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Rohitha Ananth Moudgal April 12, 2016

Evidence that pathological Tau functions through inhibition of lysine-specific demethylase 1A in tauopathy model mice

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Abstract

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The pathological aggregation of Tau protein is a known hallmark of Alzheimer's disease (AD) and is sufficient to recapitulate aspects of AD in transgenic model mice. However, the processes downstream of pathological tau have not been adequately identified. Here, we provide preliminary evidence that pathological Tau functions through lysine-specific demethylase 1A (LSD1) in a mouse model of AD. LSD1 normally represses inappropriate transcription of target genes by demethylating histone H3 lysine 4, and an earlier experiment showed that LSD1 is continuously required in adult neurons for survival. Genetic reduction of LSD1 in transgenic mice expressing mutant P301S Tau accelerates aspects of the mutant Tau phenotype, such as neurodegeneration and survival, without accelerating the spread of pathological Tau. These results suggest that the inhibition of LSD1 function may be downstream of pathological Tau. Therefore, it may be possible to target the LSD1 pathway to slow the progression of AD.

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Table of Contents

Figures and Supplemental Figures

Introduction

Alzheimer's disease (AD) is the most common form of dementia and is characterized by the degeneration of neurons leading to a loss of mental function. Typically, the disease begins with worsening memory loss and gradually progresses to severe brain atrophy. Although the precise cause of AD is unknown, aging has been shown to be a main risk factor for AD (Lindsay et al., 2002). AD afflicted an estimated 4.7 million people in the United States in 2010, and the number of patients is expected to increase to 13.8 million by 2050 (Hebert et al., 2013). The societal weight of AD is exacerbated by the immense financial burden of caring for patients. For example, expenditure on dementia in the US during 2010 was estimated at $107 billion (Hurd et al., 2013). Hallmarks of AD include Amyloid-β (Aβ) plaques and neurofibrillary tangles (NFTs) containing hyperphosphorylated Tau, whose interaction is thought to lead to neuronal death (Ittner and Götz, 2011). The Amyloid-β hypothesis of AD posits that aberrant Aβ accumulation begins the disease pathway by stressing the brain (Hardy and Selkoe, 2002) which in turn leads to pathological aggregation of tau (Supplemental Figure 1).

Amyloid-β is a peptide resulting from the cleavage of amyloid precursor protein (APP), a highly conserved integral membrane-protein. The precise primary functions of APP are currently unclear, but it is thought to be involved in synapse function (Priller et al., 2006) and iron export from the cell (Duce et al., 2010). Aβ is produced when APP is cleaved at its extracellular domain by beta secretase followed by cleavage at its intermembrane domain by gamma secretase. Aβ peptides are produced during normal cellular metabolism (Haass et al., 1992). However, formation of Aβ plaques is associated with Alzheimer's disease, possibly due to Aβ's ability to exert oxidative stress within brain cells (Lin and Beal, 2006). The hypothesis

that accumulated Aβ stresses the brain offers an explanation for why mutations in Apolipoprotein E, a regulator of Aβ, are linked to increased risk for AD (Corder et al., 1993). Interestingly, the role of Aβ in AD is often described as prion-like due to its ability to 'seed' plaques when harvested from diseased human brains and intracerebrally injected into healthy primate brains (Ridley et al., 2006).

Transgenic (Tg) model mice expressing mutant human $APP_{K670/671L}$ exhibit memory loss that correlates with the appearance of Aβ plaques (Westerman et al., 2002). This memory loss in $APP_{K670/671L}$ Tg mice can be reversed by neutralizing Aβ with antibodies (Kotilinek et al., 2002). $APP_{K670/671L}$ Tg mice do not recapitulate neurodegeneration, but Tg model mice overexpressing synthetic Aβ exhibit neuronal death in the hippocampus and neocortex (LaFerla et al., 1995). These combined findings suggest that Aβ plaque formation is involved in memory loss and neurodegeneration seen in human AD. However, Aβ plaques are observed in cognitively-normal individuals as well, albeit possibly preclinically (Reiman et al., 2009). In contrast, there is minimal neurofibrillary Tau in cognitively normal elderly control cases (Knopman et al., 2003), suggesting that pathological tau is more indicative of AD. Moreover, research in cultured neurons demonstrates that the presence of Tau protein is necessary for Aβ cytotoxicity (Rapoport et al., 2002). An experiment with model mice expressing transgenic mutant human APP revealed that genetic reduction of endogenous Tau rescues memory and learning deficits resulting from Aβ plaques without altering Aβ plaque formation (Roberson et al., 2007). Taken together, these findings suggest that Tau is downstream of Aβ in AD, which begets the analogy of Aβ and Tau acting as a "trigger" and "bullet" respectively in human AD (Bloom, 2014).

As mentioned above, the Aβ hypothesis of AD includes the formation of pathological Tau aggregates. Mutations in Tau (Microtubule Associated Protein Tau), coded by the *MAPT* gene located on chromosome 17 (Lee et al., 2001), have been linked to frontotemporal dementia (FTD). Tau protein is normally soluble and functions to stabilize microtubules within axons. However, mutations in *MAPT*, such as P301S, have revealed that Tau becomes neurotoxic when hyperphosphorylated and aggregated into insoluble neurofibrillary tangles (NFTs); these mutations in Tau can recapitulate many aspects of AD when expressed in Tg mice (Ballatore et al., 2007). *MAPT* itself is not linked to AD (Roks et al., 1999), but aberrant Tau is involved in many diseases of the brain called tauopathies. For example, NFTs comprised of hyperphosphorylated tau are a characteristic of chronic traumatic encephalopathy resulting from sports concussions (Gavett et al., 2011). Additionally, aberrant Tau has the ability to 'seed' throughout the brain, as demonstrated by an experiment involving the injection of brain extracts from transgenic mice expressing mutant human Tau into the brains of mice transgenic for wildtype human Tau (Clavaguera et al., 2009). The species of aberrant Tau capable of 'seeding' throughout a normal brain has been identified as filamentous and hyperphosphorylated (Jackson et al., 2016).

