Wallerian Degeneration Slow Mutation Does Not Alter the Amyloid Pathology of Alzheimer’s Disease

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biology

by

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The Thesis of Ann Yu-Jung Shih is approved and it is acceptable in quality and form for publication on microfilm:

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature Page</td>
<td>iii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iv</td>
</tr>
<tr>
<td>List of Figures and Tables</td>
<td>v</td>
</tr>
<tr>
<td>Abstract</td>
<td>vi</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Material and Methods</td>
<td>17</td>
</tr>
<tr>
<td>Results</td>
<td>24</td>
</tr>
<tr>
<td>Conclusion</td>
<td>39</td>
</tr>
<tr>
<td>References</td>
<td>43</td>
</tr>
</tbody>
</table>
LIST OF FIGURES AND TABLES:

Figure 1: APP proteolytic processing by α-, β- and γ-secretase ....................... 5
Figure 2: Mutation in APP ................................................................................. 11
Figure 3: Chimeric WLD³ protein ................................................................. 16
Figure 4: Kaplan-Meier Survival Curves ....................................................... 28
Figure 5: WLD;PDAPP vs PDAPP neuritic plaques in the hippocampus. .... 29
Figure 6: WLD vs wt neuritic plaques in the hippocampus ......................... 30
Figure 7: Plaque burden ............................................................................... 31
Figure 8: Immunostaining of Aβ WLD;PDAPP vs APP.......................... 32
Figure 9: Immunostaining of Aβ--WLD vs wt........................................ 33
Figure 10: Aβ burden in the hippocampus and cortex .............................. 34
Figure 11: Formic acid extraction Aβ40 and Aβ42 sandwich ELISA .......... 35
Figure 12: CHAPSO extraction Aβ40 and Aβ42 sandwich ELISA .......... 36
Figure 13: CHAPSO extraction Western blot............................................. 37
Figure 14: Characterization of total APP levels ...................................... 38
Table 1: Effectiveness of WLD³ in blocking axon degeneration .............. 42
ABSTRACT OF THE THESIS

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Wallerian Degeneration Slow (WLDs) has been found to have a neuroprotective role in many neuro-degenerative diseases, but never in the Alzheimer’s disease models. Alzheimer’s disease is the most common form of neurodegenerative disorder in the elderly afflicted with memory impairment and dementia. It is characterized by the
deposition of amyloid beta-protein (Aβ) extracellularly within neuritic plaques and neurofibrillary tangles in neuronal cell bodies. It has been hypothesized that the deposition and aggregation of Aβ in the brain initiates a cascade of events, including synaptic dysfunction that results in AD. In this study, we crossed WLD animals with PDAPP, an APP over-expressing transgenic model, and found that WLD did not influence the survival rate, the level of amyloid beta or the amount of plaque deposit in the PDADD mice. However, we can not totally rule out the possibility that WLD may have protective effects on APP transgenic models.
INTRODUCTION

Alzheimer’s disease

Alzheimer’s disease (AD) is the most common neurodegenerative disorder characterized by a progressive decline in cognitive abilities. The neuropathological hallmarks of AD are neuritic plaques and intracellular neurofibrillary tangles (NFT). Neuritic plaques consist of extracellular deposits of aggregated amyloid beta-protein (Aβ) and associated abnormal axonal and dendritic processes. Neuritic plaques, also known as senile plaques, are found in large numbers in the limbic cortex and neocortex. [1]. The dystrophic) neuritic processes are often dilated and tortuous and are marked by ultrastructural abnormalities that include enlarged lysosomes, numerous mitochondria, and NFTs. NFTs are comprised of hyperphosphorylated tau, a microtubule-associated protein and appear as pair helical filaments by ultrastrucrue. The plaques are also closely associated with reactive microglia that express activated surface antigens, are also surrounded by reactive astrocytes displaying abundant glial filaments. The microglia are usually within and adjacent to the central amyloid core of the neuritic plaque, whereas the astrocytes are often found on the outside of the plaque, with some of their processes extending centripetally toward the amyloid core. Aβ are heterogenous peptides derived from the amyloid precursor protein (see below). Much of the fibrillar Aβ found in the neuritic plaques is the species ending at amino acid 42 (Aβ 42), the slightly longer, more hydrophobic form that is particularly prone to aggregation. However, the Aβ species ending at amino acid 40 (Aβ 40) is normally more abundantly produced by cells than Aβ 42 and is usually co-localized with Aβ 42 [1].
Beta-amyloid precursor protein (APP)

The human APP gene is located on the long arm of chromosome 21, thus studies have associated AD with the trisomy 21 Downs syndrome [2]. The APP gene has 19 exons with approximately 240 kilobases spans total that encode a membrane-associated protein [3]. Alternative splicing generates APP encoding several isoforms ranging from 365 to 770 amino acid residues. The three major forms that encode the APP protein are APP 695, APP 751 and APP 770 [4]. APP 751 and APP 770 contain a Kunitz-type serine protease inhibitors (KPI) domain, which aids in blood coagulation, and these isoforms are expressed in most tissues. APP 695 isoform lacks the KPI domain and is predominately expressed in neurons [5]. Orthologous proteins have been identified in other organisms such as Drosophila, C. elegans, and all mammals [4]. The Aβ region of the protein, located near the C-terminal end and partially buried in the transmembrane domain, is not well conserved across species and has no obvious connection with APP’s native state biological functions.

APP is a type I integral membrane protein enriched in neurons. Its primary function is still unknown, though it has been proposed to have roles as a regulator of synapse formation and repair, cell signaling, neuronal plasticity, and cell adhesion [4, 6]. APP expression is up-regulated during neuronal differentiation and after neural injury. Post-translational processing of APP, specially γ-secretase cleavage, is similar to that of the surface receptor protein Notch, drawing attention to the role of cell signaling [7]. APP knockout mice are viable and have relatively minor phenotypic effects including impaired long-term potentiation and memory loss without general neuron loss [8]. On the
other hand, transgenic mice with up-regulated APP expression have also been reported to show impaired long-term potentiation [9].

