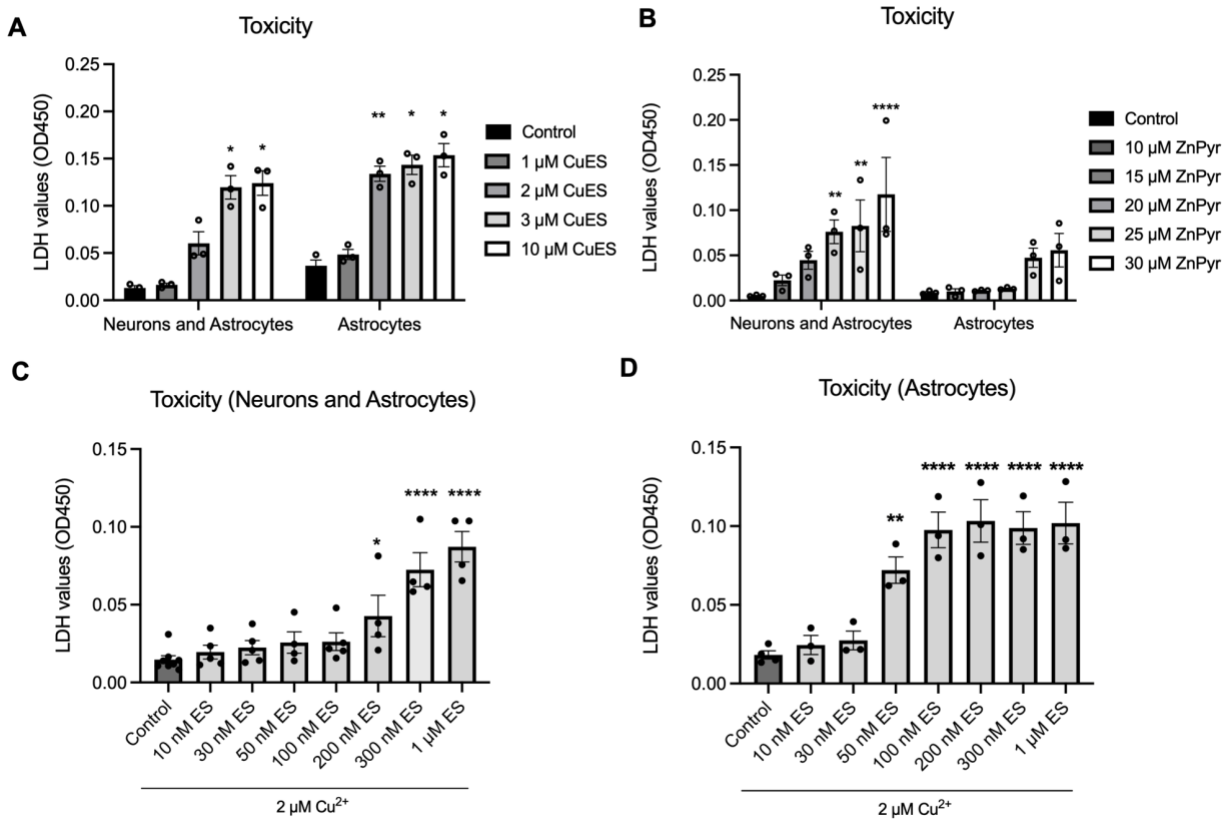

Figure 5. Astrocytes are selectively vulnerable to CuES toxicity and neurons in co-culture provide some protection against this insult. Mixed cortical cultures were first exposed to MHB in the absence or presence of 1 mM kainate (KA) overnight, with the treatment generating neuron-deprived, astrocyte cultures. Cultures-containing coverslips were then transferred to plates containing fresh MHB with various concentrations of CuES or ZnPyr. **(A)** LDH assay comparing toxicity of CuES (1 µM elesclomol) in the presence and absence of neurons. Note that in the presence of neurons, CuES does not induce a significant elevation in LDH until 3 µM copper while in the absence of neurons, CuES induces significant toxicity at 2 µM CuES (*p<0.05, **p<0.01, two-way ANOVA, Sidak post-hoc; Main effects: p<0.01 for KA; p<0.0001 for Cu^{2+} treatment; p<0.01 for interaction of KA and Cu^{2+}, n=3 biological replicates). **(B)** LDH assay comparing the toxicity of ZnPyr (250 nM elesclomol) in the presence and absence of neurons. When neurons are present, significant LDH is observed beginning at 20 µM ZnPyr while no concentration of ZnPyr tested was toxic in the absence

of neurons (*p<0.05, **p<0.01, ****p<0.0001, two-way ANOVA, Sidak post-hoc; Main effect: p=0.1047 for KA, p<0.0001 for Cu^{2+}, p=0.1158 for interaction, n=3 biological replicates). **(C)** LDH assay of elesclomol concentration response curve using mixed cerebral cultures demonstrates significant copper (2 µM) toxicity at concentrations at or above 200 nM elesclomol (*p<0.5, ****p<0.0001, one-way ANOVA, Dunnet post hoc, n=4-5 biological replicates). **(D)** LDH assay of the same elesclomol concentration curve in astrocyte cultures shows significant copper (2 µM) toxicity beginning at 50 nM elesclomol (**p<0.01, ****p<0.0001, one-way ANOVA, Dunnet post-hoc, n=3 biological replicates). Data are expressed as mean ± SEM

In a separate set of experiments, we found that astrocytic architecture was disrupted at concentrations as low as 2 μM Cu^{2+} in the presence of elesclomol concentrations as low as 30 nM, even when neurons were present in culture, as revealed by GFAP staining (Fig. 4A), further emphasizing the exquisite sensitivity of astrocytes to CuES. In contrast, most neurons had preserved architecture at 2 μM Cu^{2+}/200 nM ES, as revealed by microtubule-associated protein 2 (MAP2) staining (Fig. 4B). Very infrequently (<10% of fields viewed), a few neurons were observed to have dendritic beading following CuES treatment – a phenotype often observed with excitotoxicity (Olney et al., 1979; Hasbani et al., 1998; Weilinger et al., 2016). As dendritic beading was only observed at the highest concentrations of CuES utilized and appeared to correspond with areas of substantial astrocyte damage, we hypothesized that this phenotype was a result of a failure of damaged astrocytes to buffer excess glutamate in the cultures. Indeed, extracellular glutamate has been shown to accumulate in astrocyte-poor neuronal cultures resulting in neuronal injury (Rosenberg, 1991) and impairment of the glutamate transporter GLT-1, predominantly, but not exclusively, expressed in astrocytes (Petr et al., 2015) leads to neuronal death that can be rescued by NMDA receptor antagonism (Wang et al., 1998; Kawahara et al., 2002). To test whether the observed dendritic beading was caused by excitotoxicity or by CuES toxicity, cultures were treated with the NMDA receptor antagonist MK801. Co-treatment with 10 μM MK801 in the presence of CuES abrogated the observed beading suggesting that this form of neuronal injury was a result of excitotoxicity rather than a direct effect of CuES treatment.

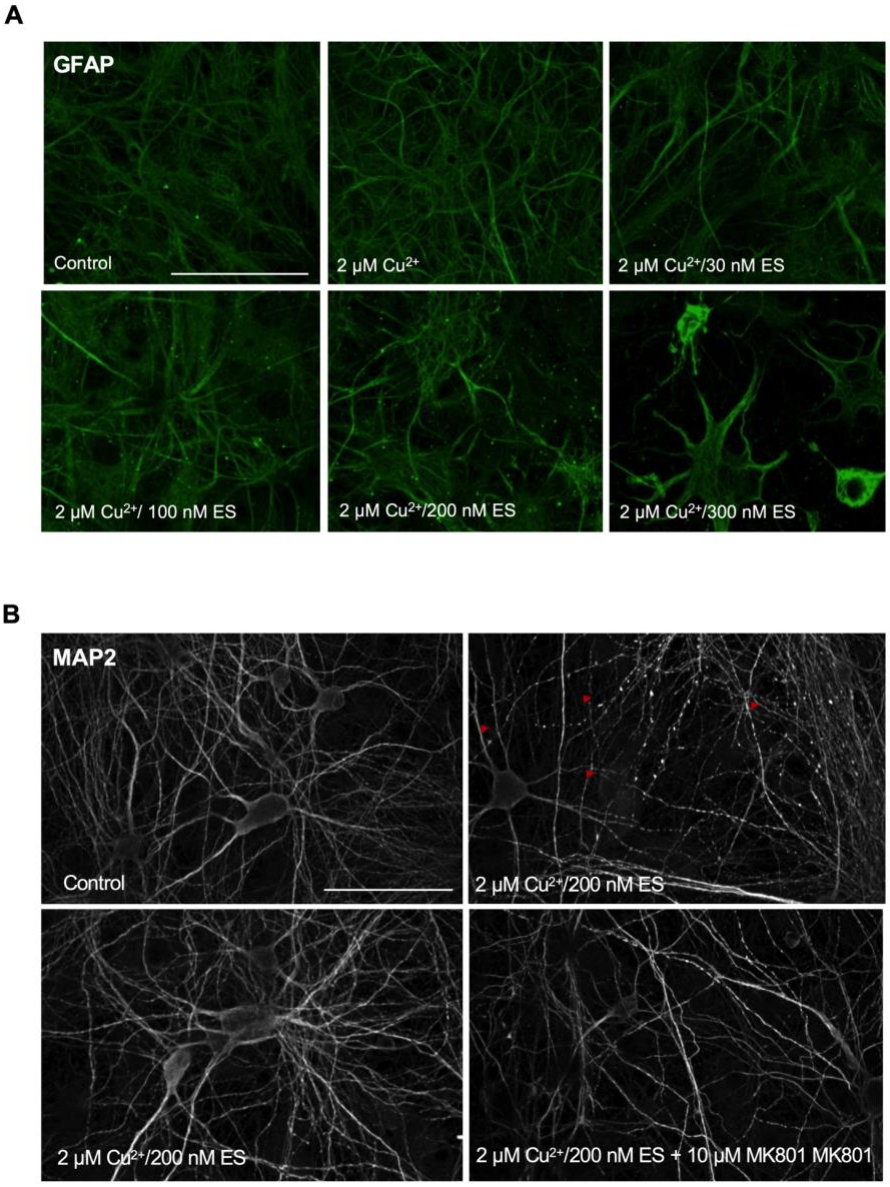

Figure 6. CuES treatment disrupts astrocyte morphology while leaving neuronal architecture intact. (A) GFAP staining of mixed cortical cultures treated with CuES shows changes in astrocyte morphology beginning at 30 nM ES and becoming more pronounced at higher concentrations of the ionophore while **(B)** neuronal architecture is preserved, even at much higher ES concentrations. Rarely, dendritic beading was observed (red arrow heads) however this phenotype could be rescued with co-treatment with the NMDA antagonist MK801 suggesting that this neuronal injury was due to a failure of astrocytes to buffer excess glutamate. Scale bars are 100 μM.

2.4.3 Immortalized human astrocytes are also sensitive to CuES treatment

In the next set of experiments, we utilized an immortalized human astrocyte cell line to confirm that human-derived astrocytes are also sensitive to copper ionophore toxicity. Indeed, a concentration response curve demonstrated that human astrocytes are also exquisitely sensitive to copper ionophore toxicity showing a significant increase in LDH signal at 2 µM Cu^{2+} in the presence of 100 nM elesclomol (Fig. 5A). Much like we observed in the C6 glioma cells, sublethal concentrations of CuES induced a dramatic change in morphology in our immortalized human astrocyte, with a similar loss of spindle-like appearance and the rounding of cells (Fig. 5B). Thus, human astrocytes are also sensitive to copper ionophore induced cell death.

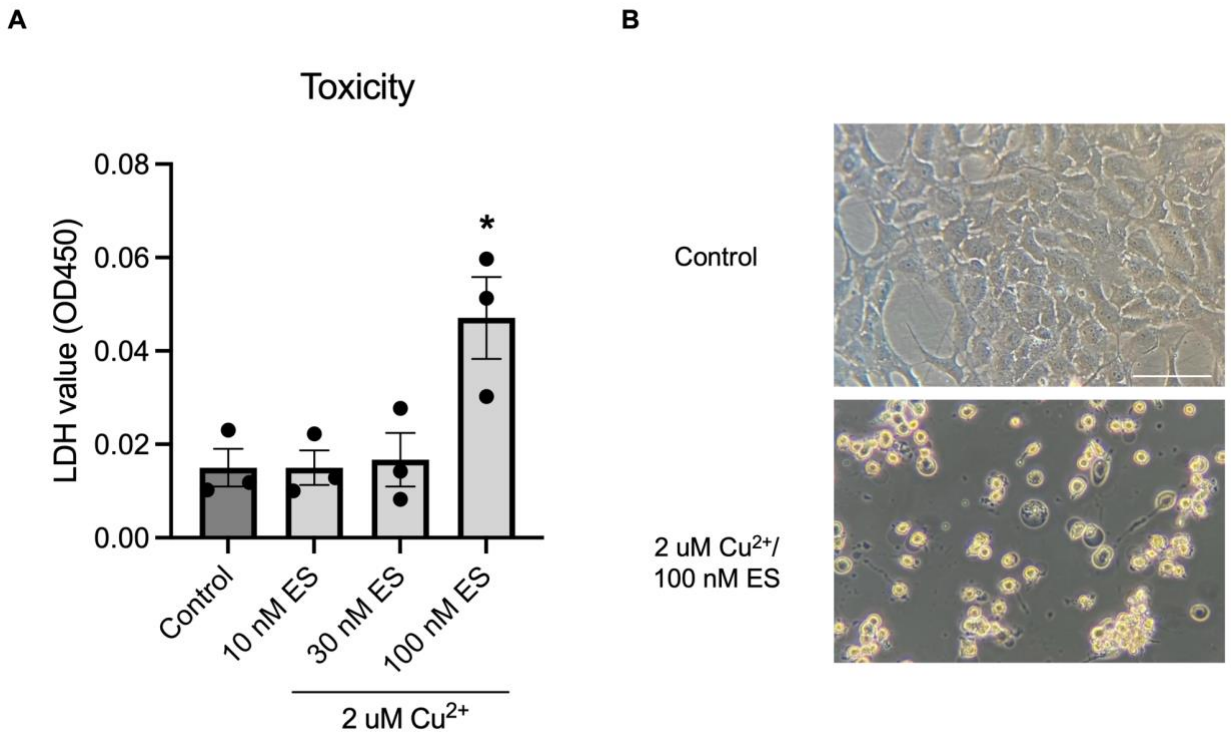

Figure 7. Human immortalized astrocytes are sensitive to copper ionophore induced cell death. **(A)** LDH assay showing concentration-dependent copper (2 µM) toxicity with increasing elesclomol

concentrations (*p<0.05, one-way ANOVA, Dunnet post-hoc, n=3 biological replicates). **(B)** Phase contrast images of immortalized astrocytes after overnight treatment with CuES showing substantially altered morphology. Data are expressed as mean ± SEM. Scale bar is 100 µM.

2.4.4 CuES-induced astrocyte death is not mediated via cuproptosis

We next aimed to determine whether the selective astrocyte cell death induced by CuES was mediated by the recently described cuproptosis pathway, which involves FDX1-mediated lipoylation and subsequent dysfunction of TCA cycle proteins, (Tsvetkov et al., 2022). As a previous study has shown that shifting metabolism away from mitochondrial oxidative respiration and towards glycolysis by inhibiting oxidative phosphorylation is protective against cuproptosis (Tsvetkov et al., 2022), we first treated astrocyte cultures with the complex III inhibitor, antimycin A. Astrocytes were pre-treated for one hour with 300 nM antimycin A before the addition of CuES and then incubated overnight with both drugs. LDH assays revealed that inhibition of complex III was not protective against CuES induced cell death (Fig. 6A). As sodium azide, a complex IV inhibitor, has been shown to be a potent inhibitor of mitochondrial respiration, we also tested whether this drug could attenuate CuES-induced cell death. Using the same treatment paradigm as described for antimycin A, we found that 300 µM sodium azide was also ineffective at rescuing astrocytes from CuES toxicity (Fig. 6B). Thus, inhibition of mitochondrial respiration, an important target of the cuproptosis pathway, is ineffective at attenuating CuES-induced death in astrocytes.

Having determined that inhibiting oxidative phosphorylation was insufficient to rescue astrocytes from CuES, we next turned our attention to FDX1, an iron sulfur protein that is a key modulator of cuproptosis (Tsvetkov et al., 2022) and asked whether knockdown of this protein would be protective against CuES toxicity. Given that our knockdown protocol required us to use cultures containing both neurons and astrocytes to avoid an extended incubation period in serum-free media, we first determined via qPCR whether *FDX1* was abundant in both cell types. To do so, we compared *FDX1* expression in cultures containing neurons and astrocytes to those containing astrocytes alone. We found no significant difference between *FDX1* expression between these cultures (Fig. 6C), consistent with the fact that neurons make up a small percentage of cells in our mixed cerebrocortical cultures. These data suggested that if knockdown (KD) were successful in mixed cultures, we would have achieved KD largely in the astrocytic component. We next tested whether we could KD *FDX1* in our cultures using siRNA. Indeed, 48 hours following siRNA treatment, we observed a significant reduction in *FDX1* expression compared to the non-targeting control (NTC) siRNA (Fig. 6D). Having demonstrated that *FDX1* was expressed in our primary cultures and could be knocked down, we treated cultures with siRNA for 48 hours after which time cells were treated overnight with CuES. LDH results demonstrated that *FDX1* KD did not attenuate CuES-induced toxicity (Fig. 6E). Of note, qPCR was also performed on sister cultures which demonstrated that this lack of rescue could not be attributed to a lack of KD as cultures treated with *FDX1* siRNA showed greater than a 90% reduction in *FDX1* expression compared to the NTC (Fig. 6F). These experiments demonstrate that *FDX1* is not a modulator of copper ionophore-induced toxicity in our cultures and that, importantly, the cuproptosis does not mediate the observed astrocyte cell death.

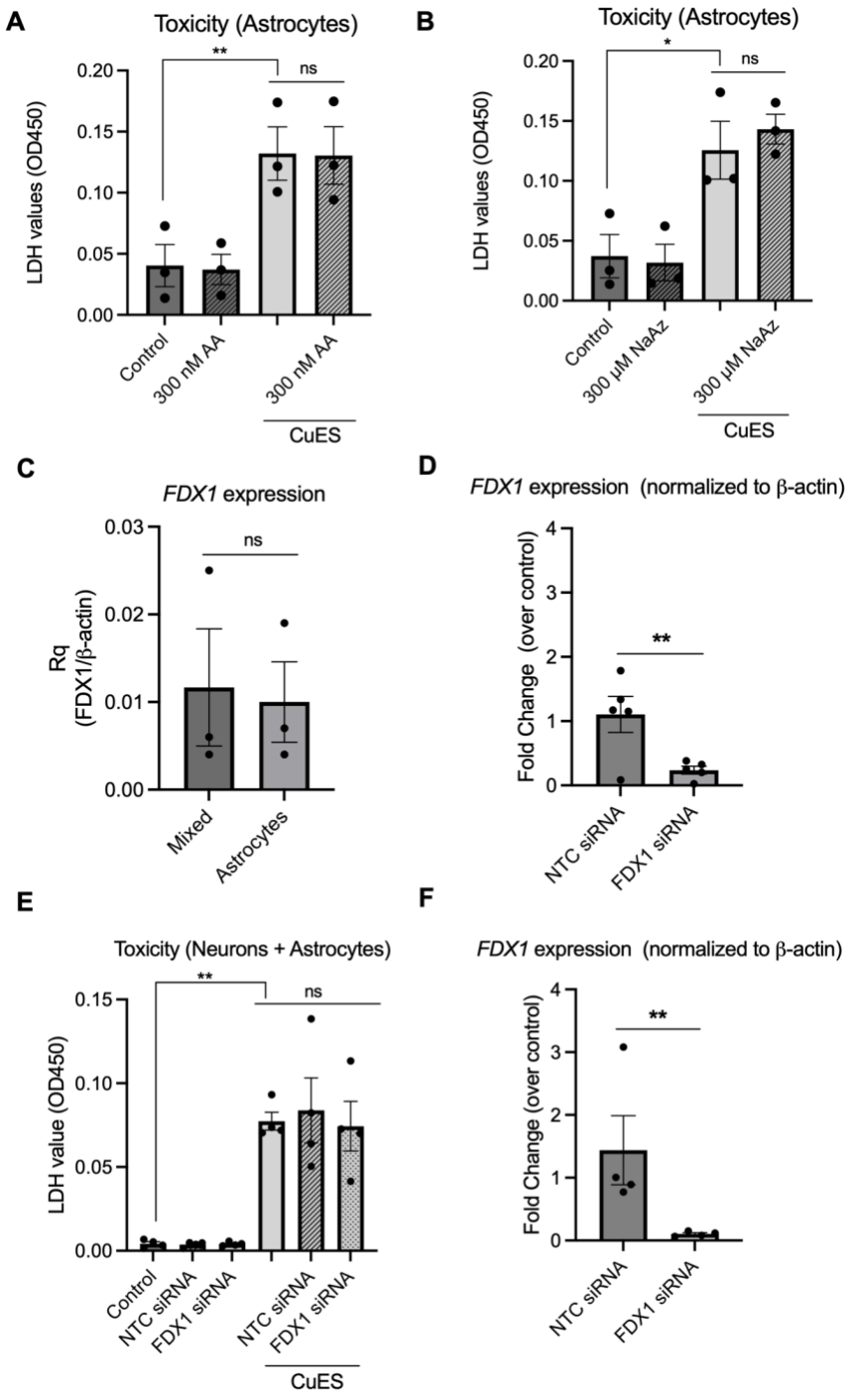

Figure 8. CuES induced astrocyte toxicity is not mediated by cuproptosis. All experiments utilized 2 µM copper. Experiments conducted in astrocyte cultures utilized 200 nM ES while those conducted in cultures containing neurons and astrocytes utilized 300 nM ES. Inhibition of mitochondrial respiration with **(A)** 300 nM antimycin A (AA), a complex III inhibitor, is insufficient to rescue CuES

toxicity in primary astrocytes (**p<0.01, repeated measures one-way ANOVA, Sidak's post hoc, n=3 biological replicates) as is **(B)** inhibition with 300 µM of the complex IV inhibitor sodium azide (NaAz) (*p<0.0.5, repeated measures one-way ANOVA, Sidak post-hoc, n=3 biological replicates). **(C)** *FDX1* is expressed at similar levels in astrocytes cultures and cultures containing both neurons and astrocytes (mixed) (paired two tailed t-test, n=3 biological replicates) and **(D)** can be knocked down in our cultures 48 hours after siRNA treatment (**p<-0.01, ratio paired two-tailed t-test, n=5 biological replicates, NTC=non-targeting control). **(E)** treatment with *FDX1* siRNA does not rescue cultures from CuES toxicity as evidenced by significant LDH release (**p<0.01, repeated measures one-way ANOVA, Sidak post-hoc, n=4 biological replicates), which **(F)** cannot be explained by a lack of knockdown in siRNA treated cultures (**p<0.01, ratio paired two-tailed t-test, n=4 biological replicates). Data are expressed as mean ± SEM.

