Specific Aims: First described in 1912 by S.A. Kinnier Wilson as "progressive lenticular degeneration," Wilson Disease (WD) is an inherited disorder of copper metabolism resulting in pathogenic copper accumulation in the brain and liver. A proposed grouping of WD neurologic symptoms includes dystonic syndrome, ataxic syndrome and Parkinsonian syndrome, though patients often present a combination of features. The disease is caused by mutation in the ATP7B gene with an estimated allele frequency of 1:100. Recent work suggests that WD may not entirely be recessive, as with one study indicating 21% of patients have only a single pathogenic mutation. Further, WD patients may present with psychiatric symptoms (often prior to WD diagnosis) including behavioral changes, personality changes, depression and anxiety.

One **major challenge** in WD treatment is that neurological symptoms often worsen when treated with standard-of-care chelators D-penicillamine and trientine as well as tetrathiomolybdate. Zinc salts are a treatment for WD, often in pre-symptomatic cases. However, the treatment efficacy of Zn salts for hepatic disease appears to be limited, and neurologic improvement is comparatively slow, though Zn treatment is less associated with neurological degradation. A major limitation in WD treatment is the lack of pre-clinical animal models to understand pathology and treatment mechanisms in neurologic WD. Therefore, ***our long-term goal is to develop a pre-clinical mouse model to improve WD treatment.***

Animal models of WD include the Long-Evans Cinnamon (LEC) rat, toxic milk (*tx*) mouse and *Atp7b*$^{-/-}$ mouse. These models have been invaluable to define hepatic mechanisms of copper toxicosis including our findings of disrupted zinc balance. Among these, the *Atp7b*$^{-/-}$ mouse is a highly characterized rodent model of WD, revealing defects in lipid metabolism as well as nuclear receptor signaling, leading to new candidate therapies. However, the utility of these models in neurologic Wilson Disease is not well explored. Although zinc is a treatment for WD, the impact of excess copper on zinc metabolism, particularly in the brain, is limited. Similarly, the impacts of copper chelators on brain zinc levels are not known. Taking these observations into account, we propose to determine the behavioral and molecular-level consequences of altered neurological copper levels and the utility of the *Atp7b*$^{-/-}$ mouse for pre-clinical studies of WD.

Why is this a good model? WD presentation in patients is diverse, as are over 650 identified pathogenic variants. A knockout mouse model is appropriate because impacts of mutation are on either function or location of the Atp7b copper exporter and *all pathogenic variants are unified in the consequence of intracellular copper accumulation*. Brain copper in the *Atp7b*$^{-/-}$ mouse continues to rise throughout life, which is accompanied by altered cholesterol metabolism and oxidative stress in brain. The spontaneous *toxic milk* Atp7b mouse mutant has been briefly described with neurological deficits, suggesting that a mouse model is appropriate for these studies. Because other important neurological syndromes including Parkinson's and Alzheimer's diseases also result in brain copper imbalance, this model has potential to inform on broader neurodegenerative pathology. The ***preliminary data*** for this proposal is largely in published work, including our own, and defines a faithful WD copper toxicity phenotype in the *Atp7b*$^{-/-}$ mouse. Our project will test the hypothesis that the *Atp7b*$^{-/-}$ mouse is an effective model for neurological WD and the impacts of WD therapies through an integrative behavioral and molecular approach in the following three ***Specific Aims***:

Specific Aim 1: Determine the extent to which *Atp7b*$^{-/-}$, *Atp7b*$^{+/-}$ mice exhibit motor and behavioral impairments. Behavioral and molecular phenotypes will be determined for male and female animals of both wild type, heterozygous and knockout (*Atp7b*$^{-/-}$) genotypes after 8 weeks treatment with early (4-14 weeks of age) or late (12-20 weeks of age) treatments as follows: untreated, D-penicillamine, or zinc.

Specific Aim 2: Determine the effects of Zn or D-penicillamine treatment on the concentration of trace metals and markers of oxidative stress in the brain for *Atp7b*$^{-/-}$, *Atp7b*$^{+/-}$ and wild type mice. This aim will identify molecular-level readouts for neurological WD and the consequences of treatment on these factors.

Specific Aim 3: Determine the changes in Cu and Zn handling protein levels in target brain regions of animals subjected to control, Zn or chelator treatments. This aim will test the sub-hypothesis that zinc-handling machinery in the brain is impacted by copper similarly to impacts in the liver in WD. If this hypothesis is supported, disruption of zinc metabolism will be identified as part of neurological WD.