**Post-translational processing of APP**

APP is post-translationally modified by N- and O-glycosylation, phosphorylation and tyrosine sulphation [4]. It is then modified by proteolytic processing to generate peptide Aβ fragments [10]. Full-length APP is sequentially processed by α-, β- and γ-secretases (Fig 1). Cleavage by α-secretase or β-secretase within the extracellular domain results in the shedding of nearly the entire extracellular domain to yield large soluble APP derivatives (APPsα and APP sβ respectively) and generating the membrane-tethered α- or β-carboxyl-terminal fragments (CTFs). Several zinc metalloproteinases, including TACE/ADAM17, ADAM9, ADAM10 and MDC9, and an aspartyl protease BACE2 can cleave APP at the α-secretase site located within the Aβ domain, essentially precluding the generation of intact Aβ. The major neuronal β-secretase is a transmembrane aspartyl protease, termed BACE1 (β-site APP cleaving enzyme). Following the extracellular cleavages, γ-secretase processes APP at the carboxyl-terminus of Aβ within the membrane-spanning domain, producing either a 3kDa product (in combination with α secretase) or Aβ (in concert with BACE1 cleavage), respectively, and the APP intracellular domain (AICD) (See Figure 1). The 40 amino acid long Aβ40 is the predominate form of Aβ, however, γ-secretase does produce longer species of Aβ42 [11].

γ-secretase is a high molecular weight complex containing at least presenilin, nicastrin, anterior pharynx defective homolog (APH1) and presenilin enhancer (PEN2). However, there are many γ-secretase substrates in addition to APP and Notch, and the list of substrate is constantly expanding. These processing events occur in various organelles
and also on the cell surface. In neurons, APP is anterogradely transported along the axons and is proteolytically processed during transit. In non-neuronal cells, APP that reaches the plasma membrane is internalized within minutes through the conserved YENPTY motif. Following endocytosis, APP is delivered to late endosomes or can be recycled back to the cell surface.

APP processing has been linked to its presence in lipid rafts. It is believed that APP is differentially cleaved by β-secretase in lipid raft micro-environment, whereas APP molecules outside a raft are more likely cleaved by the non-amyloidogenic α-secretase [12]. γ-secretase activity has also been associated with lipid rafts [13]. The role of cholesterol in lipid raft maintenance has been cited as a likely explanation for the observation that high cholesterol and an ApoE genotype are major risk factors for sporadic AD [14].

Mutations in APP that occur within the Aβ sequence and around the proteolytic cleavage sites that release Aβ are found to be the cause for some forms of FAD. These mutations are found to elevate the levels of Aβ42, which suggests that changes in APP metabolism are central to AD pathogenesis [11].
Figure 1: APP proteolytic processing by α-, β- and γ- secretase

In the non-amyloidogenic pathway, APP is cleaved by α- secretase to produce an N-terminal fragment (NTF) (sAPPα) and a membrane-bound C-terminal fragment (α-CTF or C83). On the other hand, β-secretase cleaves APP in the amyloidogenic pathway generating an NTF (sAPPβ) and β-CTF (C99). These CTF’s are then cleaved by γ-secretase to produce APP intracellular domain (AICD), p3 from a α-CTF and Aβ from β-CTF. (Adapted from http://www.alzforum.org)
**Presenilins**

Presenilins (Presenilin-1 and Presenilin-2) are integral and necessary components of the γ-secretase protease complex. The two human presenilin genes: PS1 (located on chromosome 14 encoding for presenilin 1 (PS-1)) and PS2 (located on chromosome 1 encoding for presenilin 2 (PS-2)). Both genes are closely conserved between species in vertebrates. Presenilins undergo endoproteolytic cleavage in the α helical region of one of the cytoplasmic loops to produce a larger N-terminal and a smaller C-terminal fragment which remain non-covalently attached each other. Endoproteolysis of presenilins is apparently necessary to activate γ-secretase activity. It is believed that mutations in presenilin result in a partial loss of function and this leads to increased production of Aβ42 and results in FAD.

γ-secretase can cut APP at multiple positions to generate Aβ of various lengths. Aβ 40 is the most abundant species but Aβ 42 are widely believed to be the pathogenic species that contributes to AD. Aβ 42 is more likely to aggregate, is the initial Aβ species deposited in brain and constitute the majority of the plaques in brain [15].

Presenilins are multi-pass transmembrane proteins localized mainly in the endoplasmic reticulum and Golgi membranes. The proteins are involved in several signaling pathways (such as Notch, a protein that is important for cell-cell communication involving gene regulation mechanisms that control multiple cell differentiation processes during embryonic and adult life), apoptosis and unfolded protein stress response.

**Amyloid Cascade Hypothesis**
The cause for FAD is a missense mutation in the APP or Presenilin 1 or 2 genes that presumably results in increased Aβ42 production throughout life. Unlike the familial form of AD, the cause of the sporadic form of AD is poorly understood. However, it is hypothesized that failure of Aβ clearance, such as faulty Aβ degradation or inheritance of ApoE4, could cause gradually rising Aβ42 levels in brain. There is also evidence that Aβ production can be increased, as seen in the increased BACE protein or activity in brains of AD individuals.

Alteration in APP expression or proteolytic processing or changes in Aβ stability or aggregation could result in a chronic imbalance between Aβ production and clearance that leads to the clinical-pathological syndrome of AD. Aβ has many fates: oligomerization and association with proteins such as CLAC-P or ApoE in amyloid plaques; clearance by α2-macroglobulin, ApoE, or low density lipoprotein receptor-related protein (LRP); or degradation by proteases such as insulin degrading enzyme (IDE), neprilysin (NEP) or plasminogen (PLG). The amyloid cascade hypothesis, the leading hypothesis in AD pathogenesis, states that gradual accumulation and oligomerization of Aβ42 initiates a complex, multi-step cascade that includes decrease synaptic efficacy, microglial and astrocytic activation result from inflammatory responses, alteration of neuronal ionic homeostasis, alterations of kinase and phosphatase activities that lead to tangle formation and widespread neuronal dysfunction and selective neuronal loss with attendant neurotransmitter defects.

Although the cause of the familial and sporadic forms of AD may be different, the pathogenesis was thought to be similar. According to the logic of this hypothesis, using
animals with the dominant mutation form of AD could offer insight into the general mechanisms underlying all form of AD.

**Genetic factors**

The two forms of AD are familial AD (FAD) and sporadic AD. FAD refers to the inherited form in which multiple cases of AD occur before the age of 65 years. Mutations in one of the three genes: Amyloid Precursor Protein (APP), Presenilin-1 (PS1) and Presenilin-2 (PS2), account for the majority of the known autosomal-dominant form of FAD. The majority of AD cases are sporadic with onset usually after 65 years while 2~5% of the AD cases are FAD. The role of heritability in sporadic AD is still unclear. However a significant association of the apolipoprotein (ApoE) ε4 allele with AD has been consistently demonstrated [16]. There is a close association between increasing Aβ and the genetic factors cause AD. Although the significance of genetic factors might be different for the two forms of AD, the post-mortem brain has shown that sporadic AD and familial AD have similar phenotypes. Therefore, studying the mutations underlying FAD might give insight into sporadic AD.