It is important to note that our treatment paradigm differed from the paradigm used in the characterization of cuproptosis by Tsvetkov et al. (2022) as we exposed cells to CuES overnight rather than a 2-hour pulse. As such, we next treated astrocytes for 2 hours with CuES and then incubated them in fresh media overnight. Similar to what we observed with the overnight exposure, pulsed CuES treatment led to cell death as evidenced by increased LDH release (Fig. 7A). Phase contrast microscopy revealed once again the presence of healthy appearing neurons surrounded by swollen astrocytes (Fig. 7B). To determine whether cell death induced by this shorter CuES exposure was mediated by cuproptosis, we treated astrocytes with the complex III inhibitor antimycin A and the complex IV inhibitor sodium azide, as described above. Once again, we found no difference in toxicity between groups exposed to CuES in the presence or absence of inhibitors of oxidative phosphorylation (Fig. 7C, 7D). These results suggest that both short and long exposure to CuES triggers a cell death pathway that astrocytes are selectively vulnerable to and is distinct from cuproptosis.

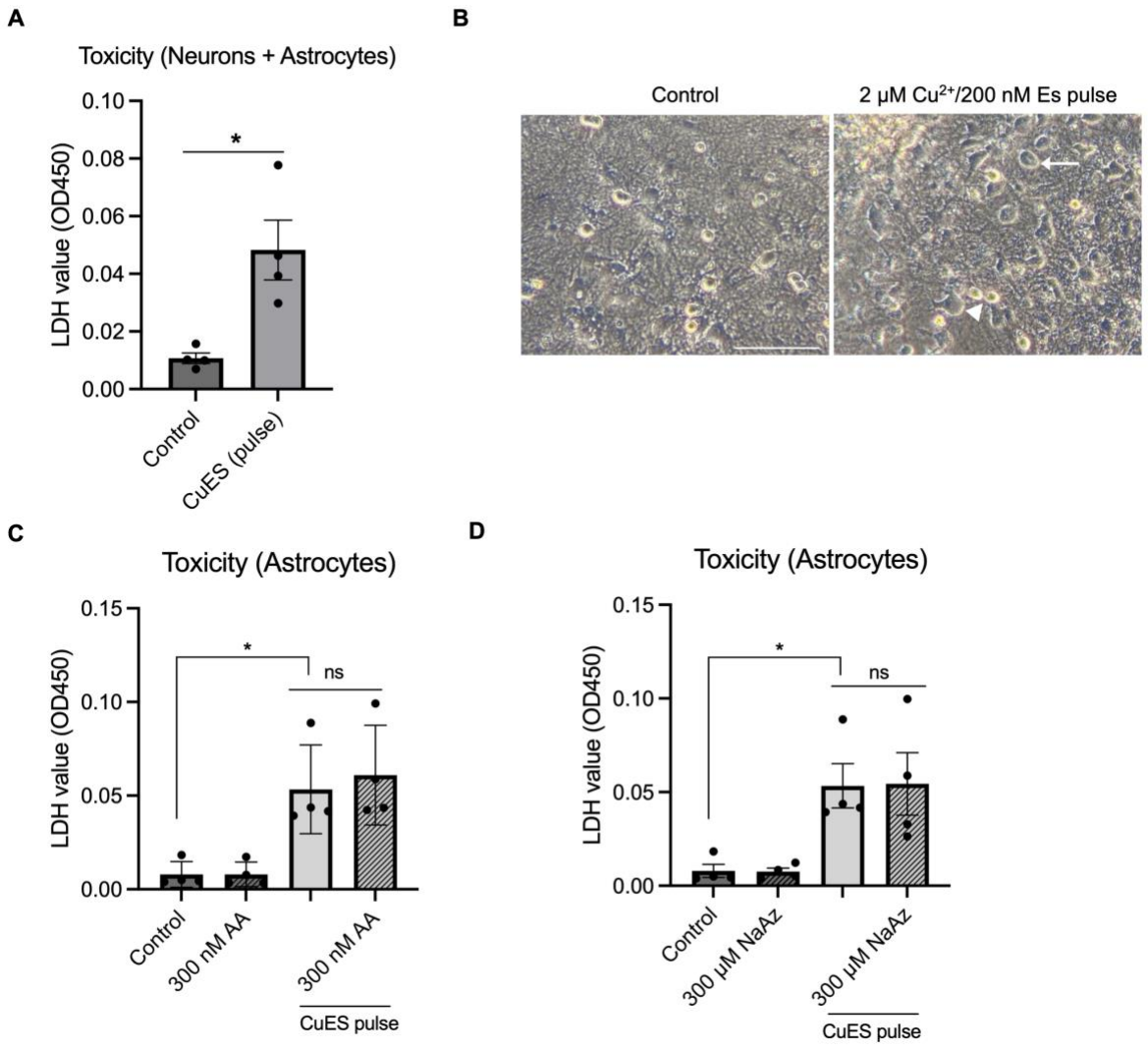

Figure 9. A CuES pulse is sufficient to induce selective astrocyte damage, which cannot be rescued by inhibition of mitochondrial respiration. A two-hour exposure to 2 μM Cu^{2+} in the presence of 300 nM elesclomol is sufficient to induce significant LDH release 18-24 hours later in mixed cortical cultures (*$p<0.05$, paired t-test, n=4 biological replicates). **(B)** This treatment causes astrocytic swelling (arrow) while healthy phase bright neurons (arrowhead) are readily apparent by phase contrast microscopy. CuES toxicity cannot be rescued by inhibition of oxidative phosphorylation with **(C)** 300 nM antimycin A (*$p<0.05$, repeated-measures one-way ANOVA, Sidak post-hoc, n=4 biological replicates) nor **(D)** with 300 μM sodium azide (*$p<0.05$, repeated measures one-way

ANOVA, Sidak post-hoc, n=4 biological replicates). Data are expressed as mean ± SEM. Scale bar is 100 μM.

Having established that astrocytes undergo copper-ionophore triggered cell death in a manner distinct from cuproptosis, we next turned our attention to other known, regulated cell death pathways such as apoptosis, necroptosis, and ferroptosis. Although there are some reports that elesclomol induces apoptosis in cancer cells and that copper and/or elesclomol can trigger ferroptosis (Hasinoff et al., 2015; Gao et al., 2021; Xue et al., 2023), we found that the pan-caspase inhibitor Z-VAD-FMK (100 μM), the necroptosis inhibitor necrostatin-1s (Nec-1s) (20 μM), and the iron chelator deferoxamine (DFO) (50 μM) all failed to abrogate cell death in astrocytes exposed to CuES both overnight ($F(1.316, 3.949)=17.26$, repeated measures one-way ANOVA, $p=0.0131$, Šídák post-hoc, CuES vs Z-VAD-FMK, $p=0.5793$; CuES vs Nec-1s, $p=0.5840$; CuES vs DFO, $p=0.1559$, n=4 biological replicates) and for a 2-hour pulse ($F(1.125, 3.374)=9.081$, repeated measures one-way ANOVA, $p=0.0482$, Šídák post-hoc, CuES vs Z-VAD-FMK, $p=0.9686$; CuES vs Nec-1s, $p=0.7346$; CuES vs DFO, $p>0.9999$, n=4 biologial replicates). Given recent evidence that copper can contribute to ferroptosis independent of iron accumulation (Xue et al., 2023), we also tested the ferroptosis inhibitor ferrostatin-1 (Fer-1) (1 μM) on astrocytes exposed to CuES overnight. As we observed with DFO treatment, 1 μM Fer-1 failed to rescue abrogate toxicity ($F(3,9)=4.547$, repeated-measures one-way ANOVA, $p=0.0013$, Šídák post-hoc, control vs CuES, $p=0.0170$; CuES vs CuES + Fer-1, $p=0.8260$, n=4 biological replicates). It must be noted that concentrations as high as 30 μM Fer-1 also did not rescue astrocytes from CuES toxicity, strongly suggesting that ferroptosis is not the cell death pathway mediating CuES induced death in astrocytes.

2.4.5 Copper-ionophore treatment induces oxidative stress in astrocytes

Given that neither cuproptosis nor other canonical regulated cell death pathways appear to mediate CuES-induced death in astrocytes, we evaluated whether oxidative stress may be involved in CuES toxicity. In cancer cells, elesclomol has been shown to transport copper to the mitochondria where redox cycling of copper results in the production of mitochondrial reactive oxygen species (ROS) (Nagai et al., 2012). Additionally, there is evidence that copper is able to bind to and form a complex with glutathione, and that this complex is capable of generating superoxide radicals (Speisky et al., 2009). Therefore, we assessed whether CuES treatment resulted in lipid peroxidation, an oxidative protein modification that can be triggered by reactive oxygen and nitrogen species (Li et al., 2022; Murphy et al., 2022). We chose to look at 4-hydoxy-2-nonenal (4-HNE) as it is both an indicator of oxidative damage as well as a mediator of oxidative stress in cells due to its reactive nature (Li et al., 2022). An increase in 4-HNE staining was observed in glial fibrillary acid protein (GFAP)-stained astrocytes was observed when cultures containing neurons and astrocytes were exposed to 2 µM Cu in the presence of 200 nM elsesclomol, consistent with an increase in oxidative stress. (Fig. 8A).

While these findings strongly suggest that CuES treatment is sufficient to induce oxidative stress in astrocytes, whether this stress mediates CuES-induced cell death was still unknown. Therefore, to determine whether attenuation of oxidative stress would also attenuate CuES-induced cell death, we treated astrocytes with the superoxide dismutase (SOD)-catalase mimic, EUK-134, which has been shown to catalytically eliminate both superoxide and hydrogen peroxide (Doctrow et al., 2002; Sharpe et al., 2002), before and during exposure to CuES. Remarkably, 30 µM EUK-134 was able to restore viability by over 40% in astrocytes treated overnight with CuES and by approximately 70% in astrocytes exposed to a CuES pulse (Fig. 8B). Thus, reactive species

are a significant mediator of CuES-induced astrocyte cell death. Given evidence that redox cycling of copper may contribute to CuES-induced ROS generation (Nagai et al., 2012), we next investigated whether pyrroloquinoline quinone (PQQ), a naturally occurring redox cofactor that has been shown to act as an antioxidant through suppression of reactive oxygen species (Zhang and Rosenberg, 2002), could also rescue astrocytes from CuES-induced death. In contrast to our findings with EUK-134, we found that 50 μM PQQ failed to protect astrocytes from CuES treatment (Fig. 8C). Given evidence that manganese-salen compounds, such as EUK-134, protect against mitochondrial oxidative stress (Melov et al., 2001; Hinerfeld et al., 2004), these results suggest that CuES induced oxidative stress may be mitochondrially generated.

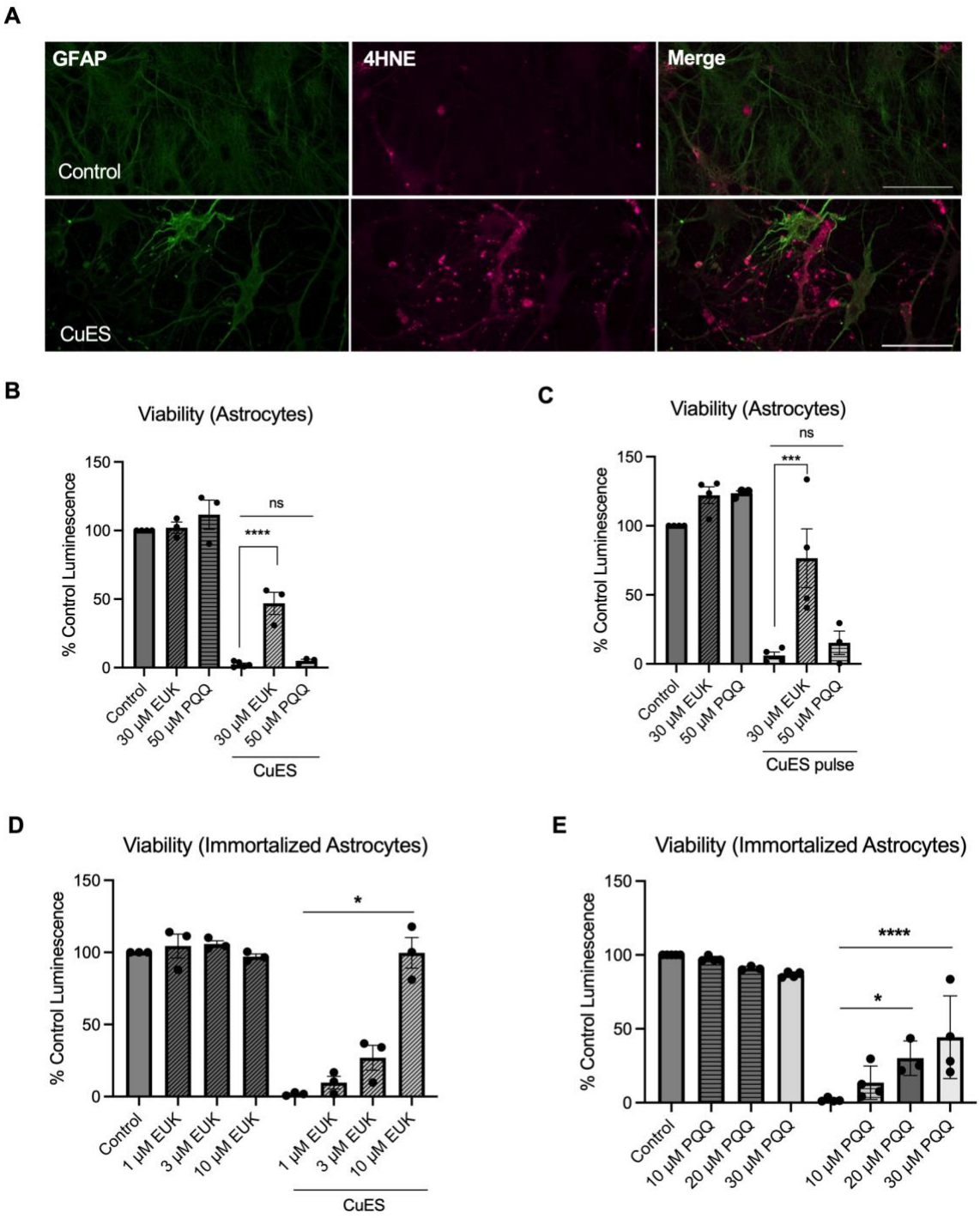

Figure 10. CuES mediates toxicity, in part, through induction of oxidative stress in astrocytes and antioxidant treatment improves viability. Cultures containing neurons and astrocytes were treated with 2 μM Cu/200 nM ES overnight and were subsequently stained with 4-HNE, a marker of lipid peroxidation. **(A)** Confocal imaging revealed an increase in 4-HNE signal in CuES treated

astrocytes as compared to controls strongly indicating increased oxidative stress following copper ionophore treatment. Treatment with the antioxidant EUK134 but not PQQ significantly increased viability of astrocyte cultures exposed to 2 µM Cu/200 nM ES both **(B)** overnight (****p<0.0001, one-way ANOVA, Sidak post hoc, n=3 biological replicates) and **(C)** for a 2-hour pulse (***p<0.001, one-way ANOVA, Sidak post-hoc, n=4 biological replicates). In contrast, immortalized human astrocytes exposed to 2 µM Cu/100 nM ES overnight were rescued by treatment with both **(D)** EUK134 (**p<0.01, repeated measures one-way ANOVA, Sidak post-hoc, n=3 biological replicates) and **(E)** PQQ (*p<0.05, ****p<0.0001, one-way ANOVA, Sidak post-hoc, n=4 biological replicates). Data are expressed as mean ± SEM.Scale bar is 50 µm.

As our prior experiments confirmed that immortalized human astrocytes are also sensitive to CuES toxicity, we next assessed whether oxidative stress mediated their susceptibility to copper ionophore treatment, as it did in primary rodent astrocytes. Indeed, treatment with EUK-134 rescued viability of immortalized human astrocytes in a concentration dependent manner with 10 µM EUK-134 restoring viability to control levels (Figure 8D). Unlike what we observed in primary cultures, however, treatment with PQQ also restored viability of CuES treated immortalized astrocytes (8E). Together, these results suggest that oxidative stress is a common mechanism underlying astrocyte cell death in response to CuES treatment.

2.4.6 Copper toxicity in C6 glioma cells is also rescued by antioxidants

Previous research has demonstrated that elesclomol induces ROS in cancer cells (Kirshner et al., 2008; Nagai et al., 2012). Moreover, there is evidence to suggest that elesclomol induces ROS in glioblastoma stem cells, cells that are crucial drivers of drug resistance and recurrence in glioblastoma (Prager et al., 2020), and that this ROS restrains their survival and growth (Buccarelli et al., 2021). Therefore, we tested whether antioxidants would rescue C6 glioma cells from CuES-induced death, as they did in our rodent astrocyte cultures. Indeed, concentration response experiments demonstrated that EUK-134 rescued C6 glioma cells from CuES toxicity in a concentration-dependent manner with 10 µM EUK-134 restoring viability of CuES exposed cells to close to 100% of control cells (Fig. 9A). Similar to what we observed in immortalized astrocytes, PQQ also rescued CuES treated C6 glioma cells in a concentration dependent manner, with 50 µM PQQ restoring viability to approximately 70% of control cells (9B). This divergence in the response of primary cultures and cell lines to rescue by PQQ may indicate a difference in mechanisms of CuES toxicity which in turn could represent an opportunity for targeted treatment.

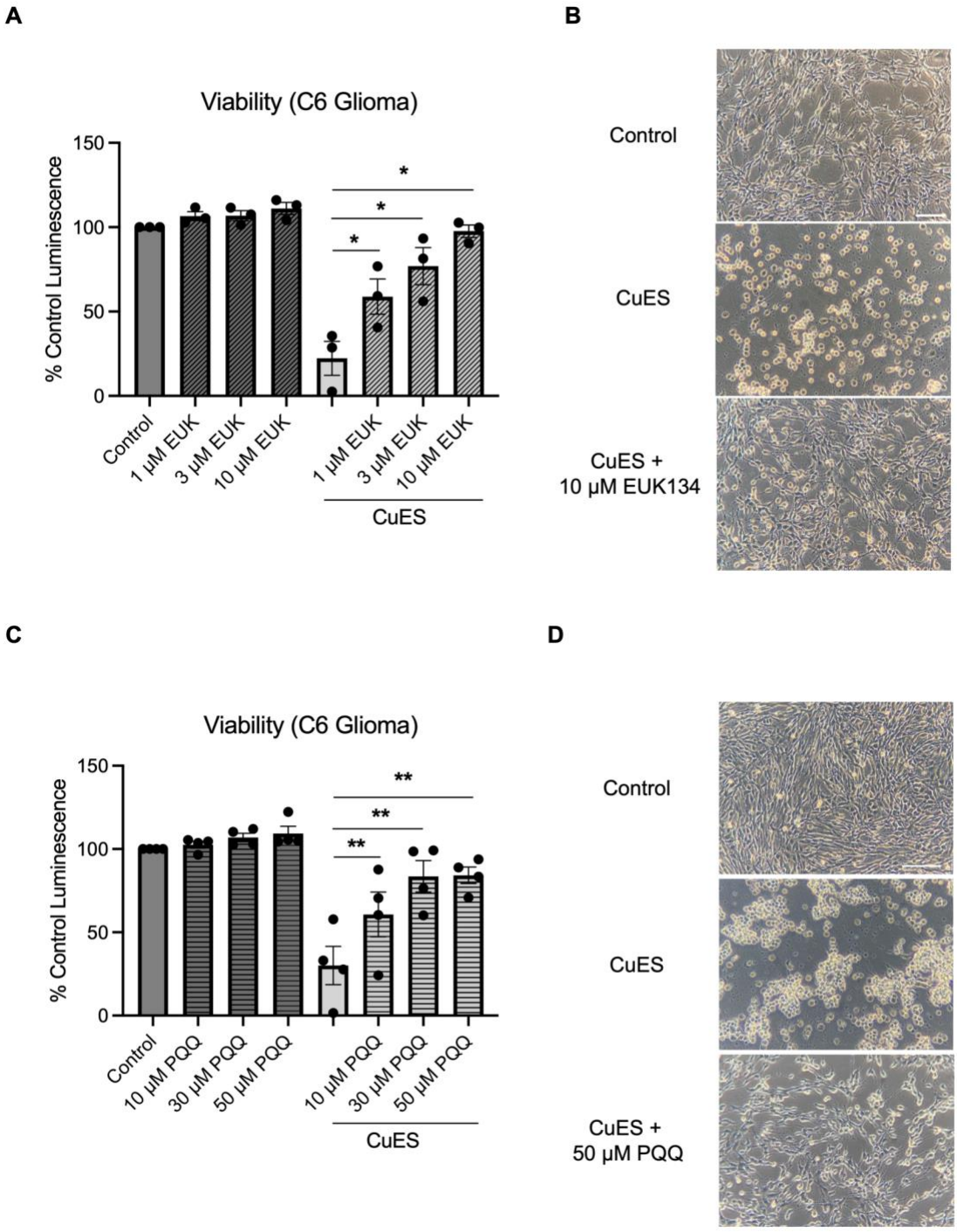

Figure 11. Antioxidants rescue C6 glioma cells from CuES induced cell death. Treatment with increasing concentrations of the antioxidant EUK-134 **(A)** rescued viability to C6 glioma cells following treatment with 2 μM Cu^{2+}/100 nM ES (*p<0.05, repeated measures one-way ANOVA, Sidak post hoc, , n=3 biological replicates). **(**B) EUK134 (10 μM) improved the morphology of C6 glioma

cells, as evidenced by phase contrast microscopy. Treatment with PQQ, another antioxidant and free radical scavenger also rescued C6 glioma cells from CuES induced toxicity in a concentration dependent manner as evidenced by **(C)** increased viability (**p<0.01, repeated measures one way ANOVA, Sidak post hoc, n=4 biological replicates) and **(D)** improved cell morphology. Data are expressed as mean ± SEM. Scale bar is 50 µm Scale bar is 50 µm.