***What will we gain*?** This project will establish a baseline neurobehavioral phenotype for the *Atp7b*$^{-/-}$ mouse and a framework to mechanistically evaluate WD treatments. This work will inform other metal-related neurodegenerative diseases with disrupted Cu metabolism such as Alzheimer's Disease (AD) and Parkinson's Disease (PD). This study will provide a foundation for treatment-focused experiments towards improved patient outcomes.

Significance - Wilson Disease (WD) is an autosomal recessive disorder of copper (Cu) metabolism with an estimated global allele frequency of 1:90 (1). The disease is caused by a loss-of-function mutation in the Atp7b Cu transporter and is subsequently characterized by pathological Cu(I) accumulation, particularly in the brain and liver, as well as by neurological deficits, often described in the literature as "neuropsychiatric symptoms of WD," which positively correlate with the severity of cerebral Cu accumulation (2). Unfortunately, neurological damage in these cases may be permanent. WD shares many symptoms with other neurological diseases such as Alzheimer's and Parkinson's—diseases that are also characterized by Cu accumulation in the brain (3). Insight into animal models of WD will likely illuminate etiologies in these more common pathologies. Therapies for WD include consumption of a Cu restricted diet, treatment with de-coppering chelators such as D-penicillamine (DPEN), trientine, tetrathiomolybdate, and zinc (Zn) supplementation. Liver transplant is necessary in WD cases with poor response to these therapies. While these treatments typically reduce hepatic Cu accumulation, they sometimes fail to improve neurological symptoms (4). For example, tetrathiomolybdate treatment was reported to have negative neurological impacts in 9% of patients in one study (5). These responses may be due to a temporary transference of Cu from the liver to the blood, worsening the overall Cu stress. Alternatively, WD treatment might negatively impact brain homeostasis of Cu or other transition metals such as Zn.

Neuropsychiatric symptoms in WD typically develop later than hepatic symptoms (6) with varying reported incidence in **15-80% of patients** (7), presenting a significant challenge in human therapeutics studies. Additionally, **pre-clinical tools to evaluate neurologic or neuropsychiatric WD and the impacts of therapies in the brain are limited.** These symptoms can be severe or fatal and significantly impact quality of life through tremor, dystonia, dysarthria, dysphagia, Parkinsonism, psychosis and cognitive impairment, among others (8, 9). Thus, the long-term goal of our proposed research is to develop a mouse model for neurological WD. The *Atp7b*$^{-/-}$ mouse is one of the most thoroughly characterized animal models of WD (10), though relatively few laboratories have substantial experience with this model.

The ***scientific premise*** and ***significance*** of this study are based on the following observations:

- Both WD patients and the *Atp7b*$^{-/-}$ mouse accumulate excess brain Cu (11, 12).
- Neurological WD manifests in movement as well as cognitive and behavioral disorders, with psychiatric symptoms in 30-64% of WD patients (13–15).
- Mice with inactivated Atp7b have neurological defects consistent with WD (preliminary data and (16)).
- Excess Zn accumulates in the cortex and specific hippocampal structures of the *Atp7b*$^{-/-}$ mouse (17).
- The lack of pre-clinical models in neurological WD presents an obstacle to closing the knowledge gap that results in poor understanding neuropsychiatric WD and its treatment.

The ***primary objective*** of this R21 project is to define behavioral and molecular quantitative phenotypes *Atp7b*$^{-/-}$ mouse that can be used to improve or develop WD treatments. This study will close important gaps in knowledge about consequences of Cu accumulation in the brain as well as WD treatment.

The ***secondary objective*** is to test the hypothesis that, as in the liver, Cu accumulation in the brain induces changes in Zn-dependent processes, identifying a novel mechanism in WD neuropathology.

Why is this mouse model appropriate? *Atp7b*$^{-/-}$ mice reportedly exhibit the most pronounced WD liver pathology of the rodent models, which closely parallels that observed in WD patients (10). *Atp7b*$^{-/-}$ mice have increased Cu (and Zn) levels in the brain in regions consistent with common WD brain lesions (11), most significantly in the gray matter of the cortex, in the hippocampus, and the cerebellum (17). PET/CT studies using the radioactive Cu isotope ^{64}Cu indicate age-dependent increases in cerebral Cu retention from the 13-21st weeks of age (18). Similarly, the Atp7b point-mutant *toxic milk* mice has Cu deposition in the hippocampus and the basal ganglia, a collection of nuclei critical for motor control (16). Dong et al. (2015) report that *Atp7b*$^{-/-}$ mice also show structural abnormalities in the basal ganglia and no significant brain Cu excess. However, this work only analyzed three samples per group at most time points, suggesting an underpowered experiment. Power analysis based on Dong et al. (2015) data indicates that a minimum of four animals per group for basal ganglia would be sufficient to test a 1.5-fold difference of means at α of 0.05, indicating an underpowered experiment.