More than 20 pathogenic mutations in APP result from codon replacements in the Aβ region, providing important insight into the mechanism of AD and have been significant in constructing animal models for AD. Mutations in APP are located within or flanking the Aβ peptide region and thus affect α-, β- or γ-secretase activity for Aβ production (Fig 2). The mutation located prior to the N-terminus to the β-secretase cleavage site in which lysine and methionine residues change to asparagine and leucine, respectively, is called the Swedish APP mutation. This mutation was found to increase β-secretase efficiency and result in more Aβ40 and Aβ42 production. Mutations located
after the $\alpha$-secretase cleavage site include the Flemish (A692G), Dutch (E693Q), Arctic (E693G), Italian (G693K), and Iowa (D694N). These mutations have decreasing $\alpha$-secretase activity while increasing $\beta$-secretase activity to cleave C-terminal fragment of APP and results in increasing formation of N-terminal fragments of A$\beta$ protein, leads to the likelihood of amyloid plaque formation. For example, the Flemish mutation has prominent cerebral amyloid angiopathy and unusually large senile plaque cores [17] and the Dutch mutation was found to have cerebral hemorrhage with amyloidosis [18]. The mutations after the $\gamma$-secretase site include the Iranian (T714A), Austrian (T714I), French (V715M), German (V715A), Florida (I716V), London (V717I), Indiana (V717L), and Australian (L723P) types. These mutations have increased $\gamma$-secretase activity to increase A$\beta$42 level. The APP mutations occurred in a small number of families characterized by excessive A$\beta$ production.

Presenilin mutations also alter APP processing and cause an increased level of A$\beta$42 production. To date, over 140 mutations have been found within the PS genes that are associated with early onset FAD. Mutations within PS1 have an age of onset between 30 and 50 years and they account for 18-50% of FAD. Mutations within PS2 have an age of onset between 40-80 years and result in increased A$\beta$ peptide production.

To date, the most important genetic risk factor for AD is the ApoE gene. The ApoE gene is located on chromosome 19q13.2. ApoE is a plasma glycoprotein with a molecular mass of 34,200 Da that contains 299 amino acids. The three polymorphisms of ApoE are ApoE2, ApoE3 and ApoE4. The E3 allele is considered normal and occurs in ~74% of population whereas the E2 and E4 alleles are less common. Individuals with ApoE4/E4 genotype were associated with the highest risk of late onset AD. An average
age of onset of 85 years is given for individuals with no E4 allele, 75 years for one E4 allele and 68 years for individuals with two copies of the E4 allele [19].
Figure 2: Mutations in APP

Missense mutations in APP are the causative factor in familial AD (FAD). The circled amino acid symbols are the mutated site. The arrows point to the consequential amino acid mutation. The name under each mutation site refers to the country each family originates and is the conventional nomenclature. For example, the Swedish mutation is usually seen in families from Sweden. (From Current Opinion in Neurobiology) [20].
Wallerian degeneration mutation in WLD<sup>s</sup> mice

Wallerian degeneration occurs in both the peripheral nervous system (PNS) and central nervous system (CNS). It is the degeneration of the distal stump of an injured axon which normally takes place over a time course of around 24 hours after a lesion, beginning with disintegration of the axonal cytoskeleton and breakdown of the axonal membrane.

The slow Wallerian degeneration mouse, C57BL/WLD<sup>s</sup>, carries a dominant mutation that markedly delays Wallerian degeneration in the distal stump of an injured axon. Injured axons from the peripheral or central nervous system of the WLD<sup>s</sup> retain the ability to transmit a compound action potential for up to 3 weeks. The WLD<sup>s</sup> gene, which maps to a single locus on mouse chromosome 4, was identified by positional cloning [21]. The gene is a chimeric 85 kb tandem triplication and encodes an in-frame fusion protein of the N-terminal 70 amino acids of ubiquitination factor Ube4b/Uf2a, an E4 ubiquitin ligase, and the entire coding sequence of NAD synthesizing enzyme Nmnat1 (nicotinamide mononucleotide adenylyltranferase 1), joined by a unique 18 amino acid sequence (Fig. 3). The protein is translated but surprisingly it remains in the nucleus and is undetectable in axons [22].

The WLD<sup>s</sup> mutant phenotype implies that axon degeneration is an active process intrinsic to the axon itself and not necessary connected to classical apoptosis mechanisms in the cell body. However, there is still controversy regarding how the WLD<sup>s</sup> protein slows Wallerian degeneration. Two hypotheses derived from the composition of WLD<sup>s</sup> gene product have been proposed: 1) dominant-negative inhibition of wild-type Ube4b by WLD<sup>s</sup> and 2) enhanced NAD biosynthesis via increased level of Nmnat1. At present,
there is more support for the dominate-negative effect of ubiquitination theory [23].
Ubiquitin is conjugated to proteins through an isopeptide linkage involving the C-
terminal carboxylate of ubiquitin and the ε-NH₂ group of a lysine side chain of the target
protein, making it for destruction by the proteasome [23]. It was observed that blocking
the ubiquitin proteasome system (UPS) with lactacystin partially mimics the delay in
Wallerian degeneration deliberated by the WLD⁸ gene [24]. There are several criticisms
about this hypothesis. First, general blockade of the UPS is toxic for axons [21]. Second,
WLD⁸ protein is mainly found in nucleus and has not been found in axons in vivo. Third
and most important, Araki et al. showed that the fusion protein of 70 amino acids Ufd2a
portion of the WLD⁸ protein does not delay axonal degradation in response to axotomy.
In contrast they demonstrated that increased Nmnat activity is most likely responsible for
the axon-rescuing activity of the WLD⁸ protein [25]. Furthermore, they demonstrated that
an increase in NAD biosynthesis or enhanced activity of the NAD-dependent deacetylase
SIRT1 protects mouse neurons from mechanical or chemical injury. A boost in NAD
levels is required to maintain SIRT1 activity in the nucleus suggesting that neuronal
survival requires the maintenance of adequate NAD, but NAD levels beyond this point
confers no additional benefit. The protective effect of WLD⁸ protein appears to be exerted
in the nucleus because addition of NAD after removal of cell bodies in the neuronal
cultures is no longer protective [26]. However, over-expression of Nmnat1 or NAD
could not replicate effect of WLD⁸ protein exerts [27]. It is possible that an alternative
program of gene expression is initiated by NAD or SIRT1 activity in the nucleus and
indirectly regulates the UPS, leading to the production of factors that actively block
Wallerian degeneration.
Studies showed that blockage of transport and axonal damage lead to increase APP proteolysis and elevate Aβ production in an axonal vesicle compartment [28]. Further, Aβ region of APP is suggested to interact with the Kinesin light chain motor protein and could link to neuronal apoptosis after disruption of axonal transport [28]. In addition, cognitive deficits in animal models of AD were found to occur before neuronal degeneration. It has been hypothesized that synapses, instead of neurons, are the first structures to be affected by toxic Aβ oligomers.