2.4.7 Zinc preconditioning is protective against CuES toxicity

Although our experiments established that CuES-induced oxidative stress mediates cell death in astrocytes, they did not reveal why neurons are able to survive this insult – especially since astrocytes are thought to have more robust systems for buffering oxidative stress as compared to neurons (Muyderman et al., 2007; Bell et al., 2015; Dringen et al., 2015). However, neurons are highly enriched in metallothionein 3 (MT3) (Aschner et al., 1997), which can bind copper with high affinity (Calvo et al., 2018; Mehlenbacher et al., 2022). Moreover, previous research has shown most of MT3 in our neuronal preparation exists in its apo-form (Aras et al., 2009; Aras and Aizenman, 2011) allowing it to bind excess metal. Although there is some data to support low levels of MT3 expression in astrocytes (Hidalgo et al., 2001), these cells primarily express metallothionein 1 (MT1) and metallothionein 2 (MT2) (Aschner et al., 1997). Importantly, MT1 and MT2 are expressed at low levels basally but their expression can be substantially induced by zinc (Silva et al., 2023). Therefore, we hypothesized that MT expression may buffer excess cytosolic copper and partially account for the differential sensitivity of neurons and astrocytes to CuES mediated injury.

To test whether the induction of MTs could protect astrocytes from CuES toxicity we preconditioned astrocyte cultures with 20 µM Zn^{2+} in the presence of 250 nM Pyr, conditions that

are not injurious to our astrocyte cultures (Fig. 3B). We first confirmed that overnight treatment with 20 µM ZnPyr was sufficient to increase expression of MT1 and MT2 in astrocyte cultures via qPCR, which was indeed the case (Fig. 10A). Having established that this concentration of ZnPyr induced a significant increase in MT1 and MT2 gene expression, we next treated astrocytes with CuES following an overnight exposure to ZnPyr. Remarkably, ZnPyr preconditioning completely abrogated CuES-induced toxicity and restored the LDH signal to that of the control group (Fig. 10B). Together these data suggest that upregulation of MTs are protective against CuES induced cell death and injury and that the differing basal levels of MTs in neurons and astrocytes may explain their differential sensitivity to CuES toxicity.

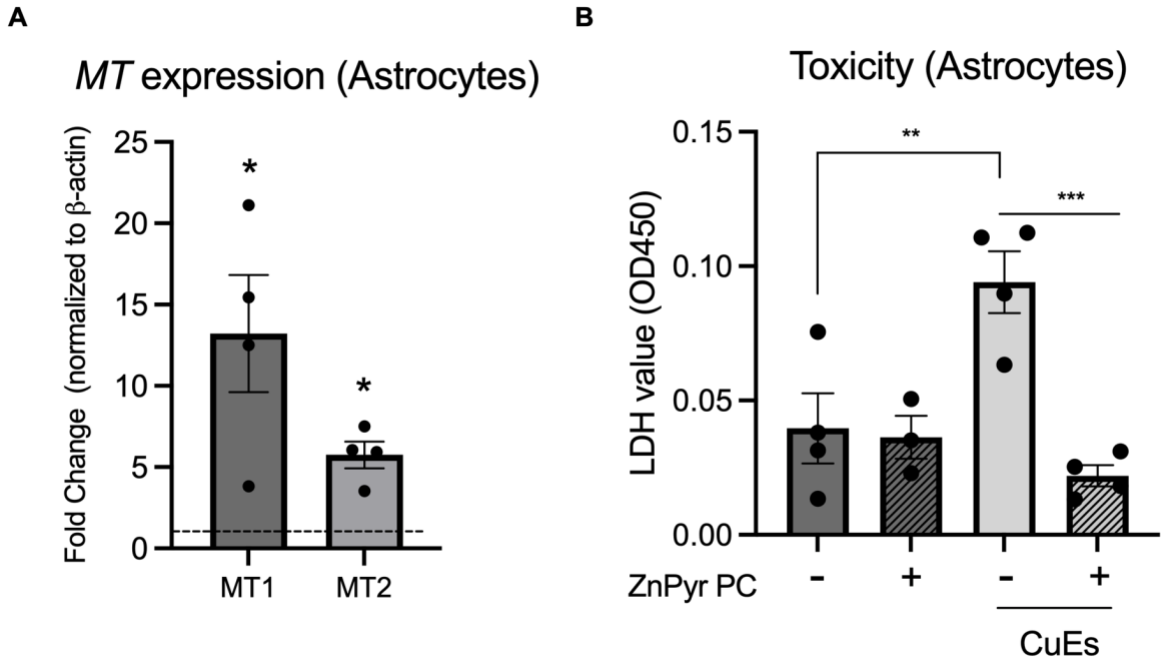

Figure 12. Preconditioning of astrocytes with ZnPyr is protective against CuES induced toxicity. Prior to exposure to 2 µM Cu^{2+}/200 nM ES, astrocytes were treated overnight with 20 µM Zn^{2+}/250 nM Pyr (ZnPyr PC= ZnPyr preconditioning), **(A)** which significantly increased expression of *MT1* and *MT2* (*<p.05, one sample t-test, *MT1* fold change vs 1, *MT2* fold change, n=4 biological replicates)

and **(B)** completely abrogated CuES induced LDH release (**p<0.01, ***p<0.001, one-way ANOVA, Sidak post hoc, n=3-4 biological replicates). Data are expressed as mean ± SEM.

2.5 Discussion

Here we demonstrate that in addition to C6 glioma, primary rodent and immortalized human astrocytes are sensitive to cell death induced by CuES treatment. Surprisingly, neurons in primary culture were highly resistant against this insult and seemingly exerted a protective effect on surrounding astrocytes. This finding is in striking contrast to the selective vulnerability of neurons to the redox-inert metal zinc, when compared to astrocytes (Dineley et al., 2000). An extensive investigation into pathways regulating copper ionophore induced toxicity in astrocytes revealed that, in contrast to the recently described cuproptosis pathway (Tsvetkov et al., 2022), CuES mediated toxicity to astrocytes in our mixed cultures is FDX1-independent and is not attenuated by treatment with inhibitors of oxidative phosphorylation. Furthermore, astrocytes cannot be rescued by inhibitors of apoptosis, ferroptosis, or necroptosis. However, consistent with previous research that has found that treatment with elesclomol, likely complexed with endogenous copper, can result in the generation of ROS (Kirshner et al., 2008; Blackman et al., 2012; Nagai et al., 2012), we have demonstrated that CuES treatment results in oxidative stress in primary rodent astrocytes and that antioxidant treatment rescues primary astrocytes, immortalized human astrocytes, and C6 glioma cells from CuES induced cell death. Importantly, we discovered that primary astrocytes can also be rescued following zinc pyrithione preconditioning, likely via the induction of metallothioneins 1 and 2, which could buffer excess intracellular copper.

One possible explanation for the divergence between Tsvetkov et al.'s (2022) findings and the results presented here is that cells that are heavily reliant on mitochondrial respiration for energy production are most vulnerable to cuproptosis (Tsvetkov et al., 2022). Astrocytes, however, are more metabolically flexible, utilizing oxidative phosphorylation, glycolysis, and β-oxidation of fatty acids, amongst other pathways, for energy production (Hertz et al., 2007; Arend et al., 2019; Juaristi et al., 2019; Harders et al., 2023). Of note, a recent study found proliferating astrocytes in culture are not dependent on oxidative phosphorylation for growth or survival (Silva et al., 2023). Moreover, respiration deficient astrocytes can survive by glycolysis *in vivo* (Supplie et al. 2017). Therefore, astrocytes may naturally shift to glycolysis in the presence of stimuli that compromise mitochondrial respiration. Furthermore, copper itself has been shown to stimulate glycolytic flux in astrocytes (Scheiber and Dringen, 2011; Bulcke and Dringen, 2015). Thus, it is possible that CuES treatment drives astrocytes further towards glycolysis rendering them less reliant on mitochondrial energy production and therefore less likely to be susceptible to cuproptotic cell death mediated by FDX1-dependent TCA cycle dysfunction, which would account for the failure of complex III and IV inhibitors to ameliorate astrocyte death in our cultures. Additionally, this lack of reliance on TCA cycle function would partially account for the lack of attenuation of CuES toxicity we observed with *FDX1* knockdown. It is important to note that copper was also found to bind to lipoylated TCA cycle proteins resulting in their oligomerization and proteotoxic stress, which was reversed by *FDX1* knockout (Tsvetkov et al., 2019, 2022). The lack of rescue by *FDX1* knockdown in our cultures thus indicates that proteotoxic stress may not be a major contributor to CuES induced cell death in astrocytes. Finally, it is important to consider that the concentration of elesclomol found to elicit cuproptosis in various cell lines was 40 nM (Tsvetkov et al., 2019, 2022) whereas this concentration of the copper ionophore was insufficient to induce

cell death in our cell cultures. Therefore, the higher concentrations of elesclomol needed in our investigation may trigger the alternative cell death-inducing pathway described here.

We found that oxidative stress, rather than perturbed mitochondrial metabolism, is a primary mediator of copper ionophore induced cell death in astrocytes. Not only did astrocytes in primary culture show increased levels of 4-HNE, an end product of lipid peroxidation (Murphy et al., 2022), but antioxidant treatment attenuated CuES-induced cell death in primary astrocytes, human immortalized astrocytes, and C6 glioma cells. The antioxidant EUK-134, a SOD-catalase mimic, rescued all three cell types from CuES mediated cell death, demonstrating that oxidative stress is a common mechanism underlying CuES-induced cytoxicity in astrocyte-like cells in our studies. To our surprise, PQQ, an essential nutrient and free radical scavenger due its ability to redox cycle (Stites et al., 2000), rescued human immortalized astrocytes and C6 glioma cells from CuES mediated cell death, but had no effect on the viability of primary astrocyte cultures. It is worth noting that while EUK-134 restored viability to nearly control levels in CuES treated cell lines, it had a less dramatic effect in primary cultured astrocytes. Thus, there may be an additional mechanism mediating CuES induced death in primary cultures that EUK-134 but not PQQ was able to partially overcome. Although the mechanism underlying the differential rescue of PQQ in these cell types is unclear, it is worth further investigation as potential differences in the type or location of reactive species production could be exploited for targeted treatment of gliomas while leaving healthy tissue unperturbed. For example, catalase has been shown to have the ability to scavenge peroxynitrite (Gebicka and Didik, 2009), a reactive nitrogen species, while PQQ can prevent the formation of peroxynitrite but cannot protect against its toxicity once formed (Zhang and Rosenberg, 2002). Despite the evidence presented here that oxidative stress mediates CuES induced cell death in astrocytes, an important limitation of this study is that the types of reactive

species induced by CuES treatment and where within the cell the formation of these species occurs are still open questions.

Given neurons' reliance on mitochondrial metabolism (Wong-Riley, 1989; Halim et al., 2010; Zheng et al., 2016), it is all the more surprising that astrocytes rather than neurons are selectively vulnerable to injury induced by this divalent metal. One clue to a potential mechanism underlying the resistance of neurons to CuES toxicity comes from our ZnPyr preconditioning experiments, which showed that ZnPyr treatment before CuES exposure completely protected astrocytes from copper ionophore induced cell death. Importantly, overnight exposure significantly upregulated *MT1* and *MT2* expression, consistent with a large body of data demonstrating that increases in intracellular free zinc drive metallothionein upregulation (Radtke et al., 1993; Heuchel et al., 1994; Andrews, 2000). As noted previously, astrocytes primarily express low levels of the MT1 and MT2 while neurons are highly enriched in MT3. Importantly, studies have demonstrated that MT3 may exist predominantly in its apo – or non-metal bound – form (Yang et al., 2001; Aras et al., 2009) and is able to bind redox active free Cu^{2+} (Meloni et al., 2007). Thus, high levels of non-metal bound MT3 in neurons may buffer the increase in intracellular copper facilitated by elesclomol and prevent the metal from redox cycling, protecting neurons from copper-induced increases in oxidative species. Although MT3 has been shown to have a high affinity for copper (Artells et al., 2014; Calvo et al., 2018; Mehlenbacher et al., 2022), it is worth noting that in *in vitro* preparations, when apo-MT2 is added to solutions containing copper-bound proteins, it is able to extract Cu^{+} from them, indicating a high affinity for the metal (Banci et al., 2010). Additionally, previous research has shown that induction of MT1 and MT2 via zinc treatment protects astrocytes from methylmercury-induced cytotoxicity (Rising et al., 1995; Aschner et al., 1998), indicating that increased MT expression is protective against a variety

of metals. Although we posit that the protective effect of MTs is mediated through its metal binding functions, research also suggests that these proteins are protective against, and can even scavenge, free radicals such as superoxide and hydroxyl radicals (Thornalley and Vašák, 1985; Chubatsu and Meneghini, 1993; Uchida et al., 2002; Ruttkay-Nedecky et al., 2013). Therefore, it is also possible that MT-facilitated rescue of astrocytes is mediated through their ability to mitigate oxidative stress, which we found to drive CuES induced cell death.

The findings described here could have significant implications for the potential treatment of gliomas with adjunct copper elesclomol therapy. Firstly, we have demonstrated that glioma cells are sensitive to CuES induced cell death although this treatment results in oxidative stress induced cell death in healthy astrocytes as well. Although proliferating glioma cells are often highly glycolytic, a number of studies have found that the TCA cycle is intact in some tumor cells and cancer stem cells (DeBerardinis et al., 2007; Dekker et al., 2020; Datta et al., 2021). Therefore, metabolic profiling of gliomas may allow for targeted therapy of tumors most susceptible to this treatment modality. The finding that metallothionein induction is protective against copper ionophore induced cytotoxicity may also have important implications for the treatment of gliomas. Malignant gliomas have been found to express higher levels of MT genes and higher expression has been associated with a more aggressive tumor type and poorer patient prognosis (Mehrian-Shai et al., 2015; Masiulionytė et al., 2019). *In vitro,* loss of MT1E and MT2A expression sensitized human glioblastoma cells to disulfiram treatment (Corsello et al., 2020), which is particularly relevant to the data presented here as disulfiram is another copper ionophore (Kannappan et al., 2021). Therefore, gliomas with lower MT expression are more likely to respond favorably to CuES treatment and MTs may represent a new target for anti-neoplastic therapeutics. In conclusion, we have demonstrated that glioma cells and astrocytes are highly susceptible to

CuES induced cell death while neurons are resistant to this insult. We have also demonstrated that this cytoxicity is FDX1-independent and mediated by oxidative stress. Additionally, we have shown that induction of metallothionein gene expression may protect against CuES toxicity.

3.0 General Discussion

Gliomas are amongst the most common and most deadly brain tumors and are often refractory to treatment. Astrocytomas, which are malignant tumors derived from astrocytes, account for over 75% of gliomas, and are extremely difficult to treat with a high rate of recurrence (Ostrom et al., 2019; Nicholson and Fine, 2021; Willman et al., 2023). Recently, there has been growing interest in the potential of copper along with the potent copper ionophore elesclomol (CuES) to treat cancers with limited effective pharmaceutical options (Buccarelli et al., 2021; Gao et al., 2021; Tsvetkov et al., 2022; Zheng et al., 2022). Here we show that C6 glioma cells are sensitive to CuES *in vitro*, in line with numerous computational studies associating the expression of cuproptosis gene signatures, such as the expression of FDX1, with prognosis (Ye et al., 2022; Zhang et al., 2022; Zhu et al., 2022). However, in contrast to these studies, we found that CuES likely exerts its toxicity through the induction of oxidative stress rather than the FDX1-dependent, cuproptosis pathway described by Tsvetkov et al. (2022). This result is consistent with several other *in vitro* investigations that found that ES induced oxidative stress in cancer cell, including glioblastoma stem cells (Kirshner et al., 2008; Nagai et al., 2012; Buccarelli et al., 2021).

A more unexpected finding from our investigation was that both primary rat astrocytes and immortalized human astrocytes were vulnerable to CuES-induced toxicity while neurons, in contrast, were resistant to CuES mediated injury and seem to provide some degree of protection to astrocytes. As with C6 glioma cells, we found oxidative stress to be a significant contributor to CuES induced astrocyte cell death. An ideal treatment modality would selectively target cancer cells while leaving healthy tissue unperturbed. Astrocytes, however, unlike neurons, are mitotic and theoretically could recover from this insult. However as oxidative stress has been associated

with astrocyte senescence and reduced proliferation (Bitto et al., 2010; Cohen and Torres, 2019), these findings may have negative therapeutic implications for the potential use of CuES in the treatment of gliomas. In addition to reducing proliferation, astrocyte senescence has been associated with an increase in neurotoxic secretions which could damage surrounding neurons unaffected by CuES treatment (Pertusa et al., 2007; Limbad et al., 2020). In fact, we observed limited excitotoxic injury to neurons as a result of astrocytic damage following CuES treatment. In addition to oxidative stress, aging has been found to contribute to astrocyte senescence (Cohen and Torres, 2019) – a point that is of particular importance in glioblastoma, a particularly aggressive glioma, as the incidence of this tumor type increases with age (Ostrom et al., 2019). Therefore, damage by CuES to healthy astrocytes may have detrimental and long-lasting consequences and, as such, protecting astrocytes may have important consequences for long-term positive patient outcomes.

Given the potential negative consequences of injuring healthy astrocytes, devising methods of glioprotection is likely to be important step in translating this *in vitro* work to pre-clinical models of glioma. One method to do so may be to delineate differences in vulnerability to CuES-mediated toxicity between glioma cells and astrocytes. Of note, we found that while the antioxidant PQQ was protective to C6 glioma cells and hTert astrocytes, it had no effect on viability of CuES-treated primary rat astrocytes. Moreover, the degree of protection differed between C6 glioma cells and hTert astrocytes. In C6 glioma cells, both 30 µM and 50 µM PQQ restored viability of CuES treated cells from ~30% of control cells to ~84%. In contrast, 30 µM PQQ restored viability of CuES treated hTert astrocytes to ~ 47% of control cells. These results suggest the possibility that oxidative stress, while a significant contributor, is not the sole mechanism underlying CuES-induced death in healthy astrocytes. It should be noted that the complete rescue of hTert astrocytes

by the antioxidant EUK-134 conflicts with this hypothesis, however, as viability of primary rat astrocytes treated with EUK-134 increased to only 47% of control cells. Therefore, primary astrocytes may behave differently to astrocyte cell lines in their response to CuES toxicity and their ability to be rescued by antioxidant treatment. An alternative explanation for the difference in response to EUK134 and PQQ treatment between these astrocyte types is that while we used primary rat astrocyte cultures at DIV 17-21, the immortalized human astrocyte cell line we used were derived from fetal brain tissue (embryonic week 18). Therefore, the stage of astrocyte development may also influence its response to CuES treatment and mechanisms of rescue. The membrane protein, aquaporin 4 (AQP4), is often used as a marker of astrocyte maturity (Baranes et al., 2023). Intriguingly, high AQP4 expression has recently been associated with poorer overall survival and poor response to chemotherapy (Wang et al., 2023; Zhu et al., 2023). Therefore, differences in astrocyte maturity should be taken into consideration when investigating molecular mechanisms of toxicity and developing glioprotective strategies.

One limitation of our study is that we do not know whether CuES localizes primarily to the cytosol or mitochondria in astrocytes and in which cellular compartment reactive species are generated. While evidence demonstrating that ES delivers copper to the mitochondria (Nagai et al., 2012; Soma et al., 2018) suggests that CuES treatment would increase mitochondrial copper in our system, the FDX1-independence of the mechanism underlying cell death suggests otherwise. A recent investigation demonstrated that FDX1 acts to release Cu(I) inside the mitochondria while FDX1-independent mechanisms of CuES toxicity occur outside this organelle (Zulkifli et al., 2023). If CuES does indeed increase mitochondrial copper content in astrocytes however, one important consideration is to determine how this increase would affect cytosolic copper levels. An elegant series of experiments using patient-derived fibroblasts demonstrated that

the mitochondrial metallochaperones SCO1 and SCO2 regulate cytosolic copper content through the generation of a mitochondrially generated redox signal (Leary et al., 2007), with SCO2 acting as a thiol oxidoreductase and oxidizing/reducing cysteines in SCO1's CxxC motif depending on mitochondrial copper levels (Leary et al., 2009, 2013). When mitochondrial copper levels are increased, the cysteines in SCO1 are primarily oxidized and COX19, a twin CX_9C motif containing protein, translocates from the intermembrane space of the mitochondria to the cytosol. Its accumulation in the cytosol indicates a state of copper overload that stimulates ATP7A to translocate to the plasma membrane to efflux copper (Leary et al., 2013; Cobine et al., 2021). In this way, mitochondrial copper levels regulate cytosolic copper levels. An open question is whether CuES delivery to the mitochondria could overwhelm and disrupt this intricate, redox-sensitive system and lead to the cellular accumulation of copper, which could, in turn, increase cytosolic oxidative stress. This system of intracellular copper regulation could also have implications for other cell types such as colorectoral cancer cells as CuES treatment increases mitochondrial copper and leads to intracellular copper retention through the degradation of ATP7A. This increase in mitochondrial and intracellular copper results in oxidative stress and subsequently triggers ferroptosis (Gao et al., 2021). Although we did not find that inhibitors of ferroptosis, including deferoxamine and ferrostatin-1, ameliorated cell death in astrocytes, the accumulation of copper in multiple cellular compartments may have relevance to our system.

Intriguingly, in yeast, the metallothionein CUP1 colocalizes to both the cytosol and the intermembrane space of mitochondria (Kim and Lindahl, 2023). Although it is generally thought that metallothioneins reside in the cytosol, this finding may have implications for our finding that pretreatment of astrocyte cultures with ZnPyr completely abrogated CuES-induced death, presumably through the upregulation of MTs, as it suggests that MTs may provide protection

against copper-mediated stress whether it is mitochondrially or cytosolically localized. The protection provided by MT upregulation can be thought of analogously to therapeutic zinc supplementation in Wilson disease. In in this disorder, oral zinc supplementation is thought to upregulate MTs in enterocytes. As MTs have a higher affinity for copper than zinc, these metal binding proteins bind copper and store them in enterocytes before the copper-containing enterocytes are eventually sloughed off, thereby keeping excess from copper from entering the circulation. This model of protection is supported by the findings that in Caco-2 cells, an immortalized cell line used to the study intestinal epithelial cells, zinc treatment activates the metal regulatory transcription factor 1 (MTF-1) which consequently results in upregulation of *MT1* and *MT2* (Hardyman et al., 2016), which are metal response element (MRE)-regulated genes (Stuart et al., 1984; Andrews, 2000). Our lab has previously shown that the ZnPyr concentrations used in this study are sufficient to activate MTF-1 and MRE-regulated gene expression in mixed cortical cultures (Krall et al., 2022), and our experiments demonstrate a significant upregulation of *MT1* and *MT2* mRNA in astrocyte cultures following zinc treatment **(**Figure 12**)**, consistent with data from Caco-2 cells. Thus, the mechanism underlying protection of astrocytes likely shares similarities to the mechanism underlying zinc treatment for Wilson disease. However, instead of buffering copper in enterocytes and avoiding its release into the circulation, MTs in astrocytes may bind copper and prevent the redox active metal from inducing oxidative stress – a mechanism we found to contribute to CuES-induced death in astrocytes. Although the primary role of MTs is to bind metals, there are data to suggest that MTs may also act to scavenge reactive species (Thornalley and Vašák, 1985; Chubatsu and Meneghini, 1993; Uchida et al., 2002; Ruttkay-Nedecky et al., 2013) and, as such, may act as antioxidants in addition to binding copper in our system.