Hepatic phenotypes in *Atp7b*$^{-/-}$ mice include the accumulation of Cu and Zn, disruption of cell cycle machinery (20, 21), and mitochondrial damage (22) as well as the overexpression of metallothionein (MT) (21)

and downregulation of Ctr1 (23), important proteins of Cu metabolism. The *Atp7b*$^{-/-}$ model also exhibits structural abnormalities in the kidney without Cu deposition (24), whereby kidney function is impaired in some WD patients (25, 26). Though Atp7b is not expressed in the adrenal gland and *Atp7b*$^{-/-}$ mice fail to show Cu deposition here, they do show decreased dopamine beta-hydroxylase (DBH), a Cu-dependent enzyme converting dopamine to norepinephrine, and therefore reduced availability of norepinephrine (27). DBH is also found in a small region of the brain known as the locus coeruleus, which houses norepinephrine-producing cells. Atp7B inactivation in these neurons similarly decreases DBH concentration (28), though behavioral impacts such as analysis of anxiety were not reported.

A knockout mouse is an accurate model for most WD variants. WD presentation in patients is diverse, though the unifying *pathognomonic characteristic* is hepatic and brain copper accumulation (29). Over 650 identified ATP7B pathogenic variants are known (1). This observation alone presents a challenge to choose one or a few variants for knock-in experiments. Functional characterization of variants has revealed differences in Atp7b biochemical and/or trafficking properties (e.g. see (30)). Since impacts of mutation are on either function or location of the Atp7b copper exporter, **all pathogenic variants are unified in the consequence of intracellular copper accumulation**. One key example is the H1069Q variant common in the European population: this variant is catalytically inactive and thus not able to transport Cu resulting in failure of biliary Cu excretion (31). Such mutations present the same biochemical outcome as a knockout in that no *functional* Atp7b is present in cells. Even mutations that are transport competent but subcellular trafficking defective are nonetheless pathogenic in that patients still accumulate excess Cu (32). Future knock-in experiments may provide a refined analysis (such as WD treatment of inactive vs. trafficking defects) once the quantifiable endpoints in the knock-out are well-defined by the proposed experiments.

Innovation - Human WD has varied clinical phenotypes even with same mutation, suggesting interaction with other genes as well as environment. One important example is a case of monozygotic twins with strikingly divergent phenotypes (33). A foundational animal model is necessary to dissect these phenotypes in controlled studies. Although it is considered the best-developed WD model, there are no detailed studies of behavior for the *Atp7b*$^{-/-}$ mouse, and few behavioral studies have been reported for WD model animals. **Other models:** The *toxic milk* mouse has mild liver disease and impaired motor coordination and spatial memory (16) as well as neuronal loss (34). This mimics neurological symptoms observed in clinical populations including dysarthria, dystonia, and tremors (35). Our project is innovative in using behavioral neuroscience and cognitive studies in the *Atp7b*$^{-/-}$ mouse model of WD. **We chose the *Atp7b*$^{-/-}$ model based on our preliminary data and published work indicating *Atp7b*$^{-/-}$-associated neurological impairment, severe liver disease, brain copper accumulation and brain Zn accumulation, consistent with human WD (20)**. The key innovation in our project is to combine behavioral assays of neurological as well as psychological phenotypes with molecular analysis to establish a pre-clinical mouse model for Wilson Disease treatment.