Several studies have shown that the molecular mechanism for the protective effects of the WLD³ protein is not restricted to the axon but also involves the synapses [29, 30]. WLD³ had already been shown to offer protection against the neurotoxin vincristine in vitro [31], retard disease progression in mouse models of motorneuron disease [30], prevents degeneration of dopaminergic fibers in the mouse model of Parkinson’s disease [32], and of peripheral sensory neurons affected by chemotherapeutic agents [33], improves to condition of progressive motor neuropathy (Pmn) mice [34] and moderately increases the survival rate in amyotrophic lateral sclerosis (ALS) mice models [35]. Thus, WLD may provide general benefit in not only acute but also chronic neuronal injury, such as neurodegenerative diseases. In this way, WLD³ protein may be beneficial in Alzheimer’s disease models. Accordingly, we are interested in testing the neuroprotective or synaptoprotective effects of the WLD³ protein in a mouse model of Alzheimer’s disease, PDAPP_{SWIND} over-expressing transgenic mice (J20). Axonopathy has been described in the AD models and since the WLD³ protein appears to be nuclear-based, there is the potential that both axons and dendrites that are subject to Aβ-induced dysfunction may be benefited from this protein. In addition, Resveratrol, a compound that
enhances SIRT1 activity, has been shown to inhibit Aβ-induced toxicity. The WLDs protein has shown promise as a protective factor in other models of neurodegeneration, but has not been tested in AD models. Therefore, this study examines the phenotype of animals resulting from crossing the WLDs mutant mice with APP transgenic mice.
Figure 3: Chimeric WLD₈ protein

WLD₈ is a chimeric protein composed of the N-terminal 70 amino acids of Ube4b, a linker region encoding 18 amino acids, and the entire coding region of Nmnat1. (From Journal of the Neurological Science) [21]
MATERIALS AND METHODS

Animals

Wld\textsuperscript{S} (+/-), APP transgenic (+/-), Wld\textsuperscript{S} (+/-)-J20 (+/-) and non-transgenic mice were used in this experiment. APP transgenic mice (line J20) express the human APP isoforms APP695, APP 751 and APP770 with the Swedish (K670N/M671L) and Indiana (V717F) mutations under the control of the PDGF-\(\beta\) promoter (Platelet Derived Growth Factor), hereon designated as PDAPP. Over-expression of the APP mutations result in amyloid deposition in hippocampus and neocortex starting around 6 months of age and quite abundant at 8 to 10 months. This transgenic line was obtained from Dr. Lennart Mucke at UCSD. Breeders for the slow Wallerian degeneration mouse, C57BL/Wld\textsuperscript{S}, were purchased from Harlan Sprague Dawley, UK. To obtain the desired genotypes, J20 APP (+/-) breeders were crossed with Wld\textsuperscript{S} (+/-) mice to generate Wld\textsuperscript{S} (+/-), J20 APP transgenic (+/-), Wld\textsuperscript{S} (+/-)-J20 (+/-) and non-transgenic mice. All of the animals are in the C57BL6 background.

Genotyping

Genomic DNA extraction

Animals were genotyped by DNA extracted from mouse tails that were digested overnight in buffer containing 100 mM tris HCl pH 8, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 60 \(\mu\)g proteinase K. After centrifugation at 13,000 RPM for 15 minutes, DNA was precipitated from the supernatant with isopropanol, gathered around a pipette tip and re-suspended in water.

Polymerase Chain Reaction (PCR)
1 μl of the re-suspended DNA was mixed with 19μl of 10X Taq DNA polymerase buffer, 25 mM MgCl2, 10 mM dNTP, 10⁻⁵ M forward primer, 10⁻⁵ M reverse primer, Taq polymerase and water. The forward and reverse primer sets to genotype Wld⁸ are (5’-CGTTGGCTCTAAGGACAGCAC-3’) and (5’-CTGCAGCCCCCACCACCCCTT-3’) respectively with the conditions: 94°C, 45 s; 60 °C, 45s; 72 °C, 45 s for 35 cycles. The forward and reverse primer sets to genotype J20 APP are (5’-GGTCATGAGAATGGGAAGAG-3’) and (5’-TGAGCATGGCTTCAACTCTG-3’) respectively with the conditions: 94 °C, 20 s; 59 °C, 30s; 72 °C, 80s for 35 cycles.

**Perfusion**

Animals were sacrificed at 12.5 to 13 months of age. The mice were anesthetized with Pentobarbital and perfused with 100 mL of ice cold 1X phosphate buffered saline (PBS). After perfusion, the brains were dissected out, the cerebellum and olfactory cortex were removed and then bisected sagittally. One hemisphere was frozen at -80°C and the other was immersed in freshly prepared 4% paraformaldehyde for 24 to 48 hours. Following fixation, the tissue was cryoprotected in 30% glycerol for 72 hours, sectioned at 40 μm, and stored in anti-freeze tissue collecting solution (20% glycerol, 30% ethylene glycole and 50 mM of disodium hydrogen phosphate) in 96 well plates with 3 tissues per well at -20°C..