The characteristics of pathological Tau aggregation in Tauopathy are exemplified by the PS19 mouse model developed by the laboratory of Virginia Lee at the University of Pennsylvania (Yoshiyama et al., 2007). These transgenic mice (hereafter referred to as Tg^{P301S}) express the P301S mutation in *MAPT* in neurons driven by mouse prion promoter at a five-fold higher rate than endogenous Tau. The phenotype of Tg^{P301S} hemizygous mice includes synaptic deficits and microglial activation by 3 months of age, hyperphosphorylated Tau aggregation by

6 months of age, significant neurodegeneration by 12 months of age, and decreased lifespan with a median survival of approximately 9 months. This mouse model recapitulates aspects of AD such as memory/learning deficits (Takeuchi et al., 2011) and severe neuronal loss within the hippocampus (Yoshiyama et al., 2007). The Tg^{P301S} mouse phenotype also includes neuropathological aspects not seen in human AD, such as motor deficits beginning with a hindlimb weakness at 3 months and ending with paralysis at 7 to 10 months of age. These motor deficits are possibly due to pathological tau aggregation in the spinal cord. These tauopathy model mice do not develop Aβ plaques and this is consistent with the disease model of pathological tau being downstream from Aβ. Yoshiyama et al. report a loss in microtubule binding by P301S Tau, but it is unlikely that the Tg^{P301S} phenotype is merely due to impaired Tau function because Tau knockout mice appear phenotypically normal (Dawson et al., 2001). The mechanism by which pathological tau functions is unknown, but it likely involves neurotoxic gain in function such as interfering with the vital cellular function of another molecule.

Lysine-specific demethylase 1 (LSD1), also known as KDM1A, is a highly conserved amine oxidase histone demethylase that recognizes and demethylates mono- and di-methyl groups on lysine-4 of histone H3 (H3K4me1/2). LSD1 functions to repress active transcription of genes by removing this active mark from the structure of chromatin (Shi et al., 2004). As a member of the coREST complex, LSD1 represses the transcription of neuronal genes in non-neuronal lineages (Chong et al., 1995). *Lsd1* is also required to silence embryonic stem cell genes during differentiation (Whyte et al., 2012). Research in *C. elegans* has shown that LSD1 is involved in epigenetically reprogramming the germline (Katz et al., 2009) and this suggests that H3K4me1/2 is involved in preserving the epigenetic memory of transcription. Taken together,

this data suggests that LSD1 is required for embryogenesis in mice (Wang et al., 2009).

Given these findings that LSD1 is involved in cell differentiation, one would not expect that LSD1 would be expressed in adult neurons. However, experiments performed in the Katz lab find that LSD1 is not only expressed in adult neurons but also necessary for their survival in mice (Christopher and Myrick et al., Submitted 2016). The induced deletion of exon 6 in both *Lsd1* alleles by *Cre-Lox* recombination ($Lsd1^{\Delta/\Delta}$) recapitulates many aspects of human AD, such as widespread neuronal loss in the hippocampus and cortex, and memory/learning deficits. Additionally, $Lsd1^{\Delta/\Delta}$ mice exhibit phenotypes that are reminiscent of the Tg^{P301S} phenotype. For example, the deletion of *Lsd1* in adult mice results in severe motor deficits 8 weeks after recombination, beginning with a hindlimb clasp and progressing to full paralysis. This paralysis aspect of the $Lsd1^{\Delta/\Delta}$ mouse phenotype is very similar to motor deficits in the Tg^{P301S} phenotype. Christopher and Myrick et al. also provide RNAseq evidence that dysregulated gene expression in $Lsd1^{\Delta/\Delta}$ hippocampal tissue has significant overlap with dysregulated pathways in prefrontal cortex tissue in human AD patients (Supplemental Figure 2). These RNAseq data raise the possibility that LSD1 function in human AD neurons could be inhibited by pTau. Furthermore, the RNAseq data shows a reactivation in stem cell gene transcription (Supplemental Figure 3), which is inappropriate in fully differentiated neurons. These combined results suggest that LSD1 is constitutively required to maintain cell fate by repressing reactivation of the stem cell program, and death results when this requirement is not met. Lastly, Christopher and Myrick et al. report that LSD1 inappropriately colocalizes with NFTs outside of neuronal nuclei in human AD cases (Supplemental Figure 4). Taken together, these findings suggest that the inhibition of LSD1 may be downstream of pTau in human AD. This

could occur through the following model: neurofibrillary tangles of pTau sequester LSD1 and prevent it from properly demethylating gene targets in the nucleus.

The many phenotypic similarities between *Lsd1*$^{\Delta/\Delta}$ mice and Tg^{P301S} hemizygous mice, such as hippocampal neuronal death and paralysis, suggest that the inhibition of LSD1 may play a role in the mutant Tau mouse phenotype. In contrast to these two animal models for AD, mice heterozygous for functioning LSD1 (*Lsd1*$^{\Delta/+}$) are viable and functionally wildtype as reported by multiple studies (Wang et al., 2007, Wasson et al., 2016). This indicates that *Lsd1*$^{\Delta/+}$ mice express sufficient LSD1 for neuronal survival. Furthermore, we do not expect that pathways downstream of LSD1 function are dysregulated in *Lsd1*$^{\Delta/+}$ mice. For this reason, we employed *Lsd1* heterozygosity to sensitize the genetic background for LSD1 in Tg^{P301S} hemizygous mice. We utilized this genetic reduction of LSD1 in Tg^{P301S} mice to test the hypothesis that the inhibition of LSD1 is downstream of pathological Tau in neurodegenerative diseases. If pathological Tau leads to neurodegeneration by interfering with LSD1, we would expect these mice to exhibit a faster, more severe neurodegeneration phenotype (Supplemental Figure 5). These experiments provide preliminary evidence that reducing LSD1 in a P301S mouse model accelerates the onset of processes downstream of pathological Tau, including premature death and earlier onset of neurodegeneration. These results indicate that it may be possible to target LSD1 therapeutically to block the progression of AD in patients.

Results

LSD1 Inappropriately Colocalizes with Aggregated Tau in Aged P301S Transgenic Mice

Christopher and Myrick et al. previously found that LSD1 is constitutively expressed in adult neuronal nuclei and abnormally associates with pathological Tau aggregates in human AD cases (Supplemental Figure 4). To determine if this is also the case in P301S Tau mice, we performed LSD1 immunofluorescence in Tg^{P301S} mice. In examining brain sections of one-year-old Tg^{P301S} hemizygous mice, we found that in addition to appearing within neuronal nuclei as expected, LSD1 inappropriately colocalizes with perinuclear Tau aggregates (Fig 1C). These results suggest that the inhibition of LSD1 may play a role in the extensively studied tauopathy phenotype of Tg^{P301S} mice, and justified our proceeding with the experiment to genetically reduce LSD1 in Tau mice.