We propose to determine ***clinically relevant*** behavioral phenotypes such as motor coordination, memory, anxiety, anhedonia and general appetite including age-dependent analysis in Aim 1 so that we may **a**) identify behavioral phenotypes in *Atp7b*$^{-/-}$ mice across time, and **b)** determine the effects of DPEN and Zn treatment on behavior. Clinical studies demonstrate that WD patients exhibit Cu accumulation and structural abnormalities in the basal ganglia (36). Thus, targeted studies must be conducted to identify the specific effects of Atp7b inactivation on Cu accumulation in the brain. In Aim 2, we propose the use of ICP-MS to quantify Cu and other trace metals in the brain and other relevant organs in parallel with molecular and histological assessment of oxidative stress and structural damage in control and treated *Atp7b*$^{-/-}$ mice. WD is progressive; Aims 1 and 2 of this study will analyze *age-dependent behavioral* phenotypes as well as Cu accumulation with brain pathology. Aim 3 will test the hypothesis that Cu accumulation in the brains of *Atp7b*$^{-/-}$ mice impacts Zn proteins and handling machinery and is a *mechanism of neurological Cu toxicity*, as we recently reported for the liver (37). Our data indicate these effects are mediated through MTs and through Cu-Zn competition. Should these analyses prompt further important questions, our laboratory has expertise in a range of molecular techniques, including transcriptomics, proteomics and metabolomics, which will be used to probe for changes in gene expression or specific metabolite concentrations.

How this work will move the field forward. This study will establish a pre-clinical screening tool for WD treatment, help understand how treatment works, provide insight into neurological Cu toxicity in WD and other neurodegenerative diseases such as PD or AD where Cu imbalance or mistargeting is also observed. This work also provides a foundational model to study genetic, epigenetic, or environmental modifiers of WD (38,

39).

Approach

Rigor and reproducibility – key testable hypotheses. Our approach applies the scientific method to test the hypothesis that defective Cu export due to loss of Atp7b function in a mouse model will present a phenotype consistent with WD. The following predicted outcomes will support the hypothesis:

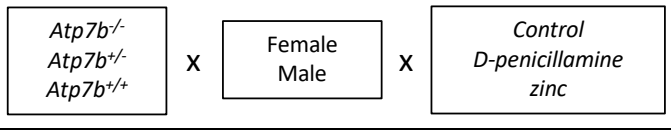

Figure 1. 3x2x3 experimental design to determine animal response to experimental treatment.

1) The *Atp7b*$^{-/-}$ mouse will exhibit quantifiable behavioral phenotypes compared to wild type.

2) Current WD treatments will impact behavioral phenotypes.

3) Zn or chelator treatment of the *Atp7b*$^{-/-}$ mouse will influence brain Cu accumulation and pathology.

4) Zn-dependent and Zn handling proteins will have different abundance in *Atp7b*$^{-/-}$ mice vs. wild type.

Our project will use a 3 x 2 x 3 design (**Fig. 1**). *Atp7b*$^{+/+}$, *Atp7b*$^{+/-}$ and (*Atp7b*$^{-/-}$) strains, male and female, will be assigned to one of three treatment conditions (untreated, DPEN, or Zn acetate) in their drinking water for 8 weeks in "early" treatment starting at 6 weeks old (timeline in **Fig. 3**) or "late" treatment starting at 4 months old (**Fig. 4**).

Each Aim will produce specific new data, while the integrative approach that includes neurobehavior, proteomics, metallomics, neurotoxicology and treatment models provides a framework to link

Rigor and reproducibility – experimental design

Power – Our study for this R21 project is the first of its kind for the *Atp7b*$^{-/-}$ mouse. Power calculations based on published studies are defined under Aims 1 and 2 and are included in the design. We have previously published meaningful data from proteomics, metabolomics and transcriptomics studies (37, 40–42).

Randomization – Mice are bred in-house and genotyped 4-5 weeks after birth. A random number list will be generated to assign each animal of each genotype and sex to a treatment group to control for selection bias.

Experimental bias — Behavioral experiments will be video recorded and scored by two reviewers who are blinded to the experimental group and genotype.

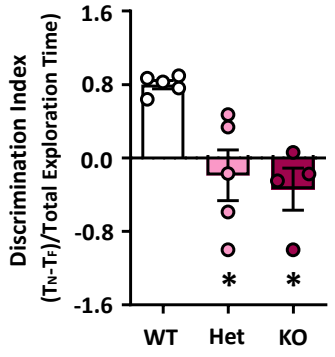

Figure 2. ***Atp7b*$^{+/-}$ and *Atp7b*$^{-/-}$ mice perform poorly compared to wild type in novel object recognition.** Preliminary discrimination index for 6-10 week-old female mice in a novel object recognition test (n=4-5). Comparison by one-way ANOVA with Tukey's HSD, * denotes P<0.05.