**Histology**

*Thioflavin S staining*

Thioflavin S staining was performed on four sections per animal taken from approximately the same anterior to posterior regions for each brain and mounted on the same glass slides to staining. The sections were placed in a slide holder, washed twice in
1X PBS for 5 minutes, placed in 0.25% Potassium Permanganate (diluted with PBS and filtered) for 10 minutes, washed three times in 1X PBS for 5-minutes, cleared in 2% Potassium disulfate and 1% oxalic acid solution (diluted wit milliQ water) in hood for 2 minutes, washed in milliQ water for 10 minutes, stained with 0.015% Thioflavin-S (diluted in 50% ethanol), washed twice in 50% ethanol for 4 minutes and finally rinsed twice in milliQ water for 5-minutes. Slides were then cover slipped with Dako Cytomation Fluorescent Mounting Media (DakoCytomation)

**Aβ immunostaining**

Aβ immunostaining was performed on four sections from each brain similar to that used for Thioflavin S staining. Slides were placed in a slide holder and rinsed in 1X PBS for 5 minutes, incubated in 10 μg/ml of proteinase K (diluted in PBS) for 15 minutes, rinsed three times in 1X PBS for 5 minutes and then individually incubated in 10% hydrogen peroxide and 1% Tween-20 (diluted in PBS) for 30 minutes. Three more rinses in 1X PBS were done in the slide holder for 5 minutes and then each was blocked for one hour in 3% normal horse serum and 0.5% Triton X-100 (diluted in PBS). Finally three more rinses in 1X PBS were done in the slide holder for 10 minutes each before each slide was incubated with 4G8 (1:1000, Signet), a mouse monoclonal antibody recognizing all Aβ isoforms, overnight at 4°C. The next day slides were placed in the slide holder and washed three times in 1X PBS for 5 minutes each, then individually incubated in the universal secondary antibody form the ABC kit (in 5% BSA and 5% goat serum) for 30 minutes at room temperature. Slides were washed three times in 1X PBS for 5 minutes, individually incubated in ABC (in PBS) for 30 minutes at room temperature, and then washed twice in 1X PBS for 10 minutes each. DAB was added to
each slide for approximately 3 minutes subsequently the reaction was stopped with milliQ water. Slides were dehydrated by placing them in 70% ethanol, 95% ethanol, 100% ethanol and lastly xylenes for 2 minutes each. The sections were then cover-slipped with Permount (Fischer Scientific).

**Image Analysis**

All sections were analyzed by light and fluorescence microscopy using a Nikon Eclipse E800 microscope. Aβ plaque detected by immunostaining or Thioflavin S staining within the hippocampus were analyzed with ImagePro software (Media Cybernetics, Inc.) from images photographed at 4X in color but converted to grayscale for quantitation. First, the total hippocampal area was outlined. Then, the threshold for Thioflavin S or Aβ immunostained images were set with reference to a histogram displaying the intensity difference. The background was flattened and manually edited to exclude blood vessel staining or artifacts. The Aβ plaque burden stained by thioflavin S or Aβ immunoreactivity was reported as percent total hippocampal area. In addition, Aβ load was quantified from a region of the neocortex overlying the hippocampus using the same procedure.

**Brain extraction**

**CHAPSO extraction**

Ice cold 1% CHAPSO with protease inhibitor (Roche Complete cocktail) was added to each hemibrain. Hemibrains were then homogenized using Dounce homogenizer over ice on speed 5 three times for ten seconds each. The homogenates were incubated for 30 minutes at 4°C in the rotator and then transferred into thick polycarbonate centrifuge tubes (Beckman 349622) and ultracentrifuged of 46,000 rpm
(100,000Xg) at 4°C for 60 minutes. After centrifugation, the supernatants aliquoted into clean microfuge tubes and stored at -80°C. The pellets were saved for formic acid extraction.

Formic acid extraction

1 ml of 70% formic acid was added to the CHAPSO resistant pellet and then homogenized three times for ten seconds each on ice. The homogenates were ultracentrifuged at 46,000 rpm at 4°C for one hour. The clear sample of supernatant between the lipid layer and pellet was collected with a 25 Ga 1 1/2 needle with 1 ml syringe. The resultant supernatant was neutralized to pH8 with nine volume of 2M Tris Base (pH 11.2). Protease inhibitor cocktail (Roche) was added to this neutralized solution, mixed and centrifuged at 3,000 rpm for 10 minutes. The supernatant from this final centrifugation was stored at -80°C.

Aβ ELISA

Aβ 40 and Aβ42 levels were determined from duplicated samples for both CHAPSO and Formic acid brain extracts ELISAs. On day one, ELISA plates (Immulon 4HBX) were coated with Ab9 (recognizing the N-terminus of Aβ) or MM26.2.1.3 (specific for Aβ42) at 50 µg/ml in PBS (100 ul/well) for detection of Aβ40 or Aβ42, respectively. Following overnight incubation at 4°C, the plates were washed extensively and then blocked overnight with Blockace (Serotec Ltd.). On day three, the plates were rinsed extensively and brain samples (CHAPSO or formic acid extracts) added to the plates. The CHAPSO samples were diluted 1:5 for both Aβ40 and Aβ42 measurements while the formic acid samples were diluted 1:4 for Aβ40 and 1:40 for Aβ42
measurements. Samples and standards were vigorously vortexed for 5 seconds prior to loading and 100 ul of samples or standards were loaded into each well. After overnight incubation at 4°C, the plates were washed again and HRP-conjugated secondary antibody was added (13.1.1 monoclonal antibody specific for Aβ40 and HRP-conjugated Ab9 antibody for Aβ42 at 1μg/ml concentration). The plates were then covered with sealing tape and incubated for four hours at room temperature. The solution was discarded, washed, and 100 ul of pre-warmed TMB membrane substrate developing solution was added to each well. The reaction was stopped with 100 ul of 2M H2SO4 in PBS. Plates were read at λ450 nm in an ELISA plate reader. Aβ40 and Aβ42 values from CHAPSO and Formic acid were normalized to the peptide standards and expressed as concentration per wet weight of brain tissue.

**Western blot**

*Tricine/Tris SDS-PAGE Urea gel*

Total Aβ was immunoprecipitated from 200 ul of CHAPSO extracts with B436 monoclonal antibody specific to the N-terminus of Aβ. After overnight incubation together with agarose anti-mouse beads and protease inhibitor cocktail (Sigma) the beads were centrifuged at 3000 RPM 4°C for 5 minutes, washed with 1% NP40 three times. The immunoprecipitates were resuspend in Laemmli sample, spun, and the supertants were fractionated in 10% Tricine/Tris SDS-urea--PAGE. Following transfer, the nitrocellulose membrane was boiled for 5 minutes and then blocked for 1 hour at room temperature with 5% milk in TBS with 0.1% Tween (TBST). The membrane was washed three times with TBST and incubated overnight with 82E1 monoclonal antibody (specific to the N-terminus of Aβ). Following washing, the membrane incubated with goat anti-
mouse horseradish peroxidase secondary antibody in 5% milk in PBST.