Genetic Reduction of LSD1 in P301S Tg Mice Decreases Lifespan

Yoshiyama et al. created line Tg^{P301S} mice and characterized their premature death with a median survival of approximately 9 months. We hypothesized that the inhibition of LSD1 is involved in this phenotype and employed a genetic strategy to investigate a potential synergistic interaction between LSD1 and P301S tau. We generated a cohort of experimental mice by crossing a male hemizygous for the P301S transgene with females heterozygous for *Lsd1*. This initial set of crosses produced three experimental mice hemizygous for P301S and heterozygous for *Lsd1* (Tg^{P301S}; $Lsd1^{\Delta/+}$), hereafter referred to as 'HemiHet mice,' in addition to control littermates either hemizygous for P301S (Tg^{P301S}) or heterozygous for *Lsd1* ($Lsd1^{\Delta/+}$) but functionally wildtype. In support of our hypothesis, we found that all three experimental HemiHet mice died suddenly between 3.5 to 4.5 months of age and were outlived by controls

(Fig 2). This shortened lifespan of HemiHet mice is in contrast with the longer 9-month median survival of Tg^{P301S} hemizygous mice (Yoshiyama et al., 2007), and suggests that reducing LSD1 accelerates deleterious processes downstream of pathological tau.

However, despite our initial promising results, the subsequently generated (F2) Tau mice displayed an attenuation or delay-in-onset of the expected phenotype, with the Tau F2 hemizygous Tg^{P301S} controls failing to develop tauopathy symptoms even past 9 months of age. Other publications about Tg^{P301S} mice have reported this variation in onset and severity of the phenotype (Iba et al., 2013, Ohia-Nwoko et al., 2014). Nevertheless, preliminary findings suggest that *Lsd1* heterozygosity still exacerbates these 'attenuated-tauopathy' Tau F2 mice. These observations are detailed later in this report.

Genetic Reduction of Lsd1 Exacerbated Brain Atrophy in a Single Tg^{P301S} Mouse

Tg^{P301S} hemizygous mice begin noticeable neurodegeneration after approximately 9 months of age and exhibit significant neuronal death in the hippocampus by 12 months of age (Yoshiyama et al., 2007). To see if this is exacerbated in the initial cohort of HemiHet mice that died around 4 months of age, we next analyzed the brains of 2 of these animals. One of these two animals exhibited severe neuronal loss. To view histological changes, we performed H&E staining of this HemiHet brain in addition to an $Lsd1^{\Delta/+}$ control and a Tg^{P301S} control. We found that the brain of the $Lsd1^{\Delta/+}$ control shows no loss of neurons (Fig 3A), while the brain of a terminal 6.4-month-old P301S hemizygous control exhibits widespread neurodegeneration (Fig 3B). In contrast to the pristine brain of the $Lsd1^{\Delta/+}$ control, the brain of the 3.8 months old HemiHet mouse exhibits severe widespread neurodegeneration, especially in the CA1 region of the hippocampus (Fig 3C). This neurodegeneration extends to the neocortex (Fig 3E) but

appears even more prominent in subcortical limbic areas (Fig 3F). We observed that many neuronal nuclei in this HemiHet brain are pyknotic, meaning that chromatin has condensed during cell death. This karyopyknosis in the HemiHet brain is reminiscent of the phenotypes of both Tg^{P301S} mice (Yoshiyama et al., 2007) and *Lsd1*-deletion mice (Christopher and Myrick et al., submitted 2016). Unlike *Lsd1*-deletion mice, both the HemiHet and Tg^{P301S} control exhibit vacuolar degeneration (empty spaces surrounding condense nuclei); this is especially prominent in the CA1 region of the HemiHet hippocampus (Fig 3D). These combined results show that a younger terminal experimental HemiHet mouse's brain exhibits brain atrophy comparable to an older terminal Tg^{P301S} hemizygous mouse's brain. These data supports our prediction that genetic reduction of LSD1 accelerates neurodegeneration downstream of pathological Tau in Tg^{P301S} mice.

P301S Tg Mouse Heterozygous for Lsd1 Lost Dendritic Projections

To further characterize the neurodegeneration that we observe in this HemiHet mouse, we performed MAP2 immunohistochemistry. MAP2 is a protein that stabilizes microtubules within dendrites and functions as a dendritic marker. In contrast to a wildtype control animal that exhibits intact hippocampal and neocortical dendritic projections (Fig 4A, 4C), the experimental HemiHet mouse's brain shows dendritic loss in the neocortex (Fig 4B) and hippocampal CA1 region (Fig 4D). This dendritic loss is consistent with our H&E results that genetic reduction of LSD1 in a Tg^{P301S} mouse resulted in neuronal cell death.

Genetic reduction of LSD1 in a Tg^{P301S} mouse did not accelerate spread of pathological Tau

Yoshiyama et al. originally reported weak human Tau IHC staining in the hippocampus of 3-month-old Tg^{P301S} mice but stronger staining with NFT formation by 6 months of age. In

accordance with our hypothesis that LSD1 inhibition is downstream of Tau tangle formation in Alzheimer's disease, we predicted that genetically reducing LSD1 would not alter human Tau levels in our HemiHet mouse. We investigated this by examining the localization of human Tau in the brains of our mice. As expected, we do not detect human Tau protein in an *Lsd1* heterozygous control mouse hippocampus (Fig 5A). Due to the complications introduced into the cohort by attenuation of the tauopathy phenotype, we were unable to immunostain human Tau aggregation in a Tg^{P301S} control age-matched to the HemiHet that died at 3.8 months. We instead immunostained human Tau in the brain of a Tg^{P301S} hemizygous mouse born in a different litter and euthanized at 7 months after it stopped exhibiting its mild hindlimb clasping and showed signs of attenuated phenotype. In this mouse, we observe human Tau staining only in the hippocampal CA3 region (Fig 5B) and neocortex (Fig 5E) of this Tg^{P301S} mouse, albeit weaker than what is characterized for its age (Yoshiyama et al., 2007). We also immunostained human Tau in the brain of a terminally paralyzed 6.4-month-old Tg^{P301S} hemizygous mouse from a different mouse colony and found human Tau aggregation and NFTs widespread throughout the brain, including the CA3 region (Fig 5C) and neocortex (Fig 5F). In contrast, we observe only weak human Tau staining in the HemiHet mouse's hippocampal CA3 region (Fig 5D) and neocortex (5G). In addition, unlike the brain of the terminal 6.4-month-old Tg^{P301S} mouse, the HemiHet brain does not exhibit NFT formation. Furthermore, in contrast to the NFTs that stain for human Tau in the 6.4-month-old Tg^{P301S} mouse's CA1 region, the 'empty spaces' surrounding condensed CA1 nuclei in the HemiHet mouse's brain (Fig 3D) do not stain for human Tau (Fig 5I). We conclude from these immunostaining experiments that human Tau aggregation is less widespread in the terminal 3.8-month-old HemiHet mouse's brain than in

the older terminal 6.4-month-old Tg^{P301S} mouse's brain. These findings suggest that decreased survival in HemiHet mice is not due to increased pathological Tau formation, and this result supports our hypothesis that inhibition of LSD1 is downstream of Tau tangles in Alzheimer's disease.