The ***preliminary data*** for this proposal is established in published work including our own that defines a faithful WD phenotype and Cu toxicity in the *Atp7b*$^{-/-}$ mouse (11, 20, 40). Studies from my lab were the first to identify direct impacts on (Zn dependent) nuclear receptors in a WD model (40) and more recent identification of impacts on Zn and Zn systems as a mechanism of Cu toxicity in WD (37). The experimental design uses an integrative behavioral and molecular approach to determine quantifiable factors in the *Atp7b*$^{-/-}$ mouse that are of value for pre-clinical studies in neurological WD. Based on previous work and our own preliminary data, we have designed an approach to determine impacts of WD-associated Cu accumulation at early and late timepoints and with short and long treatment regimes. Our group has worked with student behavioral projects in the past, although these experiments are a new direction in our WD research. **Our pilot experiments with young adult mice include a test of the novel object recognition assay** (**Fig. 1**), depicting differences in wild type (*Atp7b*$^{+/+}$), heterozygous (*Atp7b*$^{+/-}$) and knockout (*Atp7b*$^{-/-}$) in time spent with a novel vs. familiar object. These results indicate that the test is sensitive enough to detect discrimination between a novel and familiar object in a healthy, wild type mouse as well as deficits in young heterozygous and knockout mice. Sex as a biological variable is addressed in our experimental 3x2x3 design with *sex as an independent variable*.

Specific Aim 1: **Determine the extent to which *Atp7b*$^{-/-}$, *Atp7b*$^{+/-}$ mice exhibit motor and behavioral impairments.** This aim takes a systematic experimental approach to define behavioral consequences of Atp7b inactivation. It will also determine treatment impacts on behavioral phenotypes in *Atp7b*$^{-/-}$ mice and how Zn or Cu chelator (DPEN) treatment might affect wild type or *Atp7b*$^{+/-}$ mice.

Overall design. Male and female *Atp7b*$^{+/+}$, *Atp7b*$^{+/-}$ and *Atp7b*$^{-/-}$ animals will be assigned to one of three

treatment conditions (untreated, DPEN, or Zn acetate) in their drinking water for 8 weeks with early or late treatment initiation (timelines in **Fig. 3** and **Fig. 4**). WD patients are treated with Zn at 150 mg/day (approx. 2.1 mg/kg), such that a mouse allometrically-scaled dose is 0.33 mg/day (approx. 16 mg/kg). The scaled dose for DPEN is 2.2 mg/day. analysis will be implemented at specific points of the experimental timeline (denoted as blue dots in Fig. 3 and 4). 24-hour urine and fecal sampling at indicated points in the timeline (denoted as green dots in Fig. 3 and 4) will monitor Cu excretion as well as observation of food and water consumption.

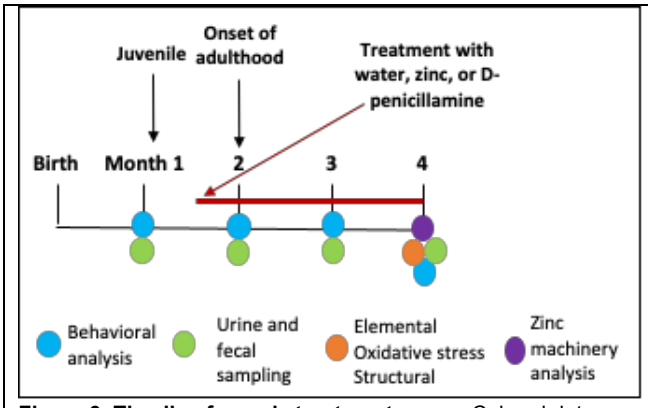

Figure 3. Timeline for early treatment group. Colored dots represent timepoints of data collection. Blue, green, orange, and purple dots correspond with behavioral analysis, urine and fecal sampling, elemental and structural analysis of tissues, and assessment of Zn transporter gene expression and enzyme function, respectively.

Treatment-induced improvement for *Atp7b*$^{-/-}$ in any behavioral deficits observed would be clinically significant, as would negative responses in any genotype. These measurements along with biochemical and pathological data provide mechanistic endpoints and candidate measurements to monitor clinical treatment.

Motor control. WD patients with neurological symptoms frequently present with severe cerebellar-dependent motor deficits, such as dystonia (6). Animals will be subjected to rotarod and beam walk tests of motor coordination. The rotarod is especially important because it is sensitive detection of cerebellum disfunction (43), where Cu accumulates in the *Atp7b*$^{-/-}$ model (17). The beam walk is a highly sensitive test that has been successfully applied in an experimental treatment with a Parkinsonian rodent model by consultant Ruben Dagda's group (see support letter) (43–45). **Expected outcomes**: Based upon preliminary findings in other WD models (16) and trial experiments in our lab, we anticipate that *Atp7b*$^{-/-}$ mice will have a decreased latency to fall in the rotarod and more foot slips in the beam walk.