Immunoreactivity was detected with SUpersignal ® chemiluminescence reagent (Pierce, Rockford, IL) for 5 minutes and then developed on autoradiography film.

**NuPage gel**

To detect full length APP, 10 μg CHAPSO extracts were fractionated by SDS-PAGE in NuPAGE™ 8% Bis-Tris gels (Invitrogen, San Diego, CA). Following transfer, the nitrocellulose membranes were blocked for 1 hour at room temperature in TBS with 0.1% Tween-20 (TBST) and 5% nonfat dry milk. The membrane was incubated with CT15, an polyclonal antibody that recognizes the C-terminus of APP, in 5% nonfat dry milk in TBST at a dilution of 1:5000 overnight at 4°C. The membrane was washed three times in TBST for 5 minutes. The primary antibody was detected with goat anti-rabbit horseradish peroxidase secondary antibody in the same buffer as the primary antibody at a dilution of 1:10,000 for two hours. The membrane was washed again three times in TBST and one last time in TBS for five minutes. The blots were treated with SuperSignal® (Pierce, Rockford, IL) for 5 minutes and then developed on autoradiography film.
RESULTS

Survival rate among all genotypes

The goal of this study is to see if one copy of WLD\(^+\) gene would have any effects in the PDAPP transgenic mouse line (J20) of amyloid deposition. Because there is a modestly prolonged survival in the SOD1 model in fALS mouse [33], we first want to see if there is a difference in survival rate between WLD; PDAPP and PDAPP animals. The PDAPP mouse is known to have a mild early lethality with about 20% premature death within the first year of life. All animals were included into this study after they were genotyped (except some mice that were weaned prematurely and were dead due to mal-nutrient were excluded from this study). All animals were past 13 months old and either terminated or used for behavior test at the time when the graph was plotted. There were 25 WLD(+/-)PDAPP (+/-), 25 WLD(+/-), 29 PDAPP (+/-) and 28 wild-type mice included for this study. Kaplan-Meier Survival Curves were plotted for this entire cohort of animals (Fig 4). Data was analyzed with paired ANOVA. There was no significance between PDAPP and WLD; PDAPP (p>0.05) or between WLD and non-Tg (P>0.05). However, there was a significant survival difference between animals with PDAPP gene and animals without (p<0.001). Thus, there was no obvious modulation of the premature death phenotype in the PDAPP APP transgenic mice by the WLDs mutant.

Amyloid Pathology

Next, we investigated the effects of WLD\(^+\) protein on amyloid pathology. We examined the brains of animals with all 4 genotypes: WLD;PDAPP, WLD, PDAPP and wild-type mice. We wanted to compare the amyloid pathology especially between
WLD;PDAPP and PDAPP groups to see if there is a significant difference to have the WLD<sup>4</sup> mutation. Multiple assessments of amyloid pathology were carried out: Thioflavin S staining, total Aβ immunostaining, Aβ<sub>40</sub>/Aβ<sub>42</sub> ELISAs and Western blots were performed on brain sections and homogenates from all four genotypes.

**Thioflavin S staining**

The purpose of Thioflavin S staining is to visualize senile plaques that appear starting age of 8~10 months in PDAPP transgenic mouse. Thioflavin S is a homogenous mixture of compounds that results from the methylation of dehydrothiotoludine with sulfonic acid. When Thioflavin S dye binds to the β-sheets of the amyloid fibrils, the dye undergoes a 120 nm red shift of its excitation spectrum, resulting in a fluorescent signal at 482 nm. This red shift is only observed if amyloid fibrils are present, and will not undergo upon binding to precursor monomers or small oligomers. Thioflavin S also stains neurofibrillary tangles but these lesions are absent in the PDAPP mice.

Animals were aged to 12.5 to 13 months and separated into WLD; PDAPP (Fig 5), PDAPP (Fig 5), WLD (Fig 6) and wild type (Fig 6) groups. First, as expected, neither the WLD nor wild type non-transgenic animals showed any amyloid deposits (p>0.05, Fig. 7). Plaques were only seen in the PDAPP and WLD;PDAPP mice. However, although there was a slight trend in lower amyloid load by thioglavin S staining in the hippocampus, there was no significant difference between these two groups (p>0.05, Fig. 7)

**Aβ immunostaining**

Thioflavin S stained plaques are dense amyloid cores consisting of highly aggregated fibrillar Aβ. To demonstrate all Aβ deposits, Aβ immunostaining with 4G8
antibody which recognizes the amino acid residues 17-26 region of Aβ, was carried out on four additional sections per animals. Similar to the results from Thioflavin S staining, there was essentially no Aβ staining in the WLD (Fig.9) and wild type (Fig. 9) control animals (p>0.05, Fig. 10a, 10b). Further, there was no difference between WLD;PDAPP (Fig. 8) and PDAPP animals in terms of plaque load fraction area in either the hippocampus or the neocortex (p>0.05, Fig. 10a, 10b)

Aβ 40 and Aβ42 ELISA results

The hypothesis of this study is that WLD8 protein might interact with the APP protein or have some unknown function at the synaptic in the PDAPP mouse model. Although there are no differences in fibril Aβ and total Aβ burden between WLD;PDAPP and PDAPP animals, we are interested to see if WLD8 protein alters the levels of soluble and insoluble Aβ. Therefore, Aβ40 and Aβ42 levels were measured from CHAPSO soluble and formic acid soluble fractions by ELISA from frozen hemi-brain samples from animals at 12.5 ~13 month old.

Insoluble Aβ40 and Aβ42 level results showed that there were no significant differences between WLD;PDAPP and PDAPP (p>0.05, Fig 11) or between WLD and wild type animals (p>0.06, Fig 11a, 11b). These two groups were significantly higher than the WLD and wild-type animals as expected (p<0.001, Fig 11). Results from CHAPSO soluble Aβ40 and Aβ42 were essentially the same as insoluble Aβ40 and Aβ42 levels (p>0.05, Fig 12). There was also no significant difference in the levels of soluble Aβ40 and Aβ42 level between WLD and wild type (p>0.05, Fig 12). As before, APP transgenic animals (WLD; PDAPP and PDAPP) showed higher levels of soluble Aβ40
and Aβ42 than WLD and wild-type animal (p<0.001, Fig 12b) (p<0.05, Fig 12a) and wild type (p<0.001, Fig 12a).