P301S Tg Mouse Heterozygous for Lsd1 Exhibits Abnormal LSD1 Localization

LSD1 functions to repress genes by demethylating histones in the nucleus. Christopher and Myrick et al. found via IHC that LSD1 appears in the nuclei of adult neurons. Since our first experiment in year-old Tg^{P301S} hemizygous mice found inappropriate localization of LSD1 outside neuronal nuclei (Fig 1D), we next utilized IHC to see if LSD1 appears mislocalized in the experimental HemiHet mouse that died at 3.8 months of age. In examining the CA1 regions of control mice, we found perfectly nuclear immunostained LSD1 in an $Lsd1^{\Delta/+}$ control (Fig 6A) and in an attenuated-tauopathy Tg^{P301S} hemizygous mouse sacrificed at 7 months of age (Fig 6B), but a lack of nuclear immunostained LSD1 in the CA1 of the terminal 6.4-month-old Tg^{P301S} mouse (Fig 6C). In contrast to these controls, the HemiHet mouse's hippocampal CA1 neurons enclosed by 'empty spaces' do not exhibit LSD1 staining (Fig 6D). This is in accordance with our suspicion that these neurons either are dead or have depleted nuclear LSD1.

We observed a second abnormal result in finding that LSD1 immunostains both within and around surviving nuclei in the HemiHet mouse's CA3 region (Fig 6E). This abnormal result may have two possible explanations. Firstly, LSD1 might be sequestered by pathological Tau in the cytosol around neuronal nuclei. This possible explanation for this abnormal staining result implies that pathological Tau physically inhibits LSD1 from entering the nucleus to demethylate

target genes. An alternative explanation is that LSD1 is simply being spilled out of nuclei that are in the process of dying.

Genetic Reduction of LSD1 results in Decreased Lifespan of Tg^{P301S} Mice Despite Attenuation of tauopathy Phenotype

As cited above, multiple laboratories have reported a phenomenon in which the mutant Tau phenotype attenuates in Tg^{P301S} mice. We first saw evidence of this 'attenuated-tauopathy' in the second (F1) generation of our PS19 line, when a P301S hemizygous mouse (Fig 3B, 4B, 5B, 6B) stopped exhibiting the mild hindlimb -clasping it had displayed for weeks. We unknowingly propagated this attenuated-tauopathy throughout our cohort by selecting another F1 generation P301S hemizygous mouse as the new founder male of the F2 generation and inbreeding it with its own mother. This F1 Tg^{P301S} founder of the F2 generation survived to 15.8 months of age, which is well past the survival previously characterized (Yoshiyama et al., 2007), and consequent offspring also exhibit attenuated-tauopathy. We suspect that despite the attenuation of the Tau phenotype, *Lsd1* heterozygosity might still decrease lifespan in these mice (Fig 7). In analyzing F2 litters that bore both HemiHet mice and Tg^{P301S} controls as littermates, we find that nine out of twelve HemiHet mice have died with an average survival of 273 days. The three surviving HemiHets have an averaged age of 259 days. In comparison, seven out of nineteen Tg^{P301S} controls have died with an average survival of 289 days. The twelve surviving Tg^{P301S} controls have an average age of 297.3 days. These preliminary results suggest that genetic reduction of LSD1 in F2 generation Tg^{P301S} hemizygous mice is sufficient to decrease survival despite attenuation of the tauopathy phenotype. We did not observe signs of neurodegeneration or Tau aggregation in examining two of these nine F2 HemiHet mice. Unlike

the three F1 generation HemiHet mice that died suddenly, the F2 HemiHet mice first began to exhibit hindlimb clasping similar to Tg^{P301S} controls before reaching terminal paralysis (Fig 8). It is possible that the two examined F2 generation HemiHet mice do not exhibit signs of cortical neurodegeneration or pathological Tau aggregation because they died from neuronal death in the spinal cord, which we did not examine.

Discussion

Pathological Tau aggregation is a hallmark of Alzheimer's Disease (AD), but downstream pharmacological targets have not been adequately identified. We hypothesized that inhibition of lysine-specific demethylase 1A (LSD1), an epigenetic repressor of transcription, is directly downstream of pathological Tau in AD. Here, we provide preliminary evidence that reducing LSD1 in transgenic mice expressing mutant human tau (Tg^{P301S}) accelerates deleterious effects downstream of pathological Tau, such as decreased survival (Fig 2) and exacerbated brain atrophy (Fig 3C, 4B) that is reminiscent of the phenotype seen in *Lsd1*$^{\Delta/\Delta}$ mice (Christopher and Myrick et al. Submitted 2016) and terminally aged Tg^{P301S} mice (Fig 3B) (Yoshiyama et al., 2007). Immunohistochemistry (IHC) staining for human tau shows that partial loss of LSD1 does not exhibit accelerated neurofibrillary tangle (NFT) formation (Fig 5D). This result suggests that pathological Tau functions through inhibition of LSD1, rather than LSD1 causing pathological tau aggregation. Finally, IHC staining shows abnormally perinuclear LSD1 in a Tg^{P301S}; *Lsd1* $^{\Delta/+}$ mouse hippocampus (Fig 6E). This perinuclear LSD1 might be due to sequestration in the cytosol by pathological Tau, as supported by our immunofluorescence (IF) finding that LSD1 and aggregated tau colocalize in neurons of one-year-old Tg^{P301S} mice (Fig 1D). Importantly, *Lsd1*$^{\Delta/+}$

mice have normal LSD1 activity and are functionally wildtype. Thus, we believe that the exacerbated phenotype of Tg^{P301S}; *Lsd1* $^{\Delta/+}$ mice is not simply the result of dysregulating two pathways. Instead, our results suggest that pathological Tau and LSD1 may be acting in a singular pathway with LSD1 being inhibited by aggregated Tau. This could occur through the following model; as neurons in the brain age, aggregated Tau fails to be cleared and sequesters LSD1 in the cytoplasm. This in turn limits the amount of LSD1 that is available in the nucleus to repress transcription by demethylating histones. In Tg^{P301S}; *Lsd1* $^{\Delta/+}$ mice, removing 50% of LSD1 enables cytoplasmic pathological Tau to more rapidly deplete LSD1 from the nucleus, resulting in the observed decrease in survival and exacerbated brain atrophy. Thus our results provide preliminary evidence that pathological Tau aggregation functions through LSD1 *in vivo* in a mouse model. If this is the case, it may be possible to target the LSD1 pathway to block the progression of Alzheimer's Disease in patients.