Learning and memory. This experiment is included because memory loss is a common cognitive deficit experienced by WD patients (46). *Atp7b*$^{-/-}$ mice accumulate Cu in the hippocampus (17), *toxic milk* mice demonstrate impairments in a related hippocampal-dependent task (16). A novel object recognition test will be used to assess the hippocampal-dependent task of learning and memory as shown in **Fig. 1**. This two-part test involves acclimating an animal to several objects in an open field, measuring their interaction with each object, and then removing one object, replacing it with a novel object and quantifying the time spent examining the novel feature (47). Animals with hippocampal lesions fail to exhibit interest in the novel object (48), whereby reduction in discernibility between novel and familiar objects is indicates hippocampal impairment. **Expected outcomes**: Based on our preliminary data, we expect *Atp7b*$^{-/-}$ and *Atp7b*$^{+/-}$ mice will fail to show discernibility (low discrimination index score) in the task compared to *Atp7b*$^{+/+}$ mice.

Anxiety. Anxiety is relevant as many WD patients report it as one of the neuropsychological symptoms they experience (50) and presently it has not been examined in the *Atp7b*$^{-/-}$ model. An elevated plus maze test will be used to test anxiety (49). In this test, animals are given 10 minutes to explore an apparatus with two dark enclosed arms and two open, unenclosed arms and the time spent in each is quantified from video recording. Animals spending more and less time in the closed and open arms, respectively, are considered to be more anxious. **Expected outcomes**: We expect *Atp7b*$^{-/-}$ mice to present decreased anxiety and consistent with decreased dopamine beta-hydroxylase activity in ATP7B deficient animals (27, 28).

Anhedonia/reward perception. Given that some WD patients present with severe depression (50, 51), but it has not been reported in animal models of WD, this is a measure worthy of exploration. Anhedonia in rodents can be achieved by both stress (52) and depression (53). Animals will be subjected to a sucrose preference test. This test is a 2-day procedure that involves simply measuring the amount of sucrose-infused water consumed by an animal. Sucrose is a strong reward for rodents and they readily prefer it to water. **Expected outcomes:** We expect reduction in sucrose preference with Atp7b inactivation.

Statistical power. We used data published for measures of anxiety in the *toxic milk* mouse (16) to provide an initial power calculation for our experiments. Based on these values, a power of 0.8 and α of 0.05, ten animals per group will be required for the initial comparison of *Atp7b*$^{-/-}$ to control phenotype and a two-tailed

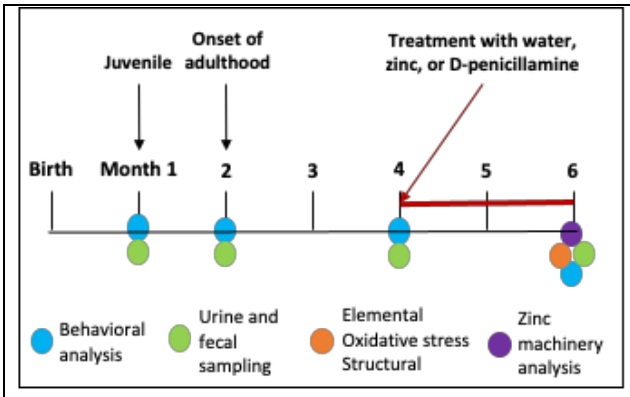

Figure 4: Timeline for late treatment group. Colored dots represent timepoints of data collection. Blue, green, orange, and purple dots correspond with behavioral analysis, urine and fecal sampling, elemental and structural analysis of tissues, and assessment of Zn transporter gene expression and enzyme function, respectively.

comparison of means, G*Power (54). We will use this preliminary power calculation for initial behavior analyses. We will use fewer animals if initial tests indicate sufficient power can be achieved with a lower number. ANOVA will compare across groups with two-way post-hoc tests for interaction of genotype and treatment.