*Western blotting of Aβ and APP levels*

The reason the ELISA result for soluble Aβ40 showed no significance between PDAPP and WLD animals might be that the ELISA system is not very sensitive to soluble Aβ40 from CHAPSO extracts. To verify the results, we performed immunoprecipitations/western blotting using CHAPSO extracts with B436 antibody, a monoclonal antibody that reacts with the amino-terminus of Aβ as well as sAPP. The immunoprecipitates fractionated in tris-tricine gels to separate Aβ40 and Aβ42 species and then blotted with 82E1, a monoclonal antibody that recognizes the free N-terminus of Aβ. This experiment showed that WLD;PDAPP and PDAPP have similar levels of soluble Aβ40 and Aβ42 but were hardly detectable in WLD and non-transgenic animals (Fig. 14).

Besides soluble and insoluble Aβ level, we also checked if there are any differences in the APP level for WLD;PDAPP and PDAPP animal brains. CHAPSO extractions were fractionated in SDS-PAGE and immunoblotted with a polyclonal antibody that recognizes the C-terminus of APP (Fig 15). Not unexpectedly, WLD;PDAPP and PDAPP have similar levels of APP while the WLD and non-transgenic animals have much lower APP expression. This is because both WLD;PDAPP and PDAPP have over expression of APP while the WLD and non-Tg animals only have the endogenous mouse APP.
The survival rate of each genotype was analyzed with repeat comparison ANOVA. The survival rate between WLD; PDAPP and PDAPP or between WLD and non-Tg are not significantly different (P>0.05). n=29 for PDAPP, n=25 for WLD; PDAPP, n=25 for WLD, and n=28 for non-Tg. The survival rate between PDAPP and non-Tg and PDAPP with WLD are highly significant (p<0.001). The survival rate difference between WLD; PDAPP with non-Tg or with WLD are also significant (p<0.01). No significance between WLD; PDAPP and PDAPP (p>0.05).

Figure 4: Kaplan-Meier Survival Curves
**Figure 5:** Thioflavin S Staining of neuritic plaques in the hippocampus – WLD; PDAPP vs PDAPP

Representative photomicrographs of Thioflavin S stained hippocampi in WLD; PDAPP and PDAPP animals. All pictures were taken at 4X and one to four sections were taken to cover the whole hippocampus and stitched up (if necessary) before plaques counting. Magnification 40X.
**Figure 6:** Thioflavin S Staining of neuritic plaques in the hippocampus – WLD vs wt

Representative photomicrographs of Thioflavin S stained hippocampi in WLD and wild type animals. All pictures were taken at 4X and one to four sections were taken to cover the whole hippocampus and stitched up (if necessary) before plaques counting. Magnification 40X.
Figure 7: Plaque burden

Plaque burden in the hippocampus of 12.5~13 month old animals were detected by Thioflavin S staining. Data was analyzed by one-way ANOVA followed by Turkey post-hoc test. There is no significance between WLD; PDAPP and PDAPP (p>0.05) or between WLD and wt (p>0.05). All genotype has n=9. WLD; PDAPP has significance from WLD or non-Tg (p<0.001). PDAPP also is significant from WLD or non-Tg (p<0.001). (Error bars:±SEM)
Figure 8: Immunostaining of Aβ in the hippocampus – WLD; PDAPP vs PDAPP

Representative photomicrographs of immunostained hippocampi in WLD; PDAP and PDAPP animals. 4G8 was used at 1:1000. All pictures were taken at 4X and one to four sections were taken per section to cover the whole hippocampus and stitched up (if necessary) before counting. Magnification 40X.
**Figure 9:** Immunostaining of Aβ in the hippocampus – WLD vs wt

Representative photomicrographs of immunostained hippocampi in WLD and wild type animals. 4G8 was used at 1:1000. All pictures were taken at 4X and one to four sections were taken per section to cover the whole hippocampus and stitched up (if necessary) before counting. Magnification 40X
Figure 10: Aβ burden in the hippocampus and cortex

Total Aβ was detected with 4G8 from 4 sections of brain slices. The percent area was calculated as Aβ stained area over total hippocampal (A) or sampled cortical area (B). Data was analyzed by one-way ANOVA followed by post-hoc test. Both hippocampal and cortical Aβ burden percent between WLD; PDAPP and PDAPP or between WLD and wt has no significance (p >0.05 for both Aβ level, n=9 for each genotypes, error bars:±SEM)
Figure 11: Formic acid extraction Aβ40 and Aβ42 sandwich ELISA

All samples were run in duplicate. Mean result were re-calculated and adjusted according to the dilution factor and weight of hemisphere. Data was analyzed using one-way ANOVA. (A) Aβ40 level (B) Aβ42 level from formic acid extraction in hemisphere of 12.5~13 month old mice. Data was analyzed using one-way ANOVA test followed by Turkey post hoc test. There is no significant difference between WLD; PDAPP and PDAPP or between WLD and non-Tg (p>0.05 for both Aβ40 and Aβ42 level, n=9 for each genotypes, error bars: ±SEM).
Figure 12: CHAPSO extraction Aβ40 and Aβ42 sandwich ELISA

All samples were run in duplicate. Mean result were re-calculated and adjusted according to the dilution factor and weight of hemisphere. Data was analyzed using one-way ANOVA. (A) Aβ40 level (B) Aβ42 level from CHAPSO extraction from hemisphere of 12.5–13 month old mice. Data was analyzed using one-way ANOVA test followed by Turkey post hoc test. There is no significance between WLD; PDAPP and PDAPP or between WLD and wt (p>0.05 for both Aβ40 and Aβ42 level, n=9 for each genotypes, error bars: ±SEM)
Figure 13: CHAPSO extraction Western blot

Aβ40 and Aβ42 were immunoprecipitated by B436 from CHAPSO extraction samples followed by immunoblotted with antibody 82E1. WLD; PDAPP and PDAPP animals have strong Aβ40 and Aβ42 expression level while WLD and wt littermates have none in Western blot.
Figure 14: Characterization of APP levels

Full length APP and tubulin were immunoblotted from CHAPSO extraction brain samples. Wt and WLD animals have less APP expressed while the PDAPP J20 and WLD; PDAPP have higher level of APP. The same blot was incubated with tubulin antibody to normalize the protein loading.
CONCLUSION

To determine whether partial preservation of axonal degeneration ameliorates AD pathology in an APP transgenic mouse line over-expressing human APP carrying two FAD mutations, WLD mutant was introduced into these APP transgenic mice. Examination of survival rate, plaque burden and soluble and insoluble Aβ levels in the hippocampus and cortex demonstrated that the presence of WLD\textsuperscript{a} protein did not affect any of these parameters.