Neurofibrillary tangles in AD are a late-stage structure of pathological Tau maturation (Bancher et al., 1989), and there is evidence that neurotoxic forms of tau precede NFT formation. For example, Tau-seeding experiments in transgenic mice expressing wildtype human Tau showed that NFTs spreading in the hippocampus did not result in neuronal loss (Clavaguera et al., 2009). Additionally, repressing mutant human P301L Tau in another tauopathy mouse model was sufficient to recover memory function and stabilize neuronal numbers despite the continued formation of NFTs (Santacruz et al., 2005). Furthermore, observations that synaptic deficits begin months before NFT formation in Tg^{P301S} mice (Yoshiyama et al., 2007) might indicate that cognitive decline begins before NFT formation in AD. Taken together, this suggests that NFTs as a late-stage form of Tau are not sufficient to

cause neurotoxicity in AD. This is consistent with our finding that Tg^{P301S}; *Lsd1* $^{\Delta/+}$ mice died before visible NFT formation (Fig 5D). We instead suggest that the severity of AD might correlate to the extent that LSD1 function is inhibited by pathological Tau (Supplemental Figure 6).

The Amyloid cascade hypothesis of Alzheimer's disease proposes that amyloid-beta (Aβ) accumulation stresses the brain and begins the disease pathway by facilitating the pathological aggregation of Tau (Hardy and Selkoe, 2002). This is reflected in mice expressing transgenic mutant APP_{751} that develop hyperphosphorylated Tau in neurites surrounding Aβ plaques (Sturchler-Pierrat et al., 1997). Consistent with this model, there is also evidence that Aβ plaques are found in cognitively normal human control cases (Braak and Braak, 1991) (Berg et al., 1998), and disease-modifying drugs targeting Aβ have been largely unsuccessful (Mullane and Williams, 2013). Furthermore, Aβ burden does not correlate with severity of brain atrophy, but the presence of neurofibrillary tangles comprised of pathological Tau does correlate with brain atrophy (Josephs et al., 2008). Together, these results suggest that Aβ burden is not sufficient to induce dementia because dementia may require Tau aggregation and events downstream of pathological Tau, such as the sequestration of LSD1. This model makes an intriguing prediction that cognitively normal cases with Aβ burden, or even rare instances of cognitively normal cases with Tau pathology, should lack LSD1 pathology. This prediction remains to be tested.

Our proposed AD model of inhibition of LSD1 by pathological Tau gives rise to speculation regarding why LSD1 might be physically sequestered by pTau. It is possible that the active site of LSD1 is inappropriately binding to pathological Tau fibrils in the cytosol. LSD1 has

been shown to demethylate non-histone protein targets such as p53 when aided by accessory proteins (Nicholson and Chen, 2009). Mass spectrometry has shown that pathological Tau is N-methylated on lysine residues in human AD (Thomas et al., 2012). This raises the possibility that this modification is recognized by the active site of LSD1, resulting in LSD1 sticking to Tau aggregates. To test this, a future experiment might involve using biochemistry to determine directly whether LSD1 can recognize and bind to methylated Tau.

The proposed inhibition of LSD1 in AD also gives rise to speculation regarding why the loss of LSD1 is neurotoxic. Christopher and Myrick et al. found that the deletion of *Lsd1* resulted in abnormal transcription of genes normally repressed in neurons. We speculate that this abnormal transcription might have two possible reasons for neurotoxicity. Firstly, the loss of LSD1 function might result in disinhibition of a molecule that kills hippocampal neurons. For example, $Lsd1^{\Delta/\Delta}$ mice exhibit upregulated *Myc* transcription, which is a stem cell gene with inherent apoptotic properties (Prendergast, 1999). LSD1 also postranslationally inhibits p53 (Huang et al., 2007), and overexpression of p53 has been shown to induce apoptosis in cultured hippocampal neurons (Jordán et al., 1997). Alternatively, neurotoxicity might result from the derepression of genes that lead to excitotoxicity in the hippocampus. For example, the loss of LSD1 function in drosophila SL2 cells resulted in increased expression of nicotinic acetylcholine receptors and sodium channel proteins (Di Stefano et al., 2007). To test whether the hippocampal $Lsd1^{\Delta/\Delta}$ neurons die from excitotoxicity, a future experiment might consist of utilizing organotypic slice culture to obtain electrophysiological recordings of $Lsd1^{\Delta/\Delta}$ CA1 neurons. This future experiment using organotypic slice culture may also provide insight into the stages of neuronal death.

Our study provides evidence in Tg^{P301S} mice that reducing LSD1 can accelerate deleterious processes downstream of pathological Tau. This gives rise to the possibility that increasing LSD1 function can slow the progression of neurodegeneration in Tg^{P301S} mice and AD patients. A genetic experiment to test this might consist of creating a strain of mice with inducible overexpression of LSD1 in neurons, and observing if LSD1 overexpression rescues survival and neurodegeneration in Tg^{P301S} mice. Similarly, drugs that antagonize the function of H3K4 methyltransferases could potentially slow down the Tg^{P301S} phenotype by preventing derepression of inappropriate transcription downstream of LSD1-inhibition. Alternatively, since H3K9me has an opposite effect on transcription from H3K4me (Nakayama et al., 2001) and is added when K3K4me is removed (Suganuma and Workman, 2008), drugs that enhance H3K9 methylation might rescue the phenotypes of $Lsd1^{\Delta/\Delta}$ and Tg^{P301S} mice. Thus, our results could potentially lead to new treatments for Alzheimer's Disease.

Experimental Procedures

Mice

All work with mice was performed in accordance with the guidelines of Emory University IACUC (approved protocol #2002534). Mice were generated by crossing a mouse heterozygous for functioning *Lsd1* (Katz Laboratory) with another mouse hemizygous for transgenic *P301S* (Weinshenker Laboratory). This breeding strategy was intended to produce offspring wildtype for both genes of interest, solely heterozygous for *Lsd1* (LSD1 controls), solely hemizygous for *P301S* (Tau controls), and experimental mice both heterozygous for *Lsd1* and hemizygous for *P301S* (HemiHets).

The first tau founder male produced 5 HemiHets, 4 wildtype mice, 8 LSD1 controls, and 2 Tau controls. One of these two Tau control mice was selected as the founder for subsequent crosses. Unfortunately, this second tau founder and all of its descendants displayed a slower onset of pathology than originally reported by Virginia Lee, and this 'phenotypic drift' greatly hindered the survival comparison between HemiHets and controls. Generation of the animal cohort was later restarted using three new P301S founder males (Jackson Laboratory, Weinshenker Laboratory).