Potential challenges – Specific Aim 1. Untreated Atp7b$^{-/-}$ mice may not show neurological deficit, especially at young ages: this would be an unexpected result, given the preliminary data in 6-10-week-old mice. However, the experiments will serve a dual purpose to test both disease and treatment-induced effects. The beam-walk test for motor control is more challenging to administer than the rotarod but is also more sensitive to mild deficits. Dr. Dagda has agreed to advise on behavioral and motor control tests. *Treatments may cause changes in behavior in wild type mice or exacerbate pathology in Atp7b$^{-/-}$*: This is possible and will help us understand how treatment impacts patients and how to mitigate side effects.

***Specific Aim 2:* Determine the effects of Zn or DPEN treatment on the concentration of trace metals and markers of oxidative stress in the brain for *Atp7b$^{-/-}$*, *Atp7b$^{+/-}$* and wild type mice.** Aim 2 will test the hypothesis that *Atp7b$^{-/-}$* mice will provide mechanistic biochemical phenotypes to evaluate WD therapies. The experimental treatment and endpoint timeline for Aim 2 will be the same as in Specific Aim 1. *Rationale:* Verified central nervous system molecular phenotypes in the *Atp7b$^{-/-}$* mouse that are consistent with WD include the following: brain Cu accumulation and ultrastructural abnormalities (11, 17–19), brain Zn accumulation (17), and progressive suppression of brain sterol metabolites and oxidative stress (56).

Neuroinflammation and pathology analysis. We will determine the consequences of *Atp7b* inactivation (*Atp7b$^{-/-}$, Atp7b$^{+/-}$*) on these phenotypes at the early or late treatment time points. Following the treatment plan in Specific Aim 1, we will quantify metal concentration and oxidative stress markers to identify **a)** impacts of Cu accumulation at early and late treatment schedules, and **b)** impacts of two key treatments for with early and late treatment initiation. Mice will be sacrificed and tissues immediately collected at the end of each experimental timeline. Brain weight will be determined along with histological brain region structure for regional atrophy and lesions as illustrated in human WD (57). Unbiased stereology will quantify potential changes in *neuron number* (58) using a commercial service (Charles River). We will leverage our quantitative microscopy expertise (58) to quantify neuronal density in target brain regions by detection of RBFOX3/NeuN, which is localized to neuronal nuclei (60).

Metals and oxidative stress determination. We will use inductively coupled plasma mass spectrometry (ICP-MS) to determine concentrations of target metals Cu, Zn and Fe in the cortex, cerebellum, basal ganglia and hippocampus of the brain. We will determine neuroinflammation by immunofluorescence assays for glial fibrillary acidic protein (GFAP) and CD11b, while glial activation will be analyzed by staining for CD40. Relative oxidative stress and reduced/oxidized glutathione ratios (GSH/GSSG), and aconitase activity, the latter of which is damaged by oxidative stress (56, 61). ***Statistical power.*** We used published brain Cu levels for initial power calculation (19). The Cohen's d for Cu in 3-month-old cerebellum comparing *Atp7b$^{-/-}$ vs Atp7b^{++}* is 2.64, suggesting a large difference in Cu in this region. Based on these values, a power of 0.8 and α of 0.05, a minimum four animals per group (we plan 10/group) will be required for the initial comparison of *Atp7b$^{-/-}$* to control for cerebellar Cu levels (two-tailed comparison of means, calculated with G*Power (54)). Cortex Cu was not different in at 3 months, while at 10 months the seven animals per group would be required for power of 0.8 and α of 0.05 (Cohen's d=1.72). **Expected outcomes**: We anticipate age-dependent and region-specific brain Cu deposition and structural abnormalities in untreated *Atp7b$^{-/-}$* mice and possibly in *Atp7b$^{+/-}$* mice. **Data collection points:** We will harvest tissues from all genotypes at the end of treatment timelines. We expect Cu redistribution in treated mice with differences in early (Fig. 3) and late treatment endpoints (Fig. 4). We expect neuroinflammation in *Atp7b$^{-/-}$* mice, including increased immunodetection of GFAP and CD11b with increased inflammatory gene expression in the hippocampus (16). We expect decreased neuron density with proliferation of astrocytes and Alzheimer-type II glial cells as reported in WD lesions (62).

Changes in these features with DPEN or Zn treatment would be clinically significant as would differences by genotype.

Potential challenges – Specific Aim 2: We expect few challenges with these assays, as elemental analysis and immunodetection are routine for our group. Alternative assays for neuroinflammation, such as inflammatory cytokine expression profiling, are complementary and can provide information about inflammatory state. Dr. Dagda has agreed to advise on these experiments and host team members for additional training.