The protection provided by induction of metallothioneins in astrocytes also provides a clue as to the differential sensitivity of neurons and astrocytes in our cultures. As noted previously, neurons express high levels of MT3, which exists predominantly in its apo form (Aras et al., 2009). The abundance of apo-MT3 in neurons could allow it to bind to CuES and prevent the toxic effects seen in astrocytes. However, it is important to note that despite supporting evidence from astrocytes cultures, this hypothesis was not directly tested in our experiments. It should be noted that we attempted to knockdown MT3 using an MT3-shRNA targeting plasmid that had previously been used successfully to show that MT3 contains free metal binding sites (Aras et al., 2009). However, we were unable to successfully knockdown the protein in our experiments without causing neuronal injury. Future experiments could directly test this hypothesis using commercially available siRNA, as we found Acell siRNA to effectively knockdown FDX1 in our cultures. As this siRNA does not require transfection, it would also avoid the confound of transfection efficiency when assessing the toxicity of CuES in MT3-depleted neurons.

As previously mentioned, another surprising finding was that in addition to being more resistant to CuES toxicity, neurons seemed to provide protection to astrocytes in our mixed cultures. This was highlighted by our elesclomol concentration-response experiments in the presence of 2 μM Cu^{2+}. While 200-300 nM ES was needed to cause significant LDH release in mixed cultured, 50 nM ES was sufficient to cause nearly maximal LDH release in astrocyte cultures (Figure 5). An unresolved question is the mechanism by which neurons provide this glioprotection. One possibility is that neurons secrete a factor that is protective to astrocytes in culture. Intriguingly, there is some data to suggest that MTs can be secreted by astrocytes and taken up by neurons. One investigation detected MT1/MT2 in the media of primary cultured astrocytes and the amount of MT1/MT2 detected in the culture media significantly increased after

co-treatment with zinc and interleukin-1 (Chung et al., 2008), presumably as a result of *MT* induction. Moreover, this investigation found increased levels of extracellular MT1/2 *in vivo* following focal cortical injury (Chung et al., 2008), suggesting that MTs may be released in response to stress or injury. Importantly *in vitro* experiments demonstrated that MT2 released from astrocytes was internalized by neurons via the endocytic receptor megalin (Chung et al., 2008). While there is not yet literature to support the secretion of MTs by neurons, neurons are capable of secreting soluble factors (März et al., 1998; Chang et al., 2003; Biber et al., 2007) and astrocytes express the megalin receptor (Bento-Abreu et al., 2008). Moreover, murine brain MT3 was found to interact with Exo84p (El Ghazi et al., 2010), a component of the exocyst complex that is critical for secretion (Guo et al., 1999). While these experiments used whole brain homogenates, making it difficult to identify the cell type from which MT3 was isolated from, given the enrichment of this MT isoform in neuronal cells, it is likely that the interaction between MT3 and Exo84p occurs in neurons. Therefore, one possible explanation for our findings is that neurons secrete MT3 or another soluble factor which is taken up by astrocytes and protects against CuES mediated stress. To test whether neurons provide glioprotection through secreted factors or through direct neuron-astrocyte communication, conditioned media from neuron-containing cultures could be used when treating astrocyte cultures or hTert immortalized astrocytes with CuES. An increase in cell viability or a shift in the ES concentration-response curve would suggest that neurons secrete a glioprotective factor. Preliminary data, using immortalized human astrocytes seeded on a transwell membrane, and placed in a well containing primary rat mixed cultures prior to CuES treatment, suggest that neurons provide protection even when not in direct contact with astrocytes, suggesting secreted factors may indeed be sufficient to rescue astrocytes. However, it is worth noting that while our experiments revealed that neurons provide protection against CuES to healthy astrocytes

in culture, whether they also provide protection to glioma cells is yet to be determined. This question could be investigated by co-culturing C6 glioma cells with our primary mixed cultures or seeding them on transwell inserts as described above.

It must be acknowledged that while a critical finding of our investigation was that astrocytes are differentially sensitive to copper, in contrast to neurons which are resistant against this stress, early work by Dennis Choi and co-workers had different results. Choi and his colleagues found, that in contrast to what we reported here, in mixed cortical cultures, neurons were vulnerable to copper toxicity while glia were unaffected (Sheline et al., 2002). However, it is important to note that while we treated cultures with a relatively low concentration of copper (2 µM) along with the potent ionophore elesclomol, Sheline et al. (2002) exposed mixed cultures to 20 µM copper alone overnight. Therefore, the rate of copper accumulation likely differed significantly in these experiments. Indeed, we found that a two-hour CuES pulse was sufficient to induce significant glial damage **(**Figure 7**)**. Previous research has also demonstrated that astrocytes are more vulnerable to copper-induced toxicity when the rate of accumulation is rapid as opposed to gradual (Scheiber and Dringen, 2013). This difference in the hypothetical rate of copper accumulation could contribute to the differences in selective cell death in these two systems, perhaps allowing glia to upregulate copper binding proteins such as the ER localized protein chaperone GRP78 (Qian et al., 2012, 2013). Supporting this idea, when Sheline & Choi (2004) compared the sensitivity of near pure neuronal cultures, glial cultures, and mixed neuronal and glial cultures to 5 µM copper in the presence of 300 nM disulfram, a copper ionophore, they found that pure neuronal cultures were the least susceptible to copper/disulfram toxicity while mixed cultures were the most susceptible, with glial cultures showing intermediate sensitivity. Moreover, mixed cultures accumulated four times the amount of copper as pure neuronal cultures (Sheline

and Choi, 2004). While we found that astrocyte cultures were significantly more sensitive in the absence of neurons, our method of generating glial cultures differed from Sheline & Choi's (2004). In our cultures, astrocytes and neurons were cultured together until we performed our experiments at which time we treated cultures with kainate to selectively eliminate neurons from cultures. In contrast, Sheline & Choi (2004) created pure glial cultures from embryonic mice. As neurons release soluble factors that regulate the expression of astrocytic receptors including the glutamate transporter GLT-1 (Gegelashvili et al., 1997), it is possible that this difference in the generation of our glial cultures contributed to our differing results. Taken together, these results suggest that treatment with copper alone, at relatively high concentrations, may be selectively toxic to neurons while copper-ionophore treatment is more toxic to glial cells. This finding may account for the extensive neuronal loss seen in disorders that result in brain accumulation of copper such as in Wilson disease (Mikol et al., 2005; Meenakshi-Sundaram et al., 2008).

Intriguingly, the putative mechanism underlying neuronal susceptibility to copper-induced cell death shares similarities to the cupropoptosis pathway defined by Tsvetkov et al. (2022). Sheline et al. (2002) found that copper treatment resulted in the accumulation of pyruvate, 2-phosphoenolpyruvate, and 2-phosphoglycerate but not glucose-6-phosphate or fructose-6-phosphate, suggesting inhibition of the pyruvate dehydrogenase (PDH) complex (Sheline et al., 2002), which regulates entry into the TCA cycle. Intriguingly, neuronal death could be rescued by addition of the PDH cofactors thiamine, dihydrolipoic acid, lipoic acid, as well as the PDH activator dichloroacetate, further suggesting that PDH inhibition underlies this selective neuronal death. This finding is consistent with recent work by Tsvetkov et al. (2022), which demonstrated that CuES treatment disrupted TCA cycle function by inhibiting PDH complex function through protein lipoylation. Although Sheline et al. (2002) posit that PDH complex inhibition is a result of

copper-induced free radicals, they similarly hypothesize that the method of inhibition involves the disruption of the lipoic acid moiety of PDH, although through the adduct of 4-HNE, a lipid peroxidation product that was previously shown to inhibit the PDH complex through modification of this moiety (Humphries and Szweda, 1998). While cuproptosis involves the binding of copper to lipoylated enzymes (Tsvetkov et al., 2022), it is unclear whether this is the case in copper-treated neurons. However, the rescue of neuronal viability by the addition of lipoic acid could theoretically act as a sink for copper ions and prevent them from binding to lipoyated PDH complex proteins or even from crossing the mitochondrial membrane.

The work described above demonstrates the intricate copper handling systems that neurons and astrocytes have developed to both utilize and protect against this redox active metal. The importance of copper is highlighted by the theory that the development of multicellular organisms coincides with the appearance of significant concentrations of O_2 in the atmosphere as it led to the ability of copper to redox cycle between Cu(I) and Cu(II) states (Crichton and Pierre, 2001). However, the same redox cycling ability that makes copper an indispensable cofactor also leads to its ability to form reactive species that lead to oxidative stress and cell death. Our work shows how this ability can be harnessed for therapeutic potential for the treatment of glioma as well as how it can lead to damage to surrounding parenchyma. As described in the introduction, copper utilization and copper-induced dysfunction in neurons has been well researched. However, there is a comparative paucity of literature regarding astrocytes and copper. Future research in this area will hopefully elucidate additional differences in the way neurons and astrocytes utilize and protect against copper induced toxicity which may allow for additional glioprotective strategies when using copper-ionophores for the treatment of disease.

Appendix: Overview of Additional Publications

While the subject of my dissertation is the investigation of mechanisms underlying the selective vulnerability of astrocytes to copper-ionophore mediated cell death, a common theme of the work I have completed throughout my graduate studies is the investigation of how neuronal dysfunction on a cellular and molecular level can have profound consequences on human health. In this appendix I have included two papers that, while not related to my dissertation goals, are related to this general theme.

Appendix A Differential Response of a Disease-linked GluN2A Mutant to NMDA Channel Blockers

Gale J. R., Kosobucki G. J., Hartnett-Scott K. A., Aizenman E. (2021) Imprecision in Precision Medicine: Differential Response of a Disease-Linked GluN2A Mutant to NMDA Channel Blockers. *Front. Pharmacol.* **12**, 773455.

Imprecision in Precision Medicine: Differential Response of a Disease-Linked GluN2A Mutant to NMDA Channel Blockers

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Abstract

Mutations in N-methyl-D-aspartate receptors (NMDAR) subunits have been implicated in a growing number of human neurodevelopmental disorders. Previously, a *de novo* mutation in *GRIN2A*, encoding the GluN2A subunit, was identified in a patient with severe epilepsy and developmental delay. This missense mutation, which leads to GluN2A-P552R, produces significant dendrotoxicity in transfected rodent cortical neurons, as evidenced by pronounced dendritic blebbing. This injurious process can be prevented by treatment with the NMDA antagonist memantine. Given the increasing use of FDA approved NMDA antagonists to treat patients with *GRIN* mutations, who may have seizures refractory to traditional anti-epileptic drugs, we investigated whether additional NMDA antagonists were effective in attenuating neurotoxicity associated with GluN2A-P552R expression. Intriguingly, we found that while treatment with memantine can effectively block GluN2A-P552R-mediated dendrotoxicity, treatment with ketamine does not, despite the fact that both drugs work as open NMDAR channel blockers. Interestingly, we found that neurons expressing GluN2A-P552R were more vulnerable to an excitotoxic insult – an effect that, in this case, could be equally rescued by both memantine and ketamine. These findings suggest that GluN2A-P552R induced dendrotoxicity andincreased vulnerability to excitotoxic stress are mediated through two distinct mechanisms. The differences between memantine and ketamine in halting GluN2A-P552R dendrotoxicity could not be explained by NMDA antagonist induced changes in MAP or Src kinase activation, previously shown to participate in NMDA-induced excitotoxicity. Our findings strongly suggest that not all NMDA antagonists may be of equal clinical utility in treating *GRIN2A*-mediated neurological disorders, despite a shared mechanism of action.

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1. Introduction

N-methyl-D-aspartate receptors (NMDARs) are ligand-gated, ionotropic glutamate receptors widely expressed in the brain, playing key roles in neuronal developmental, synaptic plasticity, and survival. NMDARs are heterotetramers composed of three main subtypes: GluN1, which are obligatory, GluN2 of which there are four subunit types (A-D), and GluN3 subunits of which there are two subunit types (A-B) (Traynelis et al., 2010; Paoletti et al., 2013). The majority of NMDARs express two glycine-binding GluN1 subunits and two glutamate-binding GluN2 subunits (Köhr, 2006). The GluN2 subtype dictates many of the NMDAR's characteristics such as its biophysical, pharmacological, and signaling properties, as well as its spatiotemporal pattern of expression (Paoletti et al., 2013; Vieira et al., 2020; Monyer et al., 1992; Watanabe et al., 1994).

Mutations in NMDAR subunits have been increasingly implicated in neurological and neurodevelopmental diseases, including intellectual disability, autism spectrum disorders, developmental delay, and epilepsy (Myers et al., 2019). The identification of *GRIN* variants in pediatric patients is significant, as individuals *GRIN* mutations and epilepsy refractory to standard anti-convulsants have been successfully treated with the FDA-approved NMDAR antagonists memantine, ketamine, and dextromethorphan (Xu et al., 2021; Li et al., 2016; Pierson et al., 2014; Amador et al., 2020). Although mutations in four genes encoding NMDAR subunits (*GRIN1, GRIN2A, GRIN2B, and GRIN2D*) have been linked to human disease (Li et al., 2019; Xu et al., 2021; Li et al., 2016; Carvill et al., 2013; Liu et al., 2017; Bahry et al., 2021), mutations in *GRIN2A* account for the majority of disease-linked variants (46%) (Myers et al., 2019; Strehlow et al., 2019). Pathogenic variants cluster in the highly conserved agonist binding domains as well as transmembrane and linker domains, which are highly intolerant to genetic variation (Strehlow et al., 2019; Ogden et al., 2017; Swanger et al., 2016).

Recently, a *de novo* missense mutation in the pre-M1 helix region – the linker between the agonist binding domain and the first transmembrane domain – was identified in a patient with profound intellectual disability, developmental delay, and epilepsy (de Ligt et al., 2012). This mutation, which results in a substitution of arginine for proline at amino acid 552 (P552R), was further characterized as leading to neurotoxicity when expressed in cultured rat cortical neurons, as evidenced by pronounced dendritic swelling (Ogden et al., 2017). This neurotoxicity could be rescued by treatment with memantine. Given the superiority of ketamine as compared to memantine in treating some *GRIN* mutations (Li et al., 2016), and its widespread use in treating status epilepticus in adult and pediatric populations (Zeiler, 2015; Ilvento et al., 2015; Mewasingh et al., 2003), we sought to determine whether ketamine treatment was equally effective as memantine in abrogating GluN2A-P552R induced neurotoxicity *in vitro*. Surprisingly, our data indicate that the P552R mutation exerts neurotoxicity through two separate mechanisms, dendritic blebbing and increased susceptibility to excitotoxic injury – both responsive to treatment with memantine but only the latter responsive to treatment with ketamine, despite a shared mechanism of action between these two drugs.

2. Methods

Materials

Primary antibodies used: rabbit anti-p38 MAPK (Cell Signaling, 9212S, 1:1000), mouse anti-phospho-p38 MAPK (Cell Signaling, 9216S, 1:1000), mouse anti-ERK (pan-ERK) (BD Transduction Lab, 610123, 1:2000), rabbit anti-phospho-p44/42 MAPK (ERK 1/2) (Cell Signaling, 910, 1:1000), mouse

anti-JNK (Santa Cruz, sc-7345, 1:1000), rabbit anti-pSAPK/JNK (Cell Signaling, 4668S, 1:1000), rabbit anti-Src (Cell Signaling, 2108S, 1:1000), rabbit anti-phospho-Src (Tyr416) (Cell Signaling, 2101S, 1:1000), and mouse anti-β-actin (Sigma, A5441, 1:10000). Mouse or rabbit secondary antibodies used: Li-cor IRDye 700 CW and Licor IRDye 800CW (LI-COR Biosciences). Chemicals were obtained from Sigma-Aldrich unless otherwise specified.

Neuronal Cultures

All procedures involving the use of animals were reviewed and approved by the University of Pittsburgh IACUC. Primary cortical cultures were prepared from embryonic day 16-17 Sprague-Dawley rats as previously described (Hartnett et al., 1997). Embryonic cortices were dissociated with trypsin and cells were plated on 12-mm, poly-L-ornithine coated glass coverslips in six-well plates at a density of 670,000 cells per well. On day 14 *in vitro* (DIV 14) cytosine arabinoside (1 to 2 μM) was used to inhibit nonneuronal cell proliferation. Cultures were used at DIV 18-25.

Transfections

Transfections were performed using Lipofectamine 2000 (Thermo Fisher). Neurons were transfected with the following plasmid mixtures (total of 1.5 μg DNA/0.5 mL): for confocal imaging, cells were transfected with a GFP-expressing plasmid (pEGFP-N1; BD Biosciences), pCI-neo vector, human wild-type GluN2A, or human GluN2A-P552R (gifts from Drs. Hongjie Yuan and Stephen Traynelis). Plasmid mixtures (per well) contained either 0.9 μg pEGFP-N1 + 0.6 μg pCI-neo vector, 0.9 μg pEGFP-N1 + 0.3 μg pCI-neo empy vector + 0.3 μg GluN2A plasmid, or 0.9 μg pEGFP-N1 + 0.3 μg pCI-neo empy vector + 0.3 μg GluN2A-P552R plasmid (Ogden et al., 2017). For luciferase viability experiments, cells were transfected with a firefly luciferase-expressing plasmid (pUHC13-3 Luciferase, gift of Dr. H. Buchard) instead of one expressing GFP. Plasmid mixtures (per well) contained either 0.375 μg pUHC13-3 Luciferase + 1.125 μg pCI-neo vector plasmid, 0.375 μg pUHC13-3 Luciferase + 0.525 μg pCI-neo vector + 0.6 μg GluN2A plasmid, or 0.375 μg pUHC13-3 Luciferase + 0.525 μg pCI-neo vector plasmid + 0.6 μg GluN2A-P552R plasmid.

Confocal Imaging and Bleb Analysis

To analyze the effect of the GluN2A-P552R mutation on dendrite morphology, cells were imaged 24 hours after transfection. All treatments were added to the cell media at the time of transfection. Images were obtained on a Nikon A1+ confocal microscope using a 20x water immersion objective. Three separate culture dates were used per experiment, and three coverslips were transfected with each plasmid mixture per condition. Each coverslip was divided into four quadrants, and one field of view was randomly selected from each quadrant resulting in four images per coverslip. Bleb counts per field were added to determine a bleb count per coverslip (blebs/CS). For these experiments, *n* refers to the number of coverslips. Laser power was sometimes adjusted between coverslips due to differences in transfection efficiency of the GFP-expressing plasmid. Nikon Instruments Software Basic Research (NIS-Elements BR) was used for non-biased analysis of dendritic blebs. All images were subjected to intensity thresholding before analysis. The object count feature was used to quantify the number of blebs in each image field. Area was restricted to 0-5 μm^2 and circularity was set to 0.5-1.0. The smooth, clean, and separate features were used to reduce background noise and settings were consistent

between control, ketamine, and MK-801 groups. Settings had to be adjusted once due to the installation of a new laser in our system

Lactate Dehydrogenase Assays

Cortical neurons were treated with 10 μM glycine, 10 μM glycine + 30 μM NMDA, or 10 μM glycine + 30 μM NMDA with either 50 μM memantine or 10 μM ketamine for 30 minutes in HEPES-buffered minimal essential media with 0.01% BSA (MHB). After 30 minutes, cells were washed with fresh MHB and then incubated with MHB or MHB containing memantine or ketamine. Twenty-four hours following glycine + NMDA treatment, medium was collected for lactate dehydrogenase (LDH) assays. Toxicity is represented by increased OD490 values. Three experiments from separate culture dates were performed, each in quadruplicate.

Luciferase Viability Assays

Cortical cultures were transfected with plasmid mixtures containing a luciferase-expressing. Twenty-four hours following transfection, cells were either left untreated, treated with 45 μM DL-*threo*-β-Benzyloxyaspartate (TBOA) (Tocris Bioscience), or co-treated with 45 μM TBOA and either 50 μM memantine or 10 μM ketamine. TBOA is a glutamate uptake inhibitor that induces an excitotoxic injury *in vitro* (Bonde et al., 2003). Twenty-four hours after drug treatment, firefly luciferase expression was measured using the SteadyLite Plus Luminescence Gene Reporter Assay System (PerkinElmer) (Aras et al., 2008). Results for TBOA-treated groups were normalized to the luminescence values (counts per second) of their respective untreated groups. This assay was performed a minimum of four times with neurons from separate culture dates.

Immunoblotting

Cortical cultures were left untreated, treated with 50 μM memantine, or treated with 10 μM ketamine for 45 minutes. Control, memantine treated-, and ketamine treated neurons were collected from the same 6 well plate for each experiment. Following treatment, neurons were rinsed twice with ice-cold PBS, exposed to cell lysis buffer (Invitrogen) supplemented with protease inhibitor cocktail (Roche Diagnostics) and phenylmethlysulfonyl fluoride, and scraped off dishes. Debris was pelleted by centrifugation at 14,000g for 10 minutes. The remaining lysates were stored at -80°C. Protein concentration of lysates was determined (Pierce BCA Protein Assay Kit; Thermo Fisher). Samples were prepared by incubating 30 μg of protein with a reducing sample buffer at 100°C for five minutes. Samples were loaded onto a 7.5% SDS-PAGE gel and proteins were separated using the Mini Protean 3 System (Biorad). Gels were transferred onto 0.2 μm nitrocellulose membranes and blocked at room temperature for one hour with 1% BSA in PBS containing 0.05% Tween 20 (PBST). Membranes were incubated overnight at 4°C in primary antibody. After washing 3x in PBST, membranes were probed with with Li-Cor IRDye-conjugated secondary antibodies labeled with IRDyes 700CW (685 nm) and 800CW (780 nm), at 1:10,000 for one hour at room temperature. Fluorescent signals were acquired (Odyssey Infrared Imaging System; LI-COR) and quantified using Fiji software.

Statistical Analysis

Data are presented as means ± SEM. All statistical analyses were performed using GraphPad Prism 9 (GraphPad). Prior to analyses, data were tested for normality using a Shapiro Wilk test. For

comparison of two sample means, a two-tailed t-test was used. For comparison of more than two sample means, a one-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons was used. For LDH assays, a two-way ANOVA with Sidak's multiple comparison test was.