All mice were housed in cages with unlimited access to food and water, and assessed for the onset of hindlimb weakness via daily tail suspension tests. With the exception of specimens found dead, mice were euthanized when paralyzed to the point of immobility.

Genotyping

Genomic DNA samples isolated from tail lysates were used as templates for PCR to verify genotypes via the following primers:

Loxp-flanked LSD1 ("Flox Ben"):

F: 5' – GCA CCA ACA CTA AAG AGT ATC C – 3'

R: 5' – CCA CAG AAC TTC AAA TTA CTA AT – 3'

P301S Trangene:

F: 5' –GGG GAC ACG TCT CCA CGG CAT CTC AGC AAT GTC TCC– 3'

R: 5' – TCC CCC AGC CTA GAC CAC GAG AAT – 3'

Tissue Fixation

With the exceptions of mice 1266, 1289, 1583, and 1584 (found dead), mice were anesthetized via isoflurane inhalation and transcardially perfused with cold 4.0%

paraformaldehyde in 0.1M phosphate buffer. Brains were removed, incubated in 4% paraformaldehyde solution for 3-5 days, then given to the Neuropathology Histochemistry Core Facility to be embedded in paraffin.

Immunohistochemical Studies

Twelve-micrometer sections of paraffin-embedded brain tissue were dewaxed with xylene and rehydrated with ethanol dilutions. Slides then underwent an antigen-retrieval treatment of being boiled in citrate buffer (microwaved) for ten minutes, then cooled to room temperature. Samples were treated with 3% hydrogen peroxide in trisbrij solution at 40°C for 5 minutes to quench endogenous peroxidases, then blocked from nonspecific antibody (Ab) binding in 2.0% goat serum in trisbrij solution at 40°C for 15 minutes. Samples were then incubated overnight with primary Ab at 4°C. Slides were then washed with TrisBrij solution and incubated with biotinylated secondary antibody plus serum at 37°C for 30 minutes, followed by a wash in TrisBrij solution. Signal amplification was performed by incubating slides with Vector Laboratories VECTASTAIN Elite ABC Kit (PK-6100) for 1 hour at 37°C. Slides were then color-developed by exposure to dilute 3,3'-Diaminobenzidine (DAB) in dilute hydrogen peroxide solution for approximately 2.5 minutes, counterstained by exposure to 30% hematoxylin solution for 1 minute, washed with brij solution and TsBrij solution, and cover-slipped in Permount™ after drying.

Table 1 | Antibodies used in IHC studies.

Target Protein	**Primary Antibody**	**1° Host**	**1° Dilution**	**Secondary Antibody Host/Target**	**2° Dilution**
LSD1	Abcam AB17721	Rabbit polyclonal	1:200 in 1% BSA	Biotinylated goat/rabbit	1:300 in 2% serum TsBrij Soln
Human tau	T14	Mouse monoclonal	1:100 in Tau Secret Formula	Biotinylated goat/mouse	1:300 in 2% serum TsBrij Soln
Hyper-phosphorylated tau	AT8	Mouse monoclonal	1:100 in Tau Secret Formula	Biotinylated goat/mouse	1:300 in 2% serum TsBrij Soln
MAP2	Millipore AB5622	Rabbit polyclonal	1:100 in 1% BSA	Biotinylated goat/rabbit	1:300 in 2% serum TsBrij Soln

Immunofluorescence

Twelve-micrometer sections of paraffin-embedded brain tissue were dewaxed with xylene and rehydrated with ethanol dilutions. Slides then underwent an antigen-retrieval treatment of being microwaved at power-70 in citrate buffer for five minutes, cooled for 30 minutes, then washed in TBS. Samples were blocked from nonspecific antibody-binding in a solution of 10% goat serum + 0.5% Triton in TBS for one hour at room temperature. Slides were incubated with primary Ab (diluted in 0.5% Tween + 10% goat serum in TBS) overnight at 4°C in a humidified chamber. Slides were then washed with TBS and incubated with secondary Ab in TBS for one hour at room temperature, followed by a wash in TBS. Lastly, samples were incubated with DAPI in TBS for 10 minutes to visualize DNA.

Table 2 | Antibodies used in fluorescence labeling studies

Target Protein	**Primary Antibody**	**1° Host**	**1° Dilution**	**Secondary Antibody**	**2° Host/Target**	**2° Dilution**
LSD1	Abcam AB17721	Rabbit polyclonal	1:200 in solution	Invitrogen Alexafluor	Goat/Rabbit	1:500 in TBS
Human tau	T14	Mouse monoclonal	1:100 in solution	Invitrogen Alexafluor	Goat/Mouse	1:500 in TBS
Hyper-phosphorylated tau	AT8	Mouse monoclonal	1:100 in solution	Invitrogen Alexafluor	Goat/Mouse	1:500 in TBS
NeuN	Millipore ABN78	Rabbit polyclonal	1:1000 in solution	Invitrogen Alexafluor	Goat/Rabbit	1:500 in TBS

Figures and Supplemental Figures

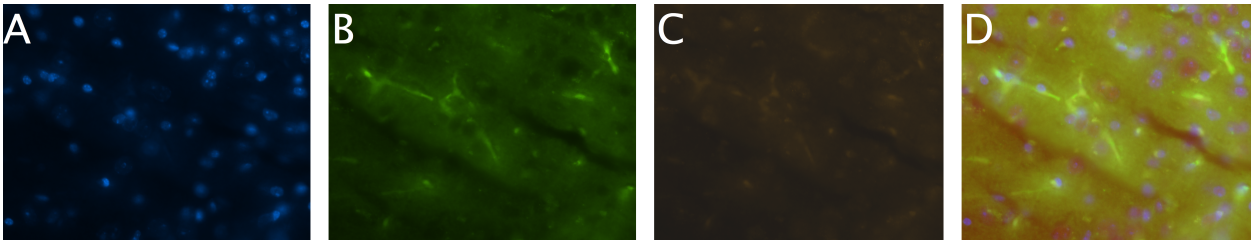


Figure 1 | P301S Transgenic mice exhibit perinuclear colocalization of LSD1 and mutant human Tau. (A, blue) Immunofluorescence labeling with the nuclear marker DAPI shows neuronal nuclei in the cortex of one-year-old Tg^{P301S} mice. **(B, green)** Labeling with human Tau-specific antibodies (T14) shows perinuclear aggregation of human Tau. **(C, red)** Labeling with LSD1-specific antibodies reveals nuclear staining as expected as well as abnormal perinuclear staining. **(D, merged)** Dual fluorescence labeling shows that LSD1 colocalizes with Tau around a minority of cortical nuclei.