First, we investigated if expression of WLD\textsuperscript{a} protein would improve the survival rate in the PDAPP mice. We found that both WLD;PDAPP and PDAPP animals showed the premature death between 4 to 6 months old. The overall survival rate was no different between these two groups while both of them have significantly lower survival rate compare to either WLD or wild type animals. This indicates that either WLD\textsuperscript{a} protein does not provide any functional protection on PDAPP mice or simply just that the synaptic protective effect does not influence survival rate in this model.

Next, we examined the plaque deposition, the hallmark of Alzheimer’s disease, and compared the level differences among all genotypes. The WLD; PDAPP and PDAPP mice had significantly different levels of both fibrillar amyloid deposits (by thioflavin S staining) and the total Aβ plaque burden (by immunostaining) as compared to either WLD or wild type animals. However, there was no significant differences of both fibril amyloid deposition and plaque burden between the WLD; PDAPP and PDAPP groups. Both soluble and insoluble Aβ40 and Aβ42 levels from all genotypes showed consistent result as the plaque burden, there was no significant variance between
animals with WLDs mutation and without from the ELISA and Western results. All of these results showed that there was no difference between WLD; PDAPP and PDAPP in terms of Aβ levels and plaque deposits. This suggests that either WLDs protein does not modulate Aβ production and plaque deposit. Previous studies have implicated neuronal injury as a trigger to increase Aβ production. However, it appears that WLDs mutant does not influence this step. Similar level of total APP level and Aβ in WLD; PDAPP and PDAPP animals implies that WLDs protein probably has no interaction with APP.

The WLDs protein does not show alteration in the amyloid pathology in PDAPP mice could be explained in two ways. First, the protective mechanism of WLDs protein might be different than the axonal atrophy caused by the malfunction of interaction of C-terminus of APP with the kinesin light chain protein. The second explanation is that WLDs protein could only show its protective effect for acute neuronal injuries, not for chronic neurodegenerative lesions. The WLDs protein has been shown to have effectiveness to delay axon degeneration for only two to three weeks after insult. From the neurodegenerative models studied through WLDs protein, those showed most effectiveness are models with acute lesion (Table 1) with shorter period of studies. For example, the myelin protein zero model showed effectiveness at 3 months but not at 5.5 months. This suggests that the protective effect may have a time dependency. It is possible that the WLDs protein might benefited during the early stage of the disease for PDAPP mice, however, as the animals aging, the protective effect wear out. Moreover, though WLDs protein may delay the degeneration of axon and slow down the elimination of synapses, it does not prevent the accumulation of APP in the injured axon [36]. As the
result, the axon and synapse could be protected while the amyloid pathology remains unaltered as the APP transgenic mice.

Studies suggest that synaptic dysfunction, rather than outright cell death, may underlie many of the deficits present in Alzheimer’s disease. Not only is synapse loss better correlated with mental decline than neuron loss, but also synaptic dysfunction. Factors that influence the health of synapses and synaptic function are fundamental areas of focus in AD research. Evidences have shown that Aβ can have impact on synaptic protein expression, synaptic transmission, synapse size and synaptic plasticity. For future experiments, we could utilize the ability of WLDs protein to delay axon degeneration and prevent synapse loss, and perform electrophysiology, cognitive behavioral testing such as fear conditioning and object recognition to determine if the WLDsPDAPP animals have rescued LTP and improved learning and memory deficits typically seen in the PDAPP mice. This is certainly possible in spite of no significant difference in amyloid pathology for these two groups.
Table 1: Effectiveness of WLD<sup>+</sup> in blocking axon degeneration
(Nature Reviews) [36]

<table>
<thead>
<tr>
<th>Insult</th>
<th>Nature of insult</th>
<th>Age of onset or acute lesion</th>
<th>Effectiveness of WLD&lt;sup&gt;6&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nerve transaction</td>
<td>Physical</td>
<td>Acute</td>
<td>2-3 week delay&lt;sup&gt;15,23&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nerve crush</td>
<td>Physical</td>
<td>Acute</td>
<td>2-3 week delay&lt;sup&gt;15,23&lt;/sup&gt;</td>
</tr>
<tr>
<td>Taxol</td>
<td>Toxic</td>
<td>Acute</td>
<td>&gt;2 week delay&lt;sup&gt;24&lt;/sup&gt;</td>
</tr>
<tr>
<td>6-hydroxydopamine</td>
<td>Toxic</td>
<td>Acute</td>
<td>Some axons preserved for at least 11 days&lt;sup&gt;57&lt;/sup&gt;</td>
</tr>
<tr>
<td>pmn</td>
<td>Genetic</td>
<td>3 weeks</td>
<td>Strong protection: 2-3 week delay&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>P0 null</td>
<td>Genetic</td>
<td>6 weeks</td>
<td>Effective at 3 months, but not at 5.5 months&lt;sup&gt;25&lt;/sup&gt;</td>
</tr>
<tr>
<td>gad</td>
<td>Genetic</td>
<td>6 weeks</td>
<td>Reduces pathology at 4 months, but no improvement in symptoms&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>SOD1 G93A transgene</td>
<td>Genetic</td>
<td>11 weeks</td>
<td>No axon protection, modest extension of lifespan&lt;sup&gt;14&lt;/sup&gt;</td>
</tr>
<tr>
<td>SOD1 G37R transgene</td>
<td>Genetic</td>
<td>4-6 months</td>
<td>No protection at 5-6 months&lt;sup&gt;13&lt;/sup&gt;</td>
</tr>
<tr>
<td>SOD1 G85R transgene</td>
<td>Genetic</td>
<td>9-10 months</td>
<td>No protection at ~1 year&lt;sup&gt;13&lt;/sup&gt;</td>
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<tr>
<td>Plp null</td>
<td>Genetic</td>
<td>8-18 months</td>
<td>No protection at 18 months&lt;sup&gt;94&lt;/sup&gt;</td>
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</table>

<sup>gad</sup>, gracile axonal dystrophy; <sup>Plp</sup>, proteolipid protein; <sup>pmn</sup>, progressive motor neuropathy; <sup>P0</sup>, myelin protein zero; SOD1, superoxide dismutase 1.
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