3. Results

A previous characterization of the GluN2A-P552R mutation found that the mutant NMDAR subunit exerted profound dendrotoxicity when expressed in primary neurons, as evidenced by dendritic swelling (blebbing) (Ogden et al., 2017). GluN2A-P552R mediated dendritic blebbing could be rescued by treating neurons with the FDA-approved NMDAR antagonist memantine (50 μM), consistent with clinical reports of NMDAR receptor antagonists being used to treat refractory epilepsy in patients with *GRIN* mutations (Li et al., 2016; Pierson et al., 2014; Amador et al., 2020). Given that ketamine, another FDA-approved NMDAR antagonist, is currently widely used to treat status epilepticus (Zeiler, 2015; Ilvento et al., 2015; Pribish et al., 2020), and has been used to treat refractory epilepsy in patient with a *GRIN2D* mutation (Li et al., 2016), we evaluated its neuroprotective profile in GluN2A-P552R mediated neurotoxicity.

We first confirmed our previous findings from the initial characterization of the GluN2A-P552R mutation (Ogden et al., 2017). As noted earlier, cortical neurons transfected with GluN2A-P552R displayed pronounced dendritic blebbing (Ogden et al., 2017) (Figure 1a and 1b). This effect was not observed in neurons transfected with the empty vector (pCI-neo) or, importantly, the wild-type subunit (GluN2A). GluN2A-P552R-mediated dendrotoxicity was attenuated by treatment with 50 μM memantine (One-way ANOVA, $p=0.3025$, $n=9$) (Figure 1a and 1c), as previously shown, confirming that FDA-approved NMDAR antagonists may be a viable treatment strategy for individual(s) with this mutation. Surprisingly, although ketamine and memantine share the same mechanism of action, similar pharmacodynamic profiles, and overlapping binding sites within NMDA receptors (Song et al., 2018; Zhang et al., 2021; Emnett et al., 2013), we found that ketamine treatment did not rescue GluN2A-P552R mediated dendritic blebbing (One-way ANOVA, $p<0.0001$; Multiple comparisons, pCI-neo vs. GluN2A, $p=0.9407$, pCI-neo vs. GluN2A-P552R, $p<0.0001$, GluN2A vs. GluN2A-P552R, $p<0.0001$, $n=9$) (Figure 2a and 2b). Indeed, an unpaired t-test revealed that there was no difference in the number of dendritic blebs per coverslip between untreated neurons and those treated with 50 μM ketamine ($p=0.7819$, $n=9$). Although there are some reports of ketamine induced neurotoxicity in neurons exposed to the drug for prolonged periods (Liu et al., 2013; Wang et al., 2017), empty vector transfected neurons and GluN2A transfected neurons, which were also treated with, did not display any dendrotoxicity, suggesting that the dendritic blebbing observed in GluN2A-P552R group was not a result of the ketamine treatment itself. It is noteworthy that ketamine's ability to inhibit GluN1/GluN2A-P552R-mediated ionic currents does not significantly differ from its ability to block GluN1/GluN2A wild type channels. Ketamine's maximum blocking efficacy for GluN1/GluN2A-P552R channels is 96% as compared to 98% for wild-type channels. Furthermore, the GluN2A-P552R mutation enhances the potency of ketamine (IC_{50} =1.3 μM for GluN2A-P552R; IC_{50}=4.7 μM for wild type GluN2A), without significantly affecting memantine's blocking actions at the receptor (IC_{50}=3.7 μM for GluN2A-P552R; IC_{50}=4.8 for wild type GluN2A) (Ogden et al., 2017). As such the lack of ketamine's ability to protect from the observed dendrotoxicity could not be accounted by an inability to antagonize the mutant channels.

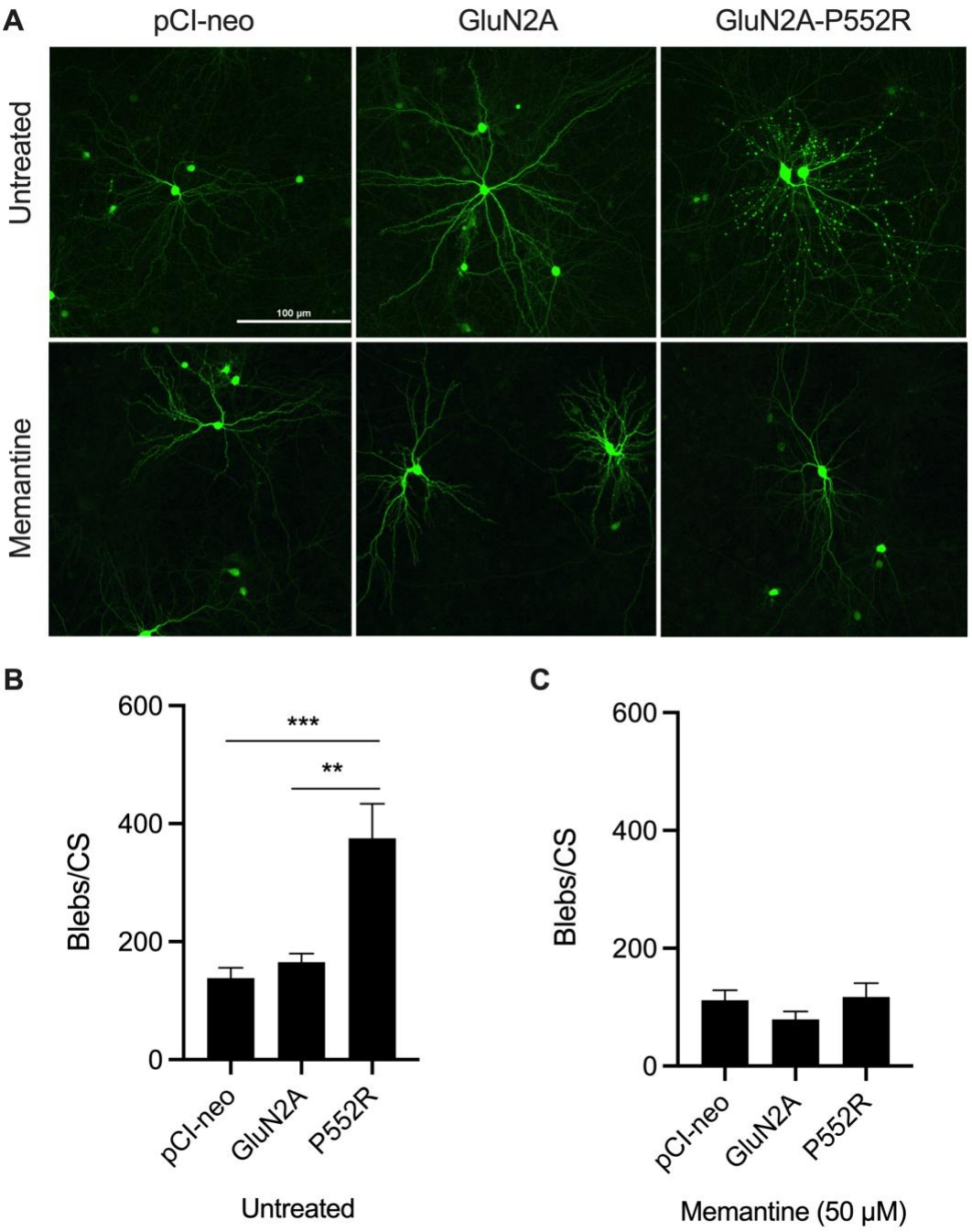

Figure 1. GluN2A-P552R expression exerts dendrotoxicity in rat primary cortical cultures which can be rescued by treatment with memantine. Representative images of cortical neurons transfected with GFP and either pCI-neo (empty vector), GluN2A, or GluN2A-P552R. Top row shows untreated neurons and bottom row shows neurons co-treated with 50 µM memantine **(A)**. Quantification of dendritic blebs per coverslip (blebs/CS) revealed significantly more blebs in GluN2A-P552R transfected neurons as compared to those transfected with pCI-neo or GluN2A **(B)**. This difference is abolished by treatment with 50µM memantine **(C)**. Data are expressed as mean ± SEM from three independent experiments with three coverslips per transfection condition in each experiment (**p<0.01, ***p<0.001, one-way ANOVA/Tukey post-hoc, n=9)

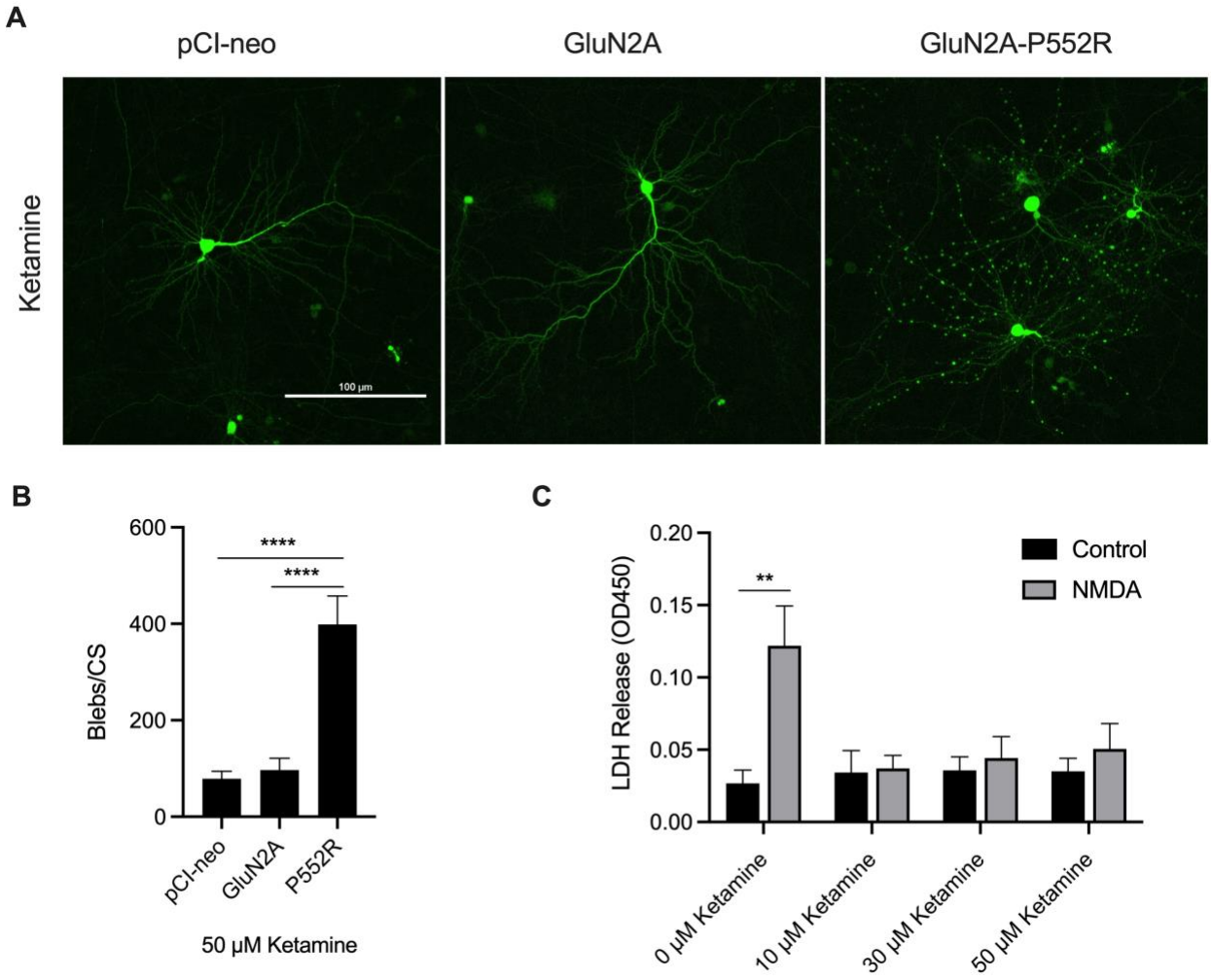

Figure 2. Ketamine treatment does not rescue GluN2A-P552R-mediated dendritic blebbing. Representative images of cortical neurons transfected with GFP and either pCI-neo, GluN2A, or GluN2A-P552R and co-treated with 50 µM ketamine show that GluN2A-P552R transfected neurons exhibit pronounced blebbing that is not rescued by the open-channel blocker **(A)**. Quantification of dendritic blebbing confirmed significantly more blebs in the GluN2A-P552R group **(B)** (****$p<0.0001$, one-way ANOVA/Tukey post-hoc, n=9). LDH assays of untransfected primary cortical neurons treated with 10 µM glycine (control) or 10 µM glycine + 30 µM NMDA (NMDA) confirmed that ketamine is protective against excitotoxic injury. **(C)** (**$p<0.01$, two-way ANOVA/Sidak post-hoc, n=3). Therefore, the failure of ketamine to rescue GluN2A-P552R dendrotoxicity is not due to inefficacy of the drug in our culture system. Data are expressed as mean ± SEM from three independent experiments. Three transfections per condition were analyzed from each independent imaging experiment.

Given these data, we sought to confirm that ketamine was indeed protective against canonical excitotoxic insults in our cell culture system. To this end, we exposed untransfected cortical cultures to either 10 μM glycine (control) or 10μM glycine and 30 μM NMDA for 30 min and co-treated with either vehicle or ketamine (10 μM, 30 μM, or 50 μM). Cell viability was assessed using LDH assays 24 hours after the exposure. Cells not treated with ketamine displayed a significant loss of viability when exposed to glycine + NMDA as compared to the control, whereas no significant differences were found between the control and glycine + NMDA groups when cells were co-treated with ketamine (Two-way ANOVA, p=0.0158 for interaction; Multiple comparisons, glycine vs glycine + NMDA: 0 μM ketamine, p=0.0021, 10 μM ketamine, p=0.9997, 30 μM ketamine, p=0.9820, 50 μM ketamine, p=0.8645, n=3) (Figure 2c). Furthermore, comparison of control neurons in these assays confirmed that ketamine treatment is not neurotoxic in our system (One-way ANOVA, p>0.05). These results indicate that ketamine is an effective excitotoxicity neuroprotectant in our preparation.

As the above data indicate that the inability of ketamine to rescue of GluN2A-P552R mediated dendrotoxicity is not due to a loss of potency or innate toxicity, we next aimed to determine whether memantine's ability to rescue this phenotype was through its classically defined role as an NMDA receptor channel antagonist or through an undefined, alternative mechanism. To this end, we exposed GluN2A-P55R expressing neurons to the open-channel blocker MK-801, which binds to the same pocket of the NMDAR as memantine and ketamine (Song et al., 2018; Zhang et al., 2021). Treatment with 10 μM MK-801 similarly abolished GluN2A-P552R mediated dendritic blebbing (One-way ANOVA, p>0.05) (Figure 3a and 3b), strongly suggesting that memantine rescues GluN2A-P552R through its known mechanism of pharmacological action.

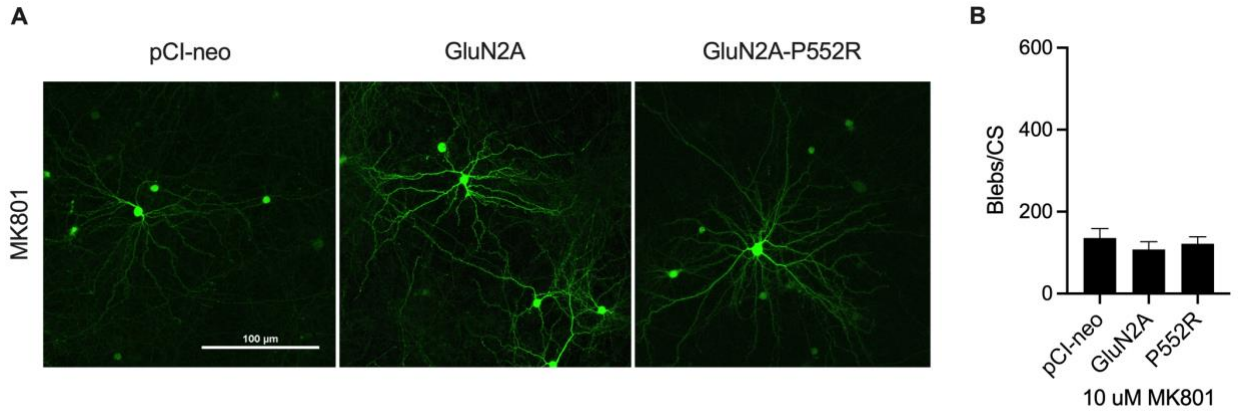

Figure 3. Dendritic blebbing is attenuated by treatment with the channel blocker MK801. Representative images of rat cortical neurons transfected with pCI-neo empty vector, GluN2A-WT, and GluN2A-P552R and co-treated with 10μM MK801(A). GluN2A-P552R expressing neurons treated with 10 μM MK801 show a significant reduction in blebbing **(B)** (one-way ANOVA, p>0.05, n=9). Data are ± SEM from three independent experiments with three coverslips analyzed per experiment.

Having established the NMDAR blockade is sufficient to rescue GluN2A-P552R mediated dendrotoxicity, we next investigated downstream pathways that may be either activated or inhibited by ketamine, but not by memantine, which could account for the differential responses observed. Activation of MAP kinases (MAPKs) have previously been shown to be part of signaling cascades initiated downstream of NMDAR activation (Chen et al., 2003; Kawasaki et al., 1997), including those implicated in excitotoxicity (Choo et al., 2012; Cao et al., 2004; Liu et al., 2014; Engin and Engin, 2021; Kawasaki et al., 1997). Therefore, we assessed via western blot whether there were differences in kinase phosphorylation in primary cortical neurons treated with either ketamine or memantine. No significant differences were found between memantine and ketamine groups in levels of phosphorylated ERK1/2 or JNK (Paired t-test, $p>0.05$, $n=5$-6) (Figure S1f and S1g). A small but significant reduction in phosphorylated p38 (p-p38) was found in ketamine treated neurons as compared to those treated with memantine (Paired t-test, $p=0.$ 0.0036, $n=5$) (Figure S1e). However, given that phosphorylation of p38 has been implicated in excitotoxic neuronal apoptosis (Liu et al., 2014; Chen et al., 2003; Kawasaki et al., 1997), it is unlikely that the reduction of p-p38 in the ketamine treated group is the mechanism underlying the persistent dendritic blebbing in ketamine treated GluN2A-P552R expressing neurons. As Src kinase has been shown to upregulate NMDAR activity (Scanlon et al., 2017; Zhao et al., 2015; Manzerra et al., 2001), we also assessed whether memantine and ketamine treatment differentially affected Src phosphorylation. However, no differences were found between groups (Paired t-test, $p>0.05$, $n=5$) (Figure S1h). Thus, the mechanism underlying the inability of ketamine to rescue GluN2A-P552R-induced dendritic blebbing is yet to be identified. Nonetheless, the data presented thus far strongly suggests that human disease-linked *GRIN* mutations may be differentially responsive to NMDAR antagonists.

Because the GluN2A-P552R mutation results in increased agonist potency and mean channel open time (Ogden et al., 2017), we hypothesized that neurons that express this mutant subunit may show enhanced vulnerability to excitotoxic injury, perhaps due to exaggerated calcium influx upon activation. To test this, we transfected neurons with a plasmid expressing firefly luciferase along with the either the empty vector, GluN2A, or GluN2A-P552R. Co-transfection of the firefly luciferase construct allowed us to compare viability of cells expressing the mutant subunit as compared to wild-type GluN2A or the empty vector (Aras et al., 2008). Twenty-four hours after transfection, neurons were exposed to a mild excitotoxic insult (treatment with a sublethal concentration of the glutamate uptake inhibitor TBOA, 45 μM) for an additional 24 hours. Indeed, following TBOA exposure, we found a significant difference in viability of GluN2A-P552R transfected neurons as compared to those transfected with the empty vector or the wild-type subunit. (One-way ANOVA, $p=0.001$; Multiple Comparisons, pCI-neo vs GluN2A, $p=0.3983$, pCI-neo vs GluN2A-P552R, $p=0.0008$, GluN2A vs GluN2A-P552R, $p=0.0181$). (Figure 4a). Co-treatment with memantine rescued the loss of viability observed in GluN2A-P552R expressing neurons (One-way ANOVA, $p>0.05$), consistent with its effect on GluN2A-P552R mediated dendrotoxicity (Figure 4b). In contrast to ketamine's failure to attenuate dendritic blebbing, co-treatment with this channel blocker also completely rescued TBOA-induced cell death in GluN2A-P552R expressing neurons (One-way ANOVA, $p>0.05$) (Figure 4c). These data strongly suggest that the P552R mutation exerts dendrotoxicity and enhances vulnerability to excitotoxic stress through two separate cell injurious signaling pathways.

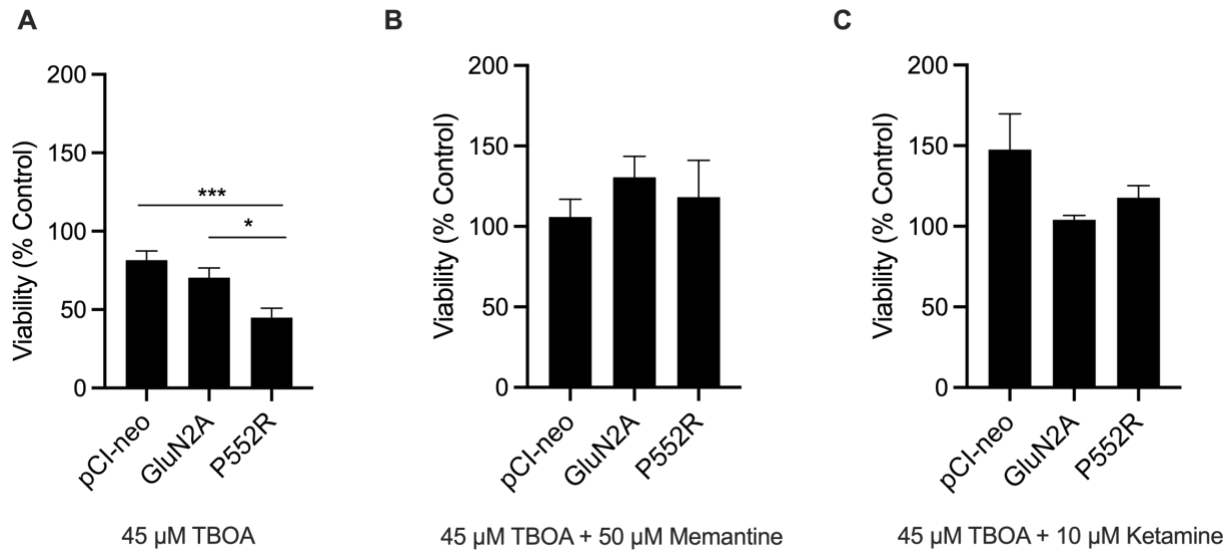

Figure 4. GluN2A-P552R enhances vulnerability to excitotoxic stress which can be rescued by both memantine and ketamine. Luciferase assays were used as a measure of cell viability. Rat cortical neurons were co-transfected with a plasmid expressing firefly luciferase and pCI-neo vector, GluN2A-WT, or GluN2A-P552R. Treatments were applied 24 hours after transfection and luciferase assays were performed 48 hours following transfection. Neurons expressing GluN2A-P552R showed significantly reduced viability after exposure to 45 μM TBOA as compared to those expressing the empty vector or wild-type receptor **(A)** (*$p<0.05$, ***$p<0.001$, one-way ANOVA/Tukey post-hoc, n=8). However, this reduction in viability was rescued by co-treatment with 50 μM memantine **(B)** (one-way ANOVA, $p>0.05$) or 10 μM ketamine **(C)** (one-way ANOVA, $p>0.05$). Luciferase values for TBOA treated neurons were normalized to their relevant vehicle-treated vector group. Data represent mean ± SEM from 4-8 independent experiments performed in triplicate or quadruplicate.