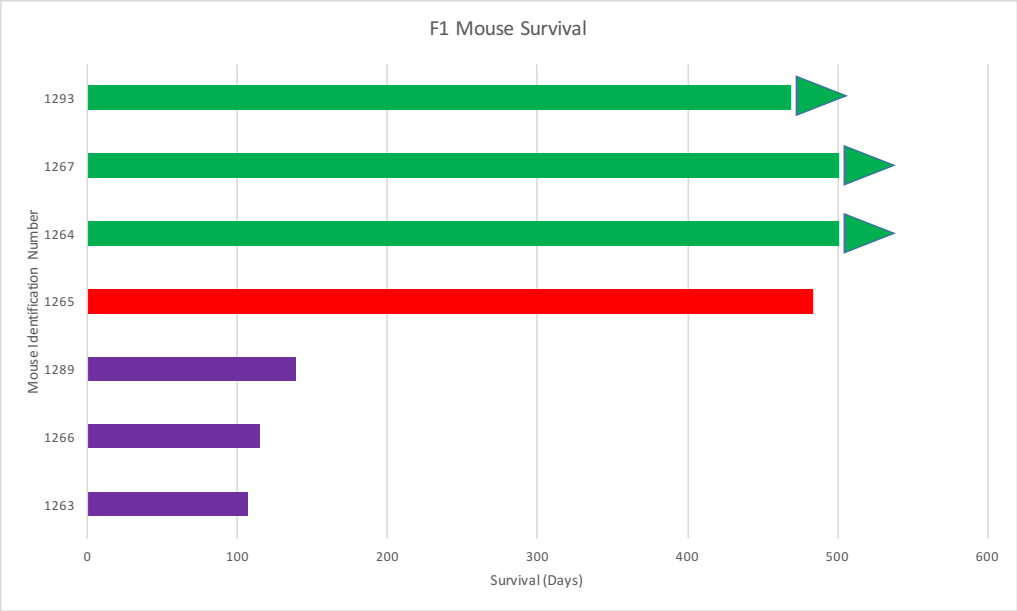


Figure 2 | P301S Tg mice heterozygous for *Lsd1* exhibit decreased lifespan. This survival graph depicts the lifespan of each individual F1 generation HemiHet mouse (purple) compared to Tg^{P301S} (red) and $Lsd1^{\Delta/+}$ (green) controls descended from the same F0 generation parents. The three HemiHets died at 3.5, 3.8, and 4.5 months of age, while the two P301S hemizygous controls survived to 6.4 and 15.9 months of age. The longer-lived P301S hemizygous control is thought to have exhibited an attenuated-tauopathy phenotype. Only mice that reached terminal paralysis or were found dead are included in this survival graph. Arrows indicate that mice are still alive at the time of this report.

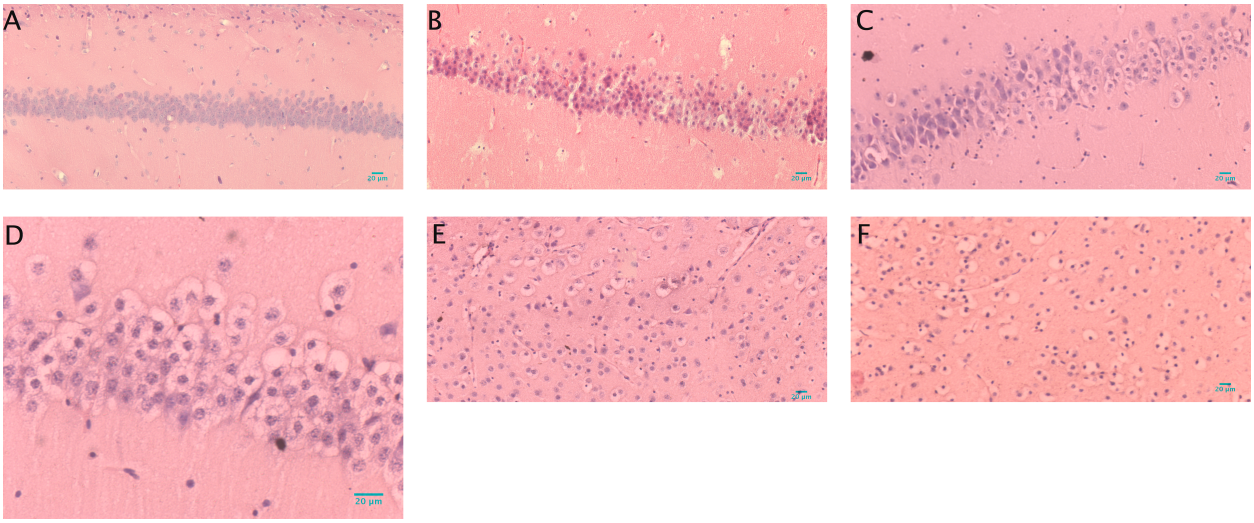


Figure 3 | Genetic reduction of LSD1 exacerbated neurodegeneration in a single Tg^{P301S} mouse. (A) H&E staining of an $Lsd1^{\Delta/+}$ control hippocampal CA1 region shows that genetic reduction of *Lsd1* in the absence of P301S does not result in a mutant phenotype. **(B)** H&E staining of the hippocampal CA1 region in a terminally paralyzed 6.4-month-old Tg^{P301S} hemizygous control shows neurodegeneration consistent with the characterized PS19 phenotype. CA1 nuclei are pyknotic and often surrounded by 'empty space.' **(C)** H&E staining of the hippocampal CA1 region in a terminal 3.8-month-old HemiHet mouse reveals severe neurodegeneration and pyknotic nuclei surrounded by 'empty spaces.' **(D)** A 40X higher magnification of the HemiHet mouse's hippocampal CA1 region shows the 'empty spaces' surrounding condensed nuclei in greater detail. **(E)** The HemiHet mouse's neocortex also shows signs of severe neurodegeneration, **(F)** as well as subcortical areas.