Specific Aim 3: **Determine the changes in Cu and Zn handling protein levels in target brain regions of animals subjected to control, Zn or chelator treatments.** *Rationale:* This aim tests the hypothesis that Zn-interacting machinery in the WD brain is impacted similarly to the liver. Since Zn is highly abundant in the brain, it is important to know if Cu accumulation and WD treatment affects Zn handling machinery or Zn-metalloproteins. Our recent work indicates increased Cu levels impact Zn distribution due to a decrease in the relative abundance of Zn-MT compared to Cu-MT. Both Cu and Zn accumulate in the *Atp7b*$^{-/-}$ and WD brain, though it is not known if Cu accumulation induces Zn-specific changes in MT, transporters or enzymes (as it does in the liver). The expected paradigm is similar to our proposed model (37), which is based on empirical MT properties, our own data, and known bio-inorganic interactions.

Proteomics approach. A bottom-up proteomics approach will target brain regions known to be affected in WD including the striatum (as noted by S.A. Kinnear Wilson (63)) included here in basal ganglia), cerebellum and cortex. This approach will provide a comprehensive protein-level profile of changes in response to Cu accumulation including potential Zn-dependent targets. In addition to broad coverage, peptide counts in proteomics provides a **more robust and quantifiable analysis** than Western blots and immunodetection with varied antibody affinities. For the limited scope of this R21 project, we will focus first on *Atp7b*$^{-/-}$ vs. wild type comparison at the end of early-onset behavior experiments. We will analyze both female and male *Atp7b*$^{-/-}$ vs. *Atp7b*$^{+/+}$ (n=5/group) for four brain regions (total 10 animals/40 samples). We will work with the IDeA National Proteomics Resource using the highly quantitative and reproducible **data-independent acquisition approach (DIA)** (64) that has been optimized at this facility (see letter of support). Key proteins of interest have previously been detected in mouse brain proteomics profiling including Zn transporters ZnT3 (which transports Zn into synaptic vesicles (71, 72)), ZnT1 or ZnT9; Zn-dependent enzymes TNAP, carbonic anhydrases and SOD1; Cu transporters Slc31A1 and ATP7A; as well as metal binding MT1, MT3 and CCS (65). These findings support a quantitative proteomics approach to define molecular responses to Cu and their relationships to Zn handling. The study will provide data on changes in key Zn machinery in the brain and indicate changes in cellular Zn status. This information is important to integrate into analysis of WD or neurodegenerative treatment in an animal model. ***Statistical approach:*** **We have experience with both proteomics and RNAseq analysis** (37, 40, 66). We will work with IDeA National Resource for Proteomics including their proteiNorm application and MSstats R package using flexible linear mixed models for statistical analysis of DIA data. Novel pathways and markers of interest will be validated by LC-SRM/MS or MRM (selected/multi reaction monitoring) with a Thermo Orbitrap Exploris in the UAA ASET lab (PI Burkhead is a co-PI for the Major Research Instrument grant that supports the Exploris LC-MS).

Expected outcomes: We expect brain Cu accumulation to induce MT expression including MT3 as a metal chelator and ROS scavenger in the central nervous system (67). We expect to observe a decrease of MTs over time in control animals, as indicated in rats (68), but increased MT3 expression as Cu accumulates in *Atp7b*$^{-/-}$ mice. Since MT3 preferentially binds Cu over Zn (69), we expect changes in brain Zn handling machinery such as exporters ZnTs 1,3,4, and 6, which are highly expressed in brain (70). Cellular Zn is also regulated by TRPML1, associated with neurodegenerative mucolipidosis IV (73). We may find indication of mitochondrial dysfunction or mitophagy as observed in multiple neurodegenerative diseases (74). We expect Cu in the brain to disrupt Zn-dependent proteins, such as those in transcription and metabolic processes as in the liver, and not 'gluzinergic' neurons with >1 mM Zn (71, 75). Given the 10-fold greater Zn in the brain compared to other tissues (75) and age-dependent brain region Cu accumulation (19) we expect Zn-interacting proteins to be impacted later in the brain than in the liver.

Potential challenges – Specific Aim 3: We expect few significant challenges with these cost-effective studies. Should quantitation by bottom-up proteomics prove uninformative, we can test the above hypothesis by analyzing these specific targets by quantitative PCR, immunofluorescence microscopy and/or Western blot. Data analysis support: PI Burkhead is AK-INBRE Bioinformatics Core Director and can lead analysis.

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