4. Discussion

The rise of next-generation and whole exome sequencing (WES) has led to the increasing identification of clinically relevant gene variants and molecular diagnoses in patients with neurological diseases. Indeed, in one large observational study, WES was able to provide a molecular diagnosis for approximately 27% of patients with neurological and developmental disorders (Yang et al., 2014). In a smaller cohort of pediatric neurological patients, WES provided a presumptive diagnosis in 41% of patients. Importantly, the results of WES affected the management of all patients with a presumptive diagnosis in this cohort including, but not limited to, the cessation and initiation of medication (Srivastava et al., 2014). Thus, genetic testing has both a high diagnostic yield and significant implications for personalized clinical management for neurological patients.

The clinical utility of genetic sequencing is especially significant for patients with epileptic encephalopathies. It is estimated that the diagnosis yield for these disorders is 15-20% (EpiPM Consortium, 2015). Intriguingly, *GRIN* mutations make up a high proportion of these disease-linked mutations, and *GRIN2A* mutations, specifically, have been identified as key drivers of epilepsy aphasia spectrum disorders (Carvill et al., 2013; Li et al., 2020; Lemke et al., 2013; Lesca et al., 2013). The identification and *in vitro* characterization of human disease-linked *GRIN* mutations has led to the successful implementation of personalized medicine for several pediatric patients. More specifically,

patients with *GRIN* mutations and intractable epilepsy have been treated with FDA-approved NMDAR blockers, including ketamine, memantine, and dextromethorphan (Table S1), which led to a reduction in seizure burden in the majority of patients treated with these targeted therapies (Xu et al., 2021; Li et al., 2016; Pierson et al., 2014; Amador et al., 2020; Platzer et al., 2017). The results of these *n* of one clinical trials have important clinical implications for patients with *GRIN*-associated epileptic encephalopathies, as refractory epileptic activity in these disorders is thought to contribute to the co-morbid, and often progressive, cognitive impairments (Auvin et al., 2016; Khan and Al Baradie, 2012).

The increasing number of individuals with identified *GRIN* mutations along with the *in vitro* characterization of NMDAR subunit variants has led to an attempt by some to predict clinical phenotypes based upon molecular diagnoses. For example, it has been suggested that *GRIN2A* mutations predominantly lead to an epileptic phenotype while *GRIN2B* variants are more likely to lead to neurodevelopmental disorders (Myers et al., 2019). However, while this may be true in broad strokes, *GRIN2B* has been identified as a causative gene in encephalitic encephalopathies (Epi4K Consortium et al., 2013; Lemke et al., 2014). Moreover, the characterization of *GRIN2B* mutations in two patients with missense mutations at the same amino acid residue revealed a divergence in multiple aspects of both the patient phenotypes and the functional properties of the mutant channels (Kellner et al., 2021), underscoring the complexity of the NMDAR and the need for complete characterizations of *GRIN* variants for targeted therapies. Similarly, characterizing *GRIN* mutations as gain-of-function or loss-of-function based on their location in the protein domain may be overly simplistic and lead to improper pharmacological treatment strategies. While a large cohort analysis of individuals with *GRIN2A* variants identified two phenotypic groups based on whether mutations occurred within the N-terminal or agonist binding domains versus the transmembrane or linker domains, with the former corresponding to loss-of function (LOF) and the latter gain-of-function (GOF) (Strehlow et al., 2019), functional analysis of disease-linked mutations within the pre-M1 linker domain of GluN2A receptors identified both LOF and GOF variants (Ogden et al., 2017). Thus, a full functional characterization of *GRIN* mutations is warranted before the initiation of treatment with channel-blockers.

In this work, we uncover another layer of complexity in the application of precision medicine to *GRIN* mutations: the differential response of a mutant subunit to channel blockers that do not differ in their potency or maximal inhibition of the receptor. Specifically, we found that a missense *GRIN2A* variant resulting in a substitution of arginine for proline at amino acid 552 (P552R) exerts neurotoxicity through two distinct mechanisms – increased sensitivity to excitotoxic insults, which is sensitive to rescue by the channel blockers memantine and ketamine, and pronounced dendrotoxicity that is sensitive to rescue by memantine and MK801, but not ketamine.

Previous characterizations of *GRIN* mutations have found differential responses to channel blockers. For example, treatment with dextromethorphan results in increased inhibition of the NMDA channel as compared to memantine in the NMDA receptor GluN2AN-N615K variant (Marwick et al., 2019). However, memantine binds to the pore by interacting with the amino acids that cluster around the area of this mutation (N612, N613, and N614) (Song et al., 2018), while dextromethorphan is thought to interact with residues in a more extracellular portion of the vestibule (LePage et al., 2005). Thus, increased inhibition of GluN2A-N615K containing receptors by dextromethorphan is consistent with the understanding of its binding site, while differences in binding sites cannot explain the observed differential dendro-protective response of GluN2A-P552R-containing NMDA receptors to memantine and ketamine, as these channel blockers bind to an overlapping site in the pore (Emnett et al., 2013; Zhang et al., 2021). To account for the noted changes, we explored whether memantine

and ketamine differentially activate or inhibit kinases downstream of neurotoxic NMDAR signaling. However, these experiments did not reveal significant differences between treatment groups. Given that memantine has been found to stabilize GluN1/GluN2A receptor desensitization in a calcium-dependent manner (Glasgow et al., 2017), one possible explanation for the discrepancy between memantine and ketamine's ability to rescue GluN2A-P552R mediated dendrotoxicity is that the mutation enhances calcium influx through NMDARs thereby enhancing memantine's potency. However, preliminary experiments showed that the IC50 of memantine in the presence of high and low intracellular calcium did not differ between wild-type and mutant receptors, suggesting this phenomenon does not account for our results (M. Phillips and J. Johnson, personal communication). Thus, the mechanism underlying the differential response of GluN2A-P552R containing NMDARs to memantine and ketamine remains to be elucidated. Nonetheless, our findings are significant in the context of personalized medicine for patients with neurological disorders attributable to *GRIN* mutations as they suggest that in addition to a functional characterization of the mutation, *in vitro* drug screening and investigation of rescue pharmacology may be necessary to identify the most appropriate therapeutic strategies to follow.

5. Data Availability Statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

6. Author Contributions

EA, JRG and GJK conceived the project and designed the experiments. JRG, GJK, and KHS performed the experiments and analyzed the data. JRG and EA wrote the paper. All authors have read and agreed to the published version of the manuscript.

7. Funding

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8. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

9. Acknowledgments

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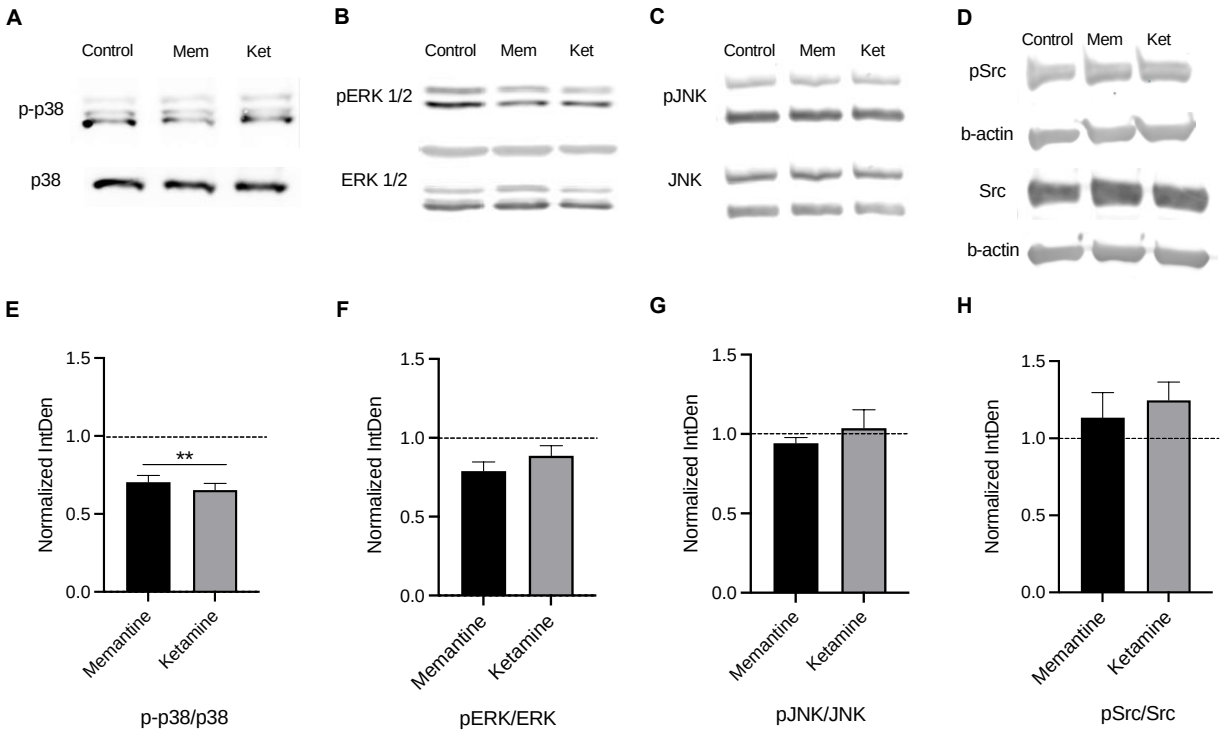
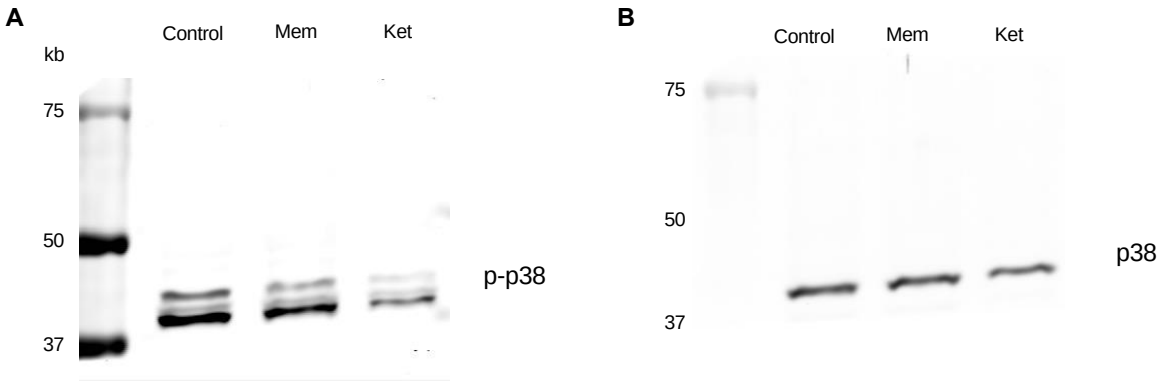
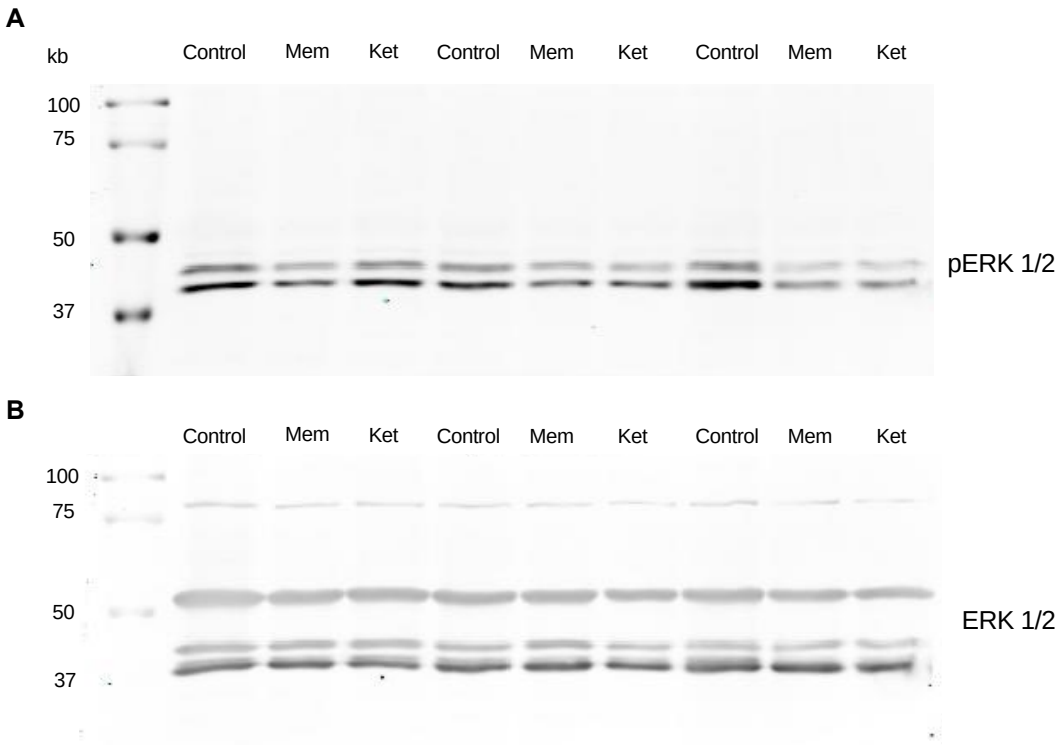
11. Supplementary Material



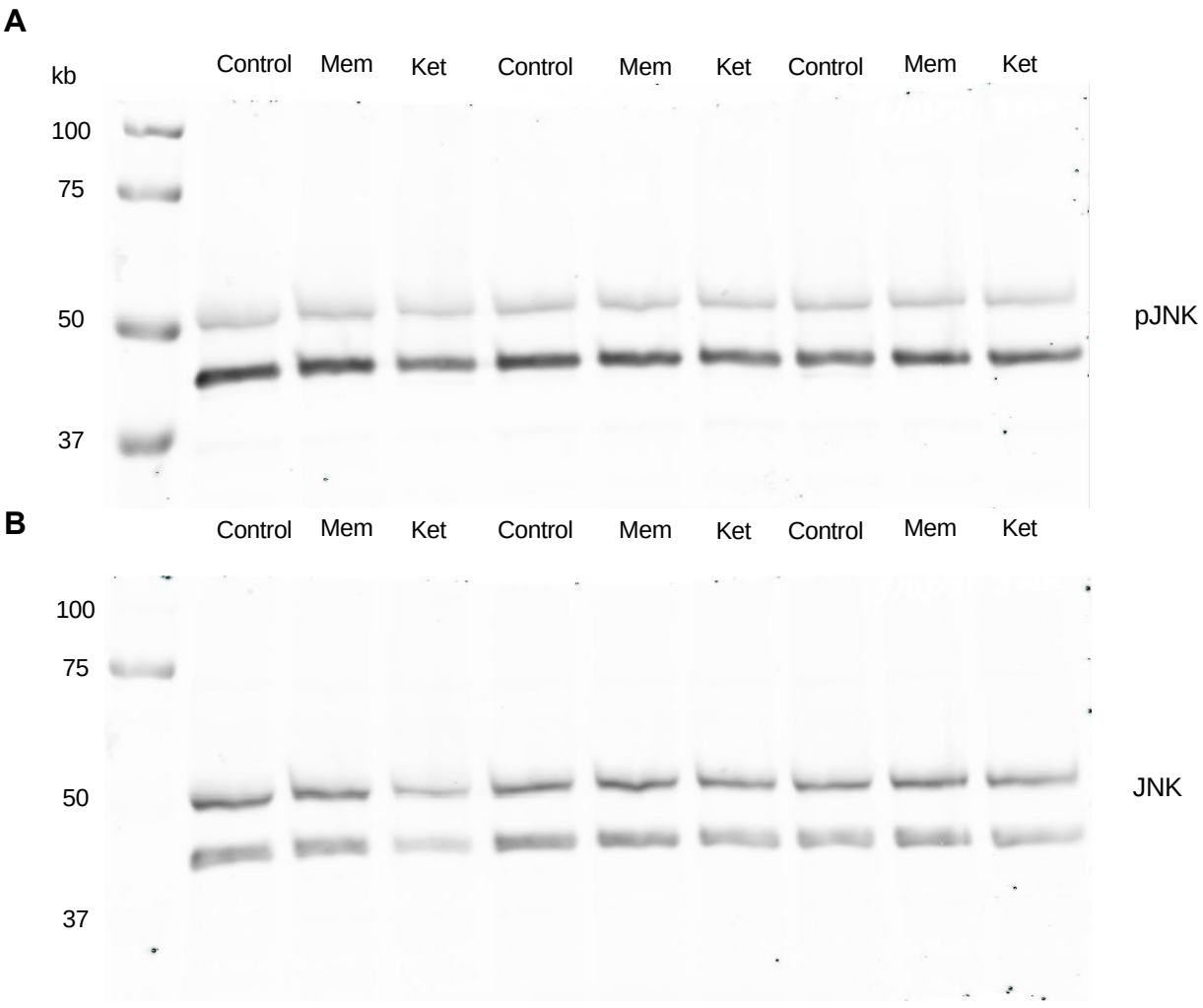
Supplementary Figure 1. Treatment with memantine and ketamine does not cause differential activation or inhibition of kinases in primary cortical neurons. Western blots samples from cortical neurons treated for 45 minutes with either vehicle, 50 μM memantine or 10 μM ketamine **(A-D)**. Quantification of memantine and ketamine treated neurons, calculated as the ratio of phosphorylated p38/p38 **(E)**, phosphorylated pERK 1/2/ERK 1/2 **(F)**, pJNK/JNK **(G)**, and pSrc/Src (after normalization to β-actin) **(H)**, normalized to ratios in vehicle treated neurons. Ketamine treatment significantly reduced p38 phosphorylation as compared to memantine treatment **(E)** (**$p<0.01$, paired t-test, $p=0.0036$), however, no other differences between treatment groups were found **(F-H)** (paired t-test, $p>0.05$). Data represent mean ± SEM from 5-6 experiments with at least 3 separate culture dates.



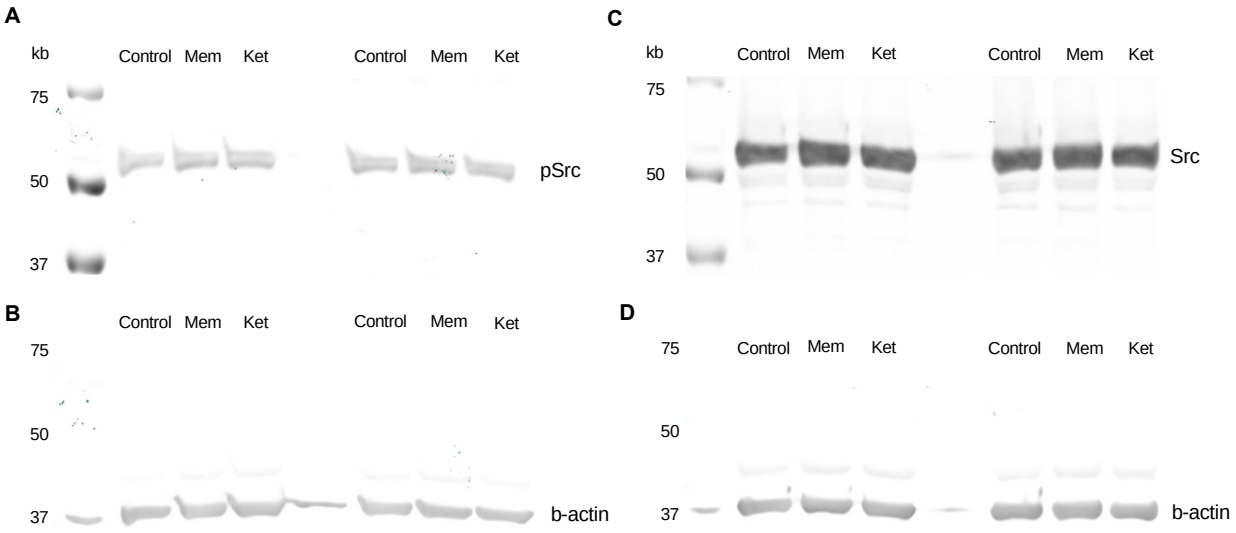
Supplementary Figure 2. Full Western blot of panel A from Supplementary figure 1. Samples from cortical neurons treated for 45 minutes with either vehicle, 50 μM memantine or 10 μM ketamine and probed for phosphorylated p38 **(A)** or p38 **(B)**.



Supplementary Figure 3. Full Western blot of panel B from Supplementary figure 1. Samples from cortical neurons treated for 45 minutes with either vehicle, 50 μM memantine or 10 μM ketamine and probed for phosphorylated ERK 1/2 **(A)** or ERK 1/2 **(B)**. Samples from 3 experiments shown from 2 separate culture dates.



Supplementary Figure 4. Full Western blot of panel B from Supplementary figure 1. Samples from cortical neurons treated for 45 minutes with either vehicle, 50 μM memantine or 10 μM ketamine and probed for phosphorylated JNK **(A)** or JNK **(B)**. Samples from 3 experiments shown from 2 separate culture dates.



Supplementary Figure 5. Full Western blot of panel A from Supplementary figure 1. Samples from cortical neurons treated for 45 minutes with either vehicle, 50 μM memantine or 10 μM ketamine and probed for phosphorylated Src **(A)** and β-actin **(B)** or Src **(C)** and β-actin **(D)**. Samples from 2 experiments shown.