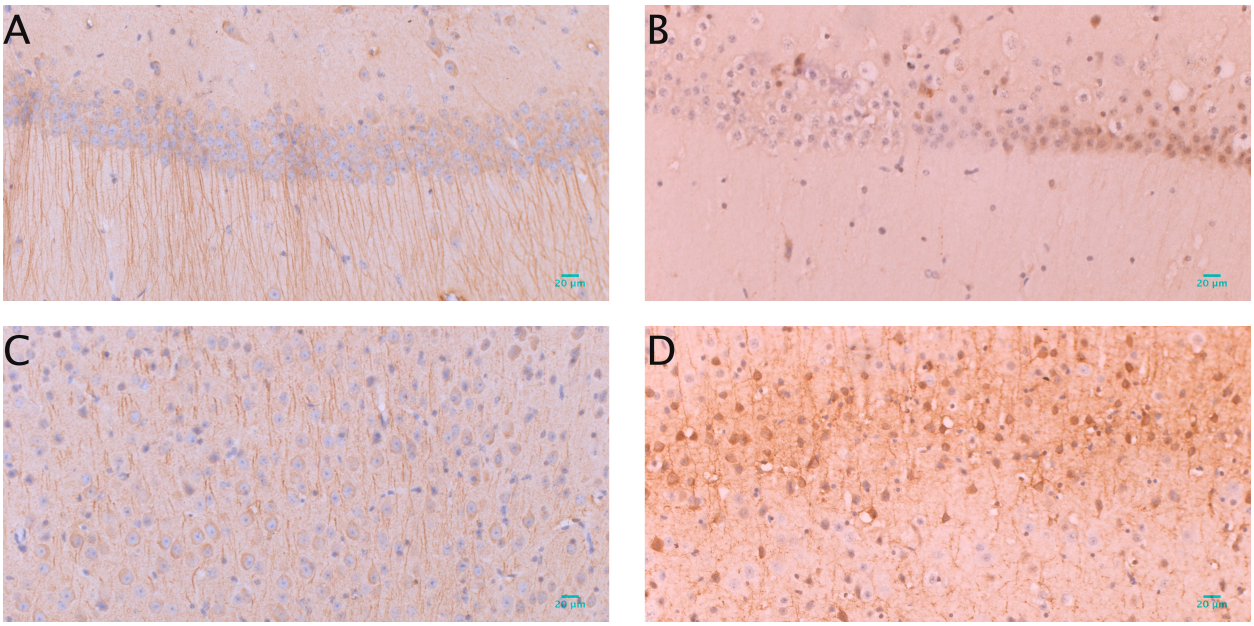


Figure 4 | HemiHet mouse lost dendritic projections within hippocampal CA1 region. (A) Immunostaining for MAP2, a dendritic marker, shows intact dendritic projections within a wildtype mouse's CA1 region. **(B)** MAP2 immunostaining reveals atrophy of dendritic projections in HemiHet mouse's CA1 region. **(C)** Dendrites within the wildtype mouse's neocortex are intact, **(D)** in contrast to the HemiHet mouse's neocortex.

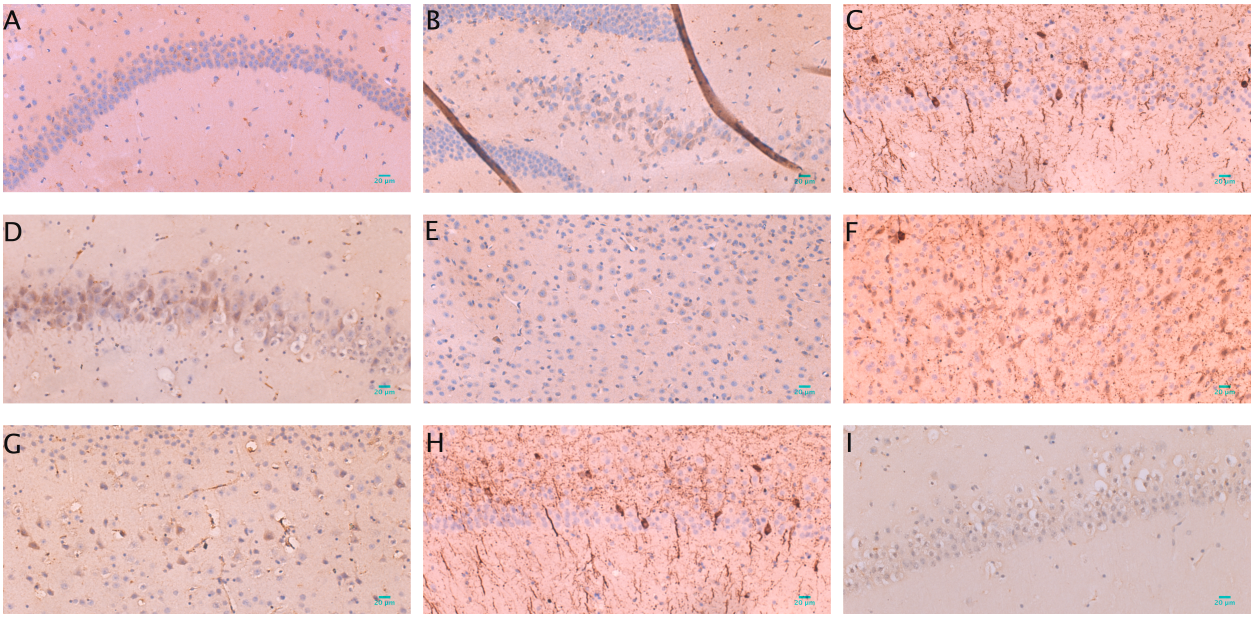


Figure 5 | Spread of human Tau in Tg^{P301S} mouse is not exacerbated by genetic reduction of LSD1. (A) The CA3 region of an *Lsd1*$^{\Delta/+}$ control mouse does not exhibit human Tau when immunostained with antibody T14, which is specific to human Tau. **(B)** There is weakly immunostained human Tau in the CA3 region of an attenuated-tauopathy Tg^{P301S} hemizygous mouse sacrificed at 7 months of age. **(C)** The CA3 region of a terminal 6.4-month-old Tg^{P301S} hemizygous mouse exhibits human Tau immunostained by T14 and evident NFTs. **(D)** The CA3 region of a terminal 3.8-month-old HemiHet mouse exhibits human Tau immunostained by T14 but not NFTs. **(E)** There is weakly immunostained human Tau in the neocortex of an attenuated-tauopathy Tg^{P301S} hemizygous mouse sacrificed at 7 months of age. **(F)** The neocortex of a terminal 6.4-month-old Tg^{P301S} hemizygous mouse exhibits human Tau immunostained by T14 as well as evident NFTs. **(G)** The CA3 region of a terminal 3.8-month-old HemiHet mouse exhibits human Tau immunostained by T14 but does not reveal NFTs. **(H)** The CA1 region of a terminal 6.4-month-old Tg^{P301S} exhibits extensive human Tau immunostained by T14 as well as

NFTs. **(I)** A terminal 3.8-month-old HemiHet mouse's CA1 region did not immunostain for human Tau and instead exhibits 'empty spaces' surrounding condensed nuclei.