Supplemental Table 1. Summary of patients with *GRIN* mutations treated with NMDAR antagonists.

Gene	Mutation	Patient Phenotype	Treatment	Clinical Response	Citation
GRIN1	p.M64I	Early onset epileptic encephalopathy, severe developmental delay	Memantine (added as an adjunct therapy)	Decrease in frequency and severity of seizures	Xu et al. (2021)
GRIN2D	p.V667I	Intractable epilepsy, global developmental delay, static encephalopathy	Memantine	Decreased seizure burden and improved developmental status	Li et al. (2016)
GRIN2D	p.Vl667I	Epilepsy resistant to standard anti-epileptic therapy, global developmental delay, microcephaly	Memantine, Ketamine and magnesium	No clinical improvement following treatment with memantine, dramatic reduction in seizures following ketamine + magnesium treatment	Li et al. (2016)
GRIN2A	p.L812M	Epileptic encephalopathy, intractable seizures, profound cognitive impairment, absent motor development	Memantine (added as an adjunct therapy)	Decrease in seizure activity, no cognitive improvement	Pierson et al (2014)
GRIN2A	p.S644G	Epileptic encephalopathy, intractable epilepsy, global developmental delay	Memantine followed by addition of dextromethorphan	Significant decrease in seizure burden, no cognitive/developmental improvement	Amador et al. (2020)
GRIN2B	p.N615I	Recurrent epileptic spasms and tonic clonic seizures, severe intellectual disability	Memantine	No improvement in seizure activity but subjective improvement in awareness	Platzer et al. (2017)
GRIN2B	p.V618G	Severe intellectual disability, seizure-free with standard anti-epileptic therapy	Memantine (added as an adjunct therapy)	Subjective improvement in awareness and decreased restlessness	Platzer et al. (2017)
GRIN2B	p.G611V	Severe intellectual disability, generalized seizures	Memantine	No change in seizure frequency but subjective improvement in walking, awareness, social interactions, and sleep pattern	Platzer et al. (2017)
GRIN2B	p.M818T	Developmental delay, focal and generalized seizures	Memantine (added as adjunct)	No change in seizure frequency but subjective improvement in awareness	Platzer et al. (2017)

Appendix B Local Translation in Primary Afferents and Its Contribution to Pain

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Title: Local Translation in Primary Afferents and Its Contribution to Pain

Short title: Local translation

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Abstract

Chronic pain remains a significant problem due to its prevalence, impact, and limited therapeutic options. Progress in addressing chronic pain is dependent on a better understanding of underlying mechanisms. While the available evidence suggests that changes within the central nervous system contribute to the initiation and maintenance of chronic pain, it also suggests that the primary afferent plays a critical role in all phases of the manifestation of chronic pain in the majority of those who suffer. Most notable among the changes in primary afferents is an increase in excitability, or sensitization. A number of mechanisms have been identified that contribute to primary afferent sensitization with evidence for both increases in pro-nociceptive signaling molecules such as voltage-gated sodium channels, as well as decreases in anti-nociceptive signaling molecules such as voltage- or calcium-dependent potassium channels. Furthermore, these changes in signaling molecules appear to reflect changes in gene expression as well as post-translational processing. A mechanism of sensitization that has received far less attention, however, is the local or axonal translation of these signaling molecules. A growing body of evidence indicates that this process is not only dynamically regulated, but also contributes to the initiation and maintenance of chronic pain. Here, we review the biology of local translation in primary afferents and its relevance to pain pathobiology.

1. Introduction

Chronic pain is a complex but common problem. According to the Global Burden of Disease 2016, pain and pain-related diseases are the leading cause of disability and disease worldwide [64]. In 2019, approximately 20.4% (50 million) of individuals in the United States alone suffer from some sort of persistent pain [204] and is estimated that this results in $635 billion in lost productivity and healthcare expenditures [29,166]. A primary cause of this burden is the relative dearth of consistently effective therapeutic interventions for pain devoid of deleterious side effects. While the opioid crisis has highlighted the devastating consequences of some of these side effects, a systematic review of non-opioid therapeutic clinical trials found only modest effect sizes and a high number needed to treat for the majority of these alternative approaches [55].

More effective therapeutic strategies will ultimately depend on a better understanding of the mechanisms that underlie persistent pain and the transition from acute to chronic pain. In this context, the primary afferent has been a focus of research for a number of compelling reasons, not the least of which is evidence that blocking afferent activity is sufficient to eliminate ongoing pain and hypersensitivity for the vast majority of pain patients, including neuropathic pain [78], visceral pain [138], osteoarthritis [167,182], and painful temporomandibular joint (TMJ) disorder [109]. Although changes throughout the central nervous system (CNS) clearly contribute to the manifestation of acute and chronic pain, the majority of these changes appear to depend on aberrant afferent activity [72,74,101] underscoring the importance of primary afferents in the development of chronic pain. An advantage of targeting primary afferents as opposed to the CNS is that peripherally restricted interventions may limit side effect liability associated with CNS-penetrant drugs. However, while effective in the short term, blocking peripheral nerves is not a viable long

term therapeutic strategy given the importance of normal afferent activity to survival. A growing body of evidence suggests that the specific mechanisms underlying aberrant afferent activity depends on a number variables including the type of injury [198], the site of injury [108], previous history [132], time after injury [73,172], sex [13,20,54], age [199], and genetics [41,160,209].

The list of potential mechanisms contributing to the initiation and maintenance of aberrant afferent activity observed in the presence of chronic pain continues to grow. These include a variety of mediators released from cells around peripheral and central terminals of nociceptive afferents, transducers responsible for converting mechanical, thermal, and chemical stimuli into neural activity, and the channels that control neural excitability [69]. Thus, much of the focus of research into the identification of mechanisms underlying chronic pain has been on afferent terminals and changes in the patterns of gene expression detected in the afferent cell body [84,107,108,153,183,186]. However, there is a growing body of evidence to suggest that local translation in the axons of these nociceptors may be effectors of some of these changes [140,148]. As a result, local translation in primary afferents may represent a viable target for alternative therapeutic strategies.

2. Methods

The purpose of this review is to summarize the results of research into the interaction between local and in particular axonal translational and pain. A primary literature search was conducted on PubMed using the search query: ((“pain”[Title/Abstract] OR “injury”[Title/Abstract] AND “axonal translation”[Title/Abstract] OR “local translation”[Title/Abstract”]. Titles and abstracts were used to screen for relevant studies. The results were then filtered manually to include studies that met at least two of the following criteria in terms of focus or purpose: 1) translation

mechanisms, 2) translation in the peripheral nervous system at site(s) distant from the cell body, 3) axonal translation in nerve injury, 4) local/axonal translation in the maintenance of pain. Studies identified using this methodology were also evaluated to determine whether they directly or indirectly investigated axonal protein synthesis. In addition, articles familiar to the authors were reviewed for relevance and included. These sources were then organized into the four sections of this review: Axonal Protein Synthesis, Initiation of Local Translation, Second Messenger Systems, and Pain Effector Molecules in Local Translation. A secondary search was conducted using each of these sections as the key words to ensure a thorough investigation of each topic. References from each primary or review source were assessed to identify additional studies missed in original searches. Reviews were omitted when possible. Sources were confined to the last 15 years, unless used for primary/historical context.

3. Axonal Protein Synthesis

Despite initial skepticism, it is now well established that most axons contain a diverse population of mRNAs for local translation that occurs within the axonal compartment [38,76,164,181]. The capacity for axonal translation appears to be one that is evolutionarily conserved as mRNA encoding protein synthesis machinery is detected in axons of species ranging from invertebrates, such as *Aplysia*, to vertebrate, such as rats [97,134], highlighting the importance of this biological process. Genome-wide microarray profiling of RNA isolated from a variety of *in vitro* culture systems, including but not limited to *Xenopus* retinal ganglion cells, rat dorsal root ganglia (DRG), and rat cortical neurons, confirmed that these neuronal compartments contain transcripts encoding protein machinery such as ribosomal proteins, translation initiation factors, and translation elongation factors [76,169,208]. Importantly, these mRNAs have been shown to be axonally translated [97] and provide the necessary protein synthetic machinery for the local translation of

the wide repertoire of mRNAs detected in these subcellular compartments [6,76,162,169,208].

Local translation of mRNA provides several advantages over protein trafficking from the cell body. First, translationally silent mRNAs can be stored locally in ribonucleoprotein (RNP granules) structures that can undergo translation in response to local stimulation, thus providing tight spatio-temporal control of local protein pools [4,58,157,175]. Consistent with this notion, axonal translation of localized mRNAs occurs in response to neuronal activity and extrinsic stimuli [21,106,111,188,201]. For example, growth cone turning in response to guidance cues, such as brain-derived neurotrophic factor (BDNF) and netrin-1, requires the localization of β-actin mRNA to distal axons and its local translation ([14,158,187,196]. Second, since axonal trafficking is an ATP-dependent process [103], local translation is more energetically efficient than the trafficking of protein from the cell body to axons. This is especially true for neurons with long axons that provide innervation to the distal appendages some of which can span a meter in length in humans. RNAs can also reach the distal portion of long axons much more quickly than many proteins and are trafficked at rates of approximately 50-400 mm/day (fast axonal transport) while a large population of proteins are transported at rates of approximately 0.2-10 mm/day (slow axonal transport) [154] and this disparity is likely explained by a reliance on different motor proteins [75]. As the sciatic nerve can exceed 1 meter in humans, these differences in transport rate and transport related-ATP consumption are of particular significance in the peripheral nervous system as compared to the CNS, where the average length of cortical axons is approximately 8 mm [65]. One can appreciate the significant temporal advantage that local translation provides when considering the example of the hind limb axons of rodents. Assuming the hind limb axons of mice may be up to 40 mm in length and those of rats may be up to 80 mm, fast axonal transport of mRNA from the DRG to these distal axons may take somewhere between 144 minutes (approximately 2.5 hours)

and 19 hours in the mouse and between 288 minutes (almost 5 hours in the rat) and 1.6 days. The trafficking of many proteins from the soma to these sites is estimated to be at least 5-fold slower. Therefore, the ability to quickly respond to extrinsic stimuli is greatly enhanced by the trafficking of mRNA and the translation of localized mRNA.

Finally, the 3' untranslated region (3'UTR) of mRNA often contains information lost in its protein form such as localization signals, translation signals, and microRNA binding sites, which function in RNA silencing [3,7,18,44,111,176]. Thus, 3'UTRs provide another layer of translation regulation. Intriguingly, neuron-enriched transcripts tend to have longer 3'UTRs and transcripts localized to neuronal processes, such as axons and dendrites, have longer 3'UTRs than those localized to the cell body [133,176]. Several alternative, elongated 3'UTR isoforms have been found to drive axonal localization and these are also enriched in microRNA binding sites [79,82,176]. Together these data suggest that the additional level of control over localization and translation of mRNA provided by 3'UTRs is particularly important to the axon function. It is also important to note that while only a small percentage of total mRNA is locally translated (approximately 5% in sensory axons), local translation appears to be critical for axon outgrowth and guidance during development [15], and maintenance and regeneration in adults [45,174,200]. Inhibition of local protein synthesis results in reduced axonal growth and loss of axonal viability [81,97]. Thus, local translation of axonal localized mRNA supports the proper function of these subcellular compartments.

4. Initiation of Local Translation

4.1. Nerve Growth Factor (NGF)

Nerve Growth Factor (NGF) is critical for the survival of subpopulations of primary afferent neurons during development, and the maintenance of the phenotype of subpopulations of afferents in the adult. However, NGF is also elevated in response to tissue injury where it will sensitize this subpopulation of afferents and therefore contribute to pain associated with injury. Indeed, NGF injected into healthy human skin can cause localized pain and hyperalgesia that develops within minutes but can last for hours to days [145]. The spatially restricted nature of this hyperalgesia suggests a peripheral rather than central mechanism of this sensitization.

NGF binds the TrkA receptor, which is found in peptidergic C fibers and Aδ fibers, sensory neurons that express the neuropeptide calcitonin gene-related peptide (CGRP) [9]. TrkA receptor activation results in the initiation of numerous different intracellular signaling cascades including at least two implicated in local translation [8,59,96]. Neuronal survival signals elicited by NGF induced TrkA receptor activation are dependent on axonally synthesized protein [35]. These same proteins have been implicated in the modulation and maintenance of pain, and therefore are thought to contribute to some of the longer lasting effects of NGF [99,113,195,205], which may be generated via local translation. Supporting the supposition that local protein translation mediates NGF- induced mechanical hypersensitivity is the finding that co-injection of NGF with a general translation inhibitor attenuates the mechanical hypersensitivity observed with intraplantar injections of NGF alone [125].

In addition to the role of NGF in promoting the local translation of downstream signaling molecules, local translation of TrkA, may also contribute to persistent pain. For example, in a rat model of trinitrobenzene sulfonic acid (TNBS)-induced colitis, there is an increase in the retrograde transport of the TrkA protein from the colon and commensurate increase in TrkA protein in the colonic afferent cell bodies. In contrast, there is an increase in the anterograde

transport of TkrA mRNA in colonic afferent axons [150]. This suggests that local translation of TrkA contributes to persistent effects of elevated NGF observed at sites of inflammation.

4.2. Other signaling molecules

Several other trophic factors have been implicated in the initiation and/or maintenance of chronic pain in addition to NGF. These include Brain Derived Tropic Factor (BDNF) and Neurotrophin-3 (NT3). A role for BDNF in pain associated with tissue injury has been most extensively characterized in the context of traumatic nerve injury, where it has been shown to mediate the decrease in inhibition observed in the spinal cord dorsal horn [32,39]. However, there is evidence for BDNF signaling in the periphery that contributes to inflammatory hypersensitivity [203]. TrkB is the high affinity receptor for BDNF, and similar to NGF, BDNF-induced activation of TrkB results in the initiation of signaling cascades shown to underlie local translation [112,168]. While the most compelling evidence in support of BDNF-induced local translation comes from CNS neurons, the fact that BDNF contributes to the sensitization of nociceptive afferents suggests that local translation in peripheral axons may contribute to the persistent pro-nociceptive effects associated with BDNF.

The high affinity receptor for NT3 is TrkC, which is primarily expressed in non-nociceptive sensory neurons. In contrast to NGF and BDNF, NT3 appears to attenuate nociceptive signaling. For example, in the chronic constriction injury (CCI) model of trigeminal nerve injury, NT3 induces a significant and long-lasting decrease in injury-induced heat hyperalgesia [46]. In the CCI model of sciatic nerve injury, NT3 significantly reduced Nav1.8 and Nav1.9 mRNA levels and attenuated neuronal upregulation of Nav1.8 and Nav1.9 protein at the site of injury [189]. Additional experiments are needed to confirm whether suppression of local translation contributes

to the anti-nociceptive effects of NT3. However, NT3 signaling has been shown to redistribute RNA granules to neurites in cortical neurons [104] and stimulates the phosphorylation of translational initiation machinery in oligodendrocytes [27] suggesting a link between NT3 signaling and the regulation of local translation.

In addition to trophic factors, several inflammatory mediators likely contribute to the initiation and/or maintenance of chronic pain via local translation. IL-6 is one of the most extensively studied of these pro-inflammatory molecules in the context of local translation, and the data suggests these signaling cascades parallel those activated by NGF [127] Indeed, there is evidence of the retrograde trafficking of locally translated proteins in the sciatic nerve within two hours after an injection of IL-6 into the mouse hindpaw [127].

Indirect evidence in support of a role for local translation contributing to the actions of a variety of other signaling molecules comes from the characterization of a phenomenon referred to as hyperalgesic priming, that is suggested to underlie the transition from acute to chronic pain. Hyperalgesic priming involves an initiating stimulus which appears to change nociceptive afferents such that the duration of the response to a subsequent challenge vastly outlasts the duration of a same challenge applied to naïve tissue [2,93,143]. For example, an intradermal injection of the inflammatory mediator prostaglandin E2 (PGE2) results in hypersensitivity that lasts for 45 to 60 minutes in naïve animals, but 24 to 36 hours in a primed animal [2,143]. While there is evidence that changes in CNS circuitry may contribute to the primed state [95], the bulk of evidence points to a long last plasticity in subpopulations of nociceptive afferents where the specific subpopulation of afferents involved depends on the nature of the priming stimulus. Intriguingly, local protein synthesis has been implicated in the establishment of hyperalgesic priming [50,51] and local administration of protein synthesis inhibitors attenuates priming-induced

hyperalgesia [51]. Of note, hyperalgesic priming may involve changes at the central as well as peripheral terminals, both of which appear to be dependent on local protein synthesis [52]. Importantly, evidence that local injection of protein synthesis inhibitors can reverse hyperalgesic priming [50] suggests local protein synthesis is not only required for the initiation of the primed state, but for its maintenance. Thus, local translation may play a critical role in the transition from acute to chronic pain. Notably, there is evidence of sex differences in the mechanisms underlying hyperalgesic priming [93], which raises the possibility that the population of locally translated proteins generated differs between sexes and, consequently, the need to consider sex when developing therapeutic interventions targeting this potential mechanism of chronic pain.

4.3 Support cells and local translation

A growing body of evidence suggests that Schwann cells may support axonal protein synthesis. Studies suggests Schwann cells have the capacity to transfer ribosomes and mRNA to the sciatic nerve after injury [22,33], and both appear to be transferred via exosomal transport [22,33,34,119,151]. Additionally, Schwann cells may provide energy substrates to support ATP production in distal axons [90,164]. Intriguingly, sensory neuron axons associate with Schwann cell-secreted exosomes in compartmentalized cultures and Schwann cell-derived exosomes promote axonal regeneration both *in vitro* and *in vivo* [117]. Highlighting the potential relevance of these exosomes to local translation is the finding that in compartmentalized cell cultures, Schwann cell derived exosome promote axonal regeneration when added to the axonal compartment but not when added to the cell body compartment [117]. Although the link between Schwann cells and local translation is still tenuous, given the presence of the machinery needed for local translation in peripheral axons, and evidence that mRNA is packaged in exosomes, it is

at least reasonable to suggest that other cells may help guide the rapid response to injury by serving as a reservoir of mRNA.

5. Second Messenger Systems in Local Translation

5.1. ERK and PI3K

Of the many signaling cascades initiated by Trk- and/or cytokine receptor activation, at least two have been implicated in local translation. One of these is phosphoinositide 3-Kinase (PI3K), a lipid kinase responsible for the phosphorylation of a number of downstream molecules involved in protein synthesis, including AKT [25,36,80,102,116]. The PI3K/AKT pathway is essential to the development and maintenance of chronic pain (for full review, see [25]). Spontaneous nociceptive behaviors induced by capsaicin and NGF injections are attenuated after intradermal injections of PI3K inhibitors wortmannin or LY294002 [193,207]. In the distal axon, this signaling pathway promotes retrograde transport of BDNF and NGF [12,67], whereas inhibition of PI3K has been shown to attenuate NGF- and IL-6-induced local translation [48,121].

The second signaling cascade implicated in local translation involves the activation of extracellular signal-regulated kinase (ERK). ERK was first implicated in nociceptive processing in the spinal cord dorsal horn, where it was demonstrated that noxious, but not non-noxious stimuli resulted in an intensity and NMDA receptor-dependent increase in the phosphorylation of ERK (pERK). More importantly, inhibition of pERK attenuated the second phase of the formalin response, which is thought to involve spinal neuron sensitization [91]. Subsequent studies showed that pERK is detectable in nociceptive afferents, where it contributes to nociceptor sensitization [37]. The rapid sensitization of nociceptive afferents was initially shown to be due to the phosphorylation of

nociceptive signaling molecules such as TRPV1 [207]. More recently, there is evidence to suggest ERK-dependent nociceptor sensitization may also be due to local translation. ERK phosphorylates several down-stream targets implicated in local translation [120,147]. Similar to PI3K, inhibition of ERK blocks local translation as well as NGF- [8,121] and IL-6- [42,43,122] induced hypersensitivity and hyperalgesic priming.

5.2 mTOR signaling

mTOR is a kinase downstream of PI3K activation. While it is involved in a variety of cellular functions including autophagy, metabolism, and transcription, its role in mRNA translation is well established [57,114,184]. For example, mTOR, as well as its downstream targets 4EBP1, S6K, and S6, are extensively expressed in a subset of myelinated primary afferent sensory fibers in the glabrous and adjacent hairy skin of the rat hindpaw [92]. 4EBP1, S6K, and S6 are involved in protein synthesis through their association with eukaryotic initiation factors and ribosomes, and constitute critical components of the local translation machinery [92]. This machinery is not only present, but functional since downstream targets for these kinases were largely phosphorylated in distal axons [66,92] and phosphorylation is blocked by intraplantar injection of rapamycin, an inhibitor of mTOR and translation [92]. The presence of translational machinery in myelinated sensory fibers does not necessarily imply that local protein translation may have a role in nociception, as it is also possible that local protein synthesis maintains the health and viability of these axons [81]. However, further investigation into this population of A-fibers demonstrated that the inhibition of protein synthesis *in vivo* increased mechanical thresholds and reduced mechanical hypersensitivity. In two rodent models of pain, capsaicin-induced secondary hyperalgesia and spared nerve injury (SNI)-induced neuropathic pain, rapamycin treatment increased mechanical thresholds in the von Frey filament and pinprick tests [66,92]. Furthermore, intrathecal

administration of rapamycin blocked mTORC1 activity in dorsal roots but not dorsal root ganglion (DRG) suggesting the effect of translation on the modulation of pain behavior occurred at the level of axons rather than the soma [66]. Nevertheless, it is important to note that while markers of mTORC1 activation were measured, resulting axonal translation was not directly demonstrated in this study.

As alluded to above, data demonstrating altered nociception in *FMR1* knockout (KO) mice supports the supposition that mTOR-mediated alterations in pain behavior are a result of the modulation of axonal translation [149]. *FMR1* encodes Fragile X mental retardation protein (FMRP), an RNA-binding protein (RBP) that has an important role in the negative regulation of local translation at synapses. Large repeat expansions in the *FMR1* gene, which encodes the FMRP protein, leads to transcriptional silencing of the gene and is a genetic cause of Fragile X syndrome (FXS), an intellectual disability that is also associated with number of co-morbid neuropsychiatric illnesses [63,190]. Although the role of mTOR signaling in FXS is not completely understood, a number of studies have identified mechanisms through which FMRP regulates mTOR signaling and vice versa [190]. Intriguingly, *FMR1* KO mice show decreased responses to ongoing nociception as evidenced by a reduction in pain-related behaviors in the second phase of the formalin test [149]. Intrathecal injections of the mTOR inhibitor rapamycin decreased second phase pain-related behaviors in wild-type (WT) mice in a dose-dependent manner without affecting the behavior of *FMR1* KO mice in response to formalin [149]. These results suggest that the mTOR pathway and localized protein synthesis may mediate formalin-induced nociception. This supposition is supported by evidence that formalin-induced neuronal hyperexcitability and second phase nociceptive behavior are mediated by rapid protein synthesis at the spinal level: these responses are attenuated by rapamycin [5], which may be due to inhibition of mRNA translation

secondary to the inhibition of mTOR signaling [10]. Additional evidence supporting the importance of axonal protein synthesis in mediating these effects includes the finding that *FMR1* KO mice exhibit a 3-week delay in the development spared nerve injury (SNI)-induced mechanical hyperalgesia [149]. This suggests that proteins involved in mediating SNI-induced mechanical hyperalgesia are axonally synthesized in WT mice but need to be trafficked from the cell body into nociceptive axons in *FMR1* KO mice [149], based on estimates of transport times for mRNA and proteins summarized above. Together, these data support the idea that translational regulation via mTOR and FMRP are an important feature of nociceptive plasticity.

5.3. Eukaryotic Initiation Factor

Eukaryotic Initiation Factor Complex (eIF4F) is a downstream target of mTOR signaling. It is comprised of eIF4E, which binds to the 5' cap protein, eIF4A, which unwinds the 5' UTR of mRNAs to expose its translation initiation sites, and eIF4G, a scaffolding protein that binds eIF4A and eIF4E [1,130]. When assembled, this complex promotes eukaryotic translation initiation and protein synthesis in primary sensory neurons and their axons [125,165]. mTORC1 indirectly affects the assembly of this complex via the phosphorylation of the 4E-binding proteins (4E-BP). These inhibitory proteins actively suppress the eIF4F complex assembly by tonically binding eIF4E which prevents its interaction with eIF4G. However, activation of mTORC1 phosphorylates 4E-BPs, causing dissociation from eIF4E and thus enabling complex assembly and translational initiation.

There is an abundance of evidence supporting the role of the eIF4F complex in regulating persistent pain states [100]. Prevention of eIF4E phosphorylation at Serine 209 abolished an increase in excitability of DRG neurons, while also mitigating mechanical and thermal

hypersensitivity, affective pain expression, and hyperalgesic priming induced by IL-6, NGF, and protease-activated receptor 2 (PAR2) [56,135]. This phosphorylation is dependent on MNK1/2 signaling, as inhibition of MNK1/2 recapitulates these behavioral and electrophysiological effects [135]. Suppression of MNK1/2 signaling was also shown to alleviate mechanical hypersensitivity in the setting of inflammatory pain [125,135]. Most relevantly, local inhibition of eIF4E phosphorylation prevents NGF- and IL-6-induced hypersensitivity [135]. Together, these data suggest MNKK1/2 and eIF4E underlie local translation driven by trophic factors and cytokines which subsequently contributes to persistent pain states and hyperalgesic priming.

6. Pain Effector Molecules in Local Translation

6.1. GAP43 and nerve regeneration

Growth-associated protein 43 (GAP-43) is a membrane protein that plays a major role in axonal pathfinding and branching for the developing nervous system. In the adult peripheral nervous system, GAP-43 is constitutively downregulated, but is upregulated following nerve injury to promote axonal sprouting [16,68,83,191]. Furthermore, intrathecal injection of IL-6 and NGF upregulates the expression of GAP-43 through the PI3K/AKT/mTOR signaling pathways [194]. Importantly, GAP-43 protein can be axonally synthesized and its local translation promotes axonal elongation and regeneration after injury [44,45]. While it is clear that GAP-43 plays a critical role in nerve regeneration following injury, there is conflicting evidence exists surrounding its role in nociceptive signaling where the interpretation of the results associated with manipulations of GAP-43 on nociceptive behavior have been confounded by the potential impact of these manipulations on glial cells as well as regenerating axons.

More compelling evidence exists for a pain-related role for GAP-43 in humans. GAP-43 is

detected in cutaneous NGF+ and CGRP+ nerve fibers [49,179]. In patients with diabetes, GAP-43 expression levels have not only been shown to increase [19,61,62,197], but there appears to be a correlation between the level of GAP-43 expression and pain, and this correlation is independent of fiber loss [19,61,62]. Similar results were reported in patients with chronic pancreatitis [40].

6.2. CREB

As discussed above, both NGF and IL-6 are important signaling molecules in the initiation of local translation. One interesting point of convergence between IL-6 and NGF-mediated protein translation is their effect on axonal translation of cyclic AMP response element binding protein (CREB), a transcription factor implicated in the modulation and maintenance of pain [113,195,205]. It was previously established that CREB mRNA is present is distal axons of sensory neurons and that NGF stimulates its local translation [35]. Importantly, when distal axons were severed from their respective cell bodies, *in vitro*, the presence of axonal CREB protein was dependent on both NGF and translation demonstrating that CREB mRNA is locally translated in NGF-dependent manner. Moreover, this axonally synthesized CREB was retrogradely trafficked to the soma and was also found to be present in its phosphorylated form in the nucleus [35]. Retrogradely trafficked CREB was also found to be colocalized with TrkA-signaling endosomes following NGF application [35], which is significant because TrkA-containing signaling endosomes mediate the activation of a kinase that is required for phosphorylation of CREB in response to axonal application of neurotrophins [185]. Intriguingly, axonal CREB was able to induce cyclic response element (CRE)-dependent transcription [35] raising the possibility that axonally translated CREB could induce transcription of pain-related genes in response to noxious stimuli. Supporting this possibility, more recent studies demonstrate that local translation and retrograde transport of CREB contributes to IL-6-induced nociceptor plasticity. One study

demonstrated that intraplantar injection of IL-6 in mice resulted in the detection of CREB protein in the sciatic nerve within 2 hours. Click chemistry was used to confirm that the increased CREB protein detected in the sciatic nerve was nascently synthesized at the site of injection. Significantly, disruption of retrograde transport of CREB protein with microtubule disrupting agents prevented IL-6 mediated mechanical sensitivity and hyperalgesic priming and inhibited IL-6 mediated BDNF upregulation in the DRG [127], which was previously shown to be an important component of nociceptor sensitization [126]. The use of a cAMP response element (CRE) consensus sequence as an oligonucleotide decoy to disrupt CREB's action as a transcription factor, prevented IL-6 mediated mechanical hypersensitivity and hyperalgesic priming as well as IL-6 mediated increases in BDNF expression [126,127]. Together, these data demonstrate the intimate link between CREB's local translation and retrograde trafficking, its role as a transcription factor, and IL-6-induced plasticity. It is tempting to speculate that local translation of CREB, or other yet to be identified proteins, may effect transcriptional changes in DRGs that are important for the maintenance of persistent pain, especially since retrograde signaling effects transcriptional changes in the soma [131]. Support for this comes from research in regenerating sensory neurons. After nerve crush injury, an axonally localized *Importin β1* mRNA isoform, a classical nuclear import receptor, is upregulated and locally translated. Importantly, this locally translated Importin β1 associates with the motor protein dynein to coordinate the trafficking of an injury-signaling complex containing transcription factors that are axonally translated and retrogradely trafficked to the nucleus [17,77] Conditional Cre/lox mediated knockout of the axonally localizing element of Importin β1 mRNA attenuates the transcriptional response to nerve injury detected at the cell body and delays recovery indicating that local translation of this transcript and its retrograde signaling, and presumably nuclear translocation, is critical for functional plasticity of the sensory neuron

after injury [144].

6.3. CGRP

In addition to the local translation and retrograde transport of transcription factors such as CREB, there is also evidence that pain-related proteins are locally synthesized. Calcitonin gene-related peptide (CGRP) is a protein widely expressed in central and peripheral nociceptive pathways where it plays a role in orchestrating the response to tissue injury and infection [26,88,110], and is involved in migraine pathophysiology, as well as somatic, visceral, neuropathic, and inflammatory pain [159]. CGRP is also hypothesized to play a key role in peripheral sensitization [87]. Interestingly, CGRP mRNA and protein levels increase in regenerating sensory axons after peripheral nerve injury both in cultured DRG and intact nerves [174]. αCGRP mRNA is also concentrated at growth cones and its local translation may play a role in nerve regeneration [174]. Indeed, CGRP was found to be locally translated in peripheral sensory nerve axons and knockdown of CGRP mRNA by local administration of siRNA reduced the capacity for nerve regeneration [174]. The local translation of CGRP may also have relevance to nociceptive signaling [141,142,174].

6.4. Voltage-gated sodium channels (VGSCs)

Elevated protein and mRNA levels of several ion channels, including Nav1.8, have been detected in peripheral sensory axons after injury [70,156,171]. Increased Nav1.8 resurgent currents are thought to contribute neuronal hyperexcitability and hypersensitivity associated with peripheral neuropathies [192] and Nav1.8 expression is suggested to be necessary for the expression of spontaneous, inflammatory, and neuropathic pain after injury [70,94,155]. Thus, the increased axonal levels of Nav1.8 mRNA and subsequent locally synthesized protein is highly relevant to

multiple types of persistent pain. As soma levels of Nav1.8 are reduced in some models of neuropathic pain [70,163], one interpretation of these findings is that pre-formed Nav1.8 protein is redistributed to axons following injury rather than being locally translated [70,139]. However, the upregulation of axonal Nav1.8 mRNA following nerve injury has been replicated by several investigators [28,82,171], and suggests that increased axonal Nav1.8 protein is due to local translation. Moreover, an alternative, elongated Nav1.8 3'UTR has been recently identified in sensory axons following sciatic nerve entrapment [82]. As mRNAs with extended 3'UTR have been shown to preferentially localize to axons [79,176] and distinct 3'UTR isoforms can differentially mediate activity dependent translation of mRNA in neurons [111], it is possible that this alternative Nav1.8 mRNA isoform is preferentially localized and axonally translated in states of hyperexcitability like those seen in neuropathic pain.

While Nav1.8 mRNA and protein is consistently found to be upregulated in the axon following peripheral nerve injury [82,156,171], it is highly likely that other VGSCs are axonally translated following injury and contribute to the initiation and/or maintenance of persistent pain states. For example, following infraorbital nerve entrapment (IoNE), an animal model of trigeminal neuralgia, there is a significant increase in Nav1.3, Nav1.7, and Nav1.8 mRNA in the infraorbital nerve of the IoNE group as compared to the sham group [137]. Conversely, in the trigeminal ganglion (TG) of IoNE animals, there was a reduction in Nav1.1, Nav1.6, and Nav1.8 mRNA. That the pattern of changes local Nav subunit translation may be injury specific is suggested by our recent observation that Nav1.1, but not Nav1.3, Nav1.7, or Nav1.8 is increased in the infraorbital nerve following chronic constriction injury [146]. Together, these observations suggest that there is a redistribution of specific Nav subtype mRNA from TG to peripheral axons following injury. It is important to note that axons contain the necessary secretory machinery, such

as protein components of the signal recognition particle, endoplasmic reticulum, and Golgi apparatus, to traffic axonally translated VGSCs to the plasma membrane of axons [71,118,128,129,180]. Consistent with these data are the observations that nerve entrapment [137] and constriction injury [146] result in changes in the sensitivity of action potential propagation in the infraorbital nerve to Nav subtype selective blockers. Importantly, local pharmacological block of Nav1.1 at the site of the constriction injury of the infraorbital nerve attenuated injury-induced mechanical hypersensitivity, suggesting that these axonally translated proteins are not only functional but may serve as therapeutic targets for the treatment of pain.

6.5 MMP-9

Matrix metalloproteinase-9 (MMP-9) has been implicated in pain and inflammation [177]. Although it has traditionally been thought to be secreted in its pro-, or inactive form, and subsequently operate in the extracellular space to cleave proteins such as growth factors, cell surface receptors, and cell adhesion molecules, a growing body of literature supports its role in the intracellular space as well [30,177,178]. Of note, MMP-9 has emerged as an important molecule in the control of synaptic plasticity. In the CNS, MMP-9 mRNA is trafficked into dendrites and locally translated in an activity dependent manner [47,89,105,210]. Although there is no direct evidence for local translation of MMP-9 in the peripheral nervous system, MMP-9 mRNA has found to be upregulated in the sciatic nerve following multiple models of sciatic nerve injury [24,82], suggesting it could be axonally translated and locally secreted following injury, where it could contribute to pain via the cleavage of several targets including myelin basic protein [24,115]. It is likely that in addition to the cleavage of proteins directly involved in the control of nociceptor excitability, MMP-9 contributes to injury-induced pain via indirect actions. For example, there is evidence that MMP-9 induced cleavage of cytokines, such as TNF-α and IL1-β, as well as

upregulation of chemokines are important components of MMP-9 mediated neuropathic pain [98,206]. Further supporting this point is the finding that intrathecal injections of an MMP-9 targeting antibody not only decreases mechanical allodynia associated with chemotherapy induced neuropathy but downregulates inflammatory mediators such as IL-6 and TNF-α in DRG neurons [173] suggesting that these cytokines may be the pain effector molecules.

7. Conclusions and Future Directions

A general model of the steps associated with local translation in primary afferents is illustrated in Figure 1. As highlighted throughout this article, while the phenomenon of local translation is well-established, much of the evidence in support of its role in chronic pain is indirect. A variety of tools are now available, however, to visualize the trafficking of mRNA and translation machinery, as well as local changes in protein levels. Thus, in the immediate future, many of the steps involved in local translation can and should be directly demonstrated in the context of tissue injury and pain.

In the context of future directions and potential therapeutic implications of local translation for the treatment of pain, there are critical questions to be answered with respect to each step of the process outlined in Figure 1. For example, while there is abundant evidence that neural activity is sufficient to trigger local translation [47,53,111,175], less is known about the link between injury-induced patterns of activity and the patterns of local proteins translated, let alone the site(s) throughout the neuron where local translation occurs. Similarly, while we highlighted several of the mechanisms implicated in the initiation of local translation, this is likely to involve more than a focal increase in NGF or IL6. That said, while focal increases in pro-nociceptive mediators may explain why local translation occurs at one point in a neuron, but not another, little is known about the

mechanisms controlling site(s) of local translation in response to injury. Recent evidence that organelles called ribosome associated vesicles (RAVs), a structure that may prove to be critical for local translation, are transported along microtubules [23], suggests a mechanism for local translation at the site of nerve injury. That is, if the nerve injury is associated with microtubule disruption, RAVs will necessarily cluster at the site of damage.

The trafficking and release of mRNA in RNA binding proteins (RPBs) would provide another mechanism influence site(s) of local translation. It remains to be determined which RBPs transport pain-related mRNAs to axons let alone whether they differ from those that regulate the transport of mRNAs that are critical in the regeneration of axons following injury. For example, the RBP zipcode-binding protein 1 (ZBP1) transports both GAP-43 and β-actin mRNA to axons to support axonal outgrowth [44,45] and the RBP nucleolin forms transports mTOR mRNA to sensory axons where it is locally translated following nerve injury [170]. However, whether these RBPs are also implicated in the axonal localization of pain-related mRNAs remains to be demonstrated. Additionally, determining whether RBPs coordinate and transport pain-related mRNAs in the same RNA granule, akin to how the RBP splicing factor proline and glutamine rich (SFPQ) orchestrates and transports an RNA regulon that includes mRNAs whose local translation is critical for the viability of sensory axons [31,60], could be of clinical significance as it could provide an opportunity to target the axonal translation of multiple pain-related mRNAs at once. If a common RNA binding motif is identified in multiple mRNAs that are locally translated in the context of chronic pain, therapeutics such as antisense oligonucleotides (ASOs) could be developed to prevent the trafficking of these mRNAs to distal axons. Supporting this approach is the finding that competitively inhibiting the RBP poly(A) binding protein (PABP), a protein important in RNA

stability, reduces axonal protein synthesis *in vitro* and injection of the competitive oligonucleotide into the hindpaw of mice attenuates pain in multiple models of inflammatory pain including NGF- and IL6- induced hyperalgesic priming [11].

If the emergence of ectopic activity generation observed at sites proximal to that of nerve injury [101,172] not only contribute to neuropathic pain but are due to local translation, it will be critical to identify mechanisms controlling the site(s) or local translation to target this mechanism therapeutically. However, if the site of local translation contributing to pain associated with injury is known, targeted application of inhibitors of steps critical for local translation may provide pain relief with minimal side effects. Indeed peripheral administration of translational inhibitors has been shown to reduce pain related symptoms in animal models of chronic pain [50,123] and metformin, an AMPK activator, which acts upstream of mTOR, corrects abnormal mRNA translation and reverses enhanced neuronal excitability and behavioral hypersensitivity in several models of chronic pain [85,86,202]. This finding is particularly significant as metformin is already FDA approved for the treatment of type 2 diabetes and could potentially be repurposed for the treatment of chronic pain or alleviate pain associated with diabetic peripheral neuropathy.

Based on a strategy analogous to that proposed for the therapeutic efficacy of metformin, eFT508 is another molecule that should provide pain relief via the block of local translation. eFT508 is an inhibitor that selectively blocks MNK-mediated phosphorylation of the translation initiation factor eIF4E [152]. As phosphorylation of 4E-BP by mTOR relieves inhibition of eIF4E, eFT508 can inhibit translation of molecules downstream of mTOR activation. The MNK-eIF4E signaling axis has already been established as an important player in the development of chronic pain [135,136]

and systemic administration of eFT508 has been shown to alleviate neuropathic pain in rodent models [124,161]. Excitingly, eFT508 is currently in phase II clinical trials for the treatment of solid tumors. If approved, this drug could potentially serve as another treatment strategy to target the transition from acute to chronic pain.

Thus, while there is still much to be learned about local translation, together the available data suggests that local translation is not only important for nerve regeneration but plays a critical role in chronic pain. That this process is dynamically regulated suggests it may contribute to the initiation of the chronic pain state and/or the transition from acute to chronic pain. Furthermore, evidence suggests that targeting points of convergence such as mTOR and eIF4 may be viable treatment strategies. As more is learned about local translation, it should be possible to develop even more specific therapeutic strategies. However, as is becoming clear for all known therapeutic targets, the relative contribution of any specific mechanism is likely to be context dependent, and will vary as a function of a variety of factors including time, site of injury, and nature of the injury. For example, the subsets of mRNAs translated in response to inflammation are likely to differ from those translated in response to nerve injury. As such, targeted treatment approaches are likely to be necessary to adequately target chronic pain in patients with osteoarthritis and post-surgical pain versus those with traumatic nerve injury. Nonetheless, the emerging evidence strongly suggests that inhibition of axonal translation has the capacity to be both a personalized and effective therapeutic strategy for the treatment of chronic pain.

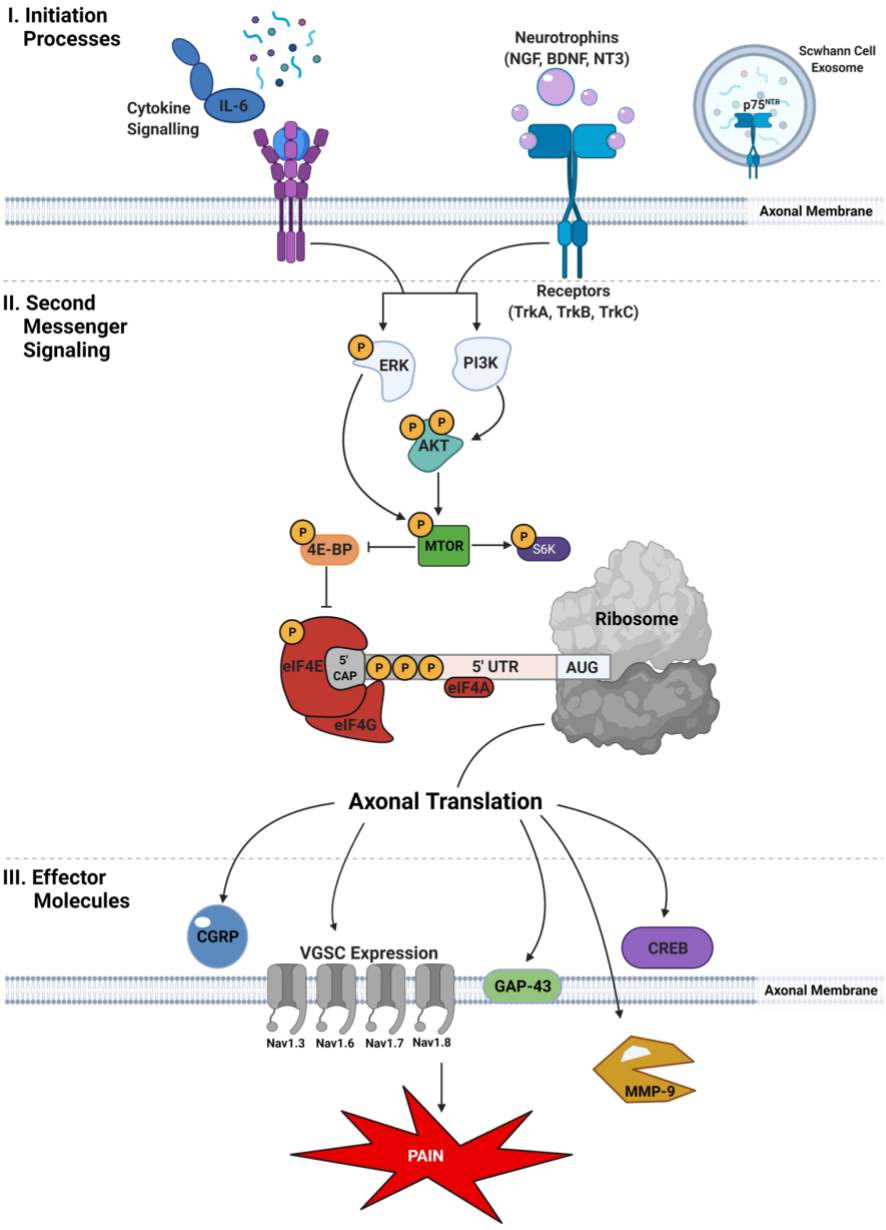

Figure 1: Summary of steps associated with local translation in primary afferent axons. The process is initiated (Initiation Process) via neural activity (not shown) and the activation of receptors for cytokines and neurotrophins. There is also evidence that the process may be started by the transfer of mRNA and translation machinery via exosomes released from local cells such as Schwann cells. Receptor activation initiates second messenger cascades (Second Messenger

Signaling) ultimately resulting in the translation of mRNA. The proteins locally translated (Effector Molecules) may contribute to nociceptive signaling (CGRP), the facilitation of action potential propagation (voltage-gated sodium channels, VGSCs), and proteins trafficked back to the cell body that influence gene expression (CREB).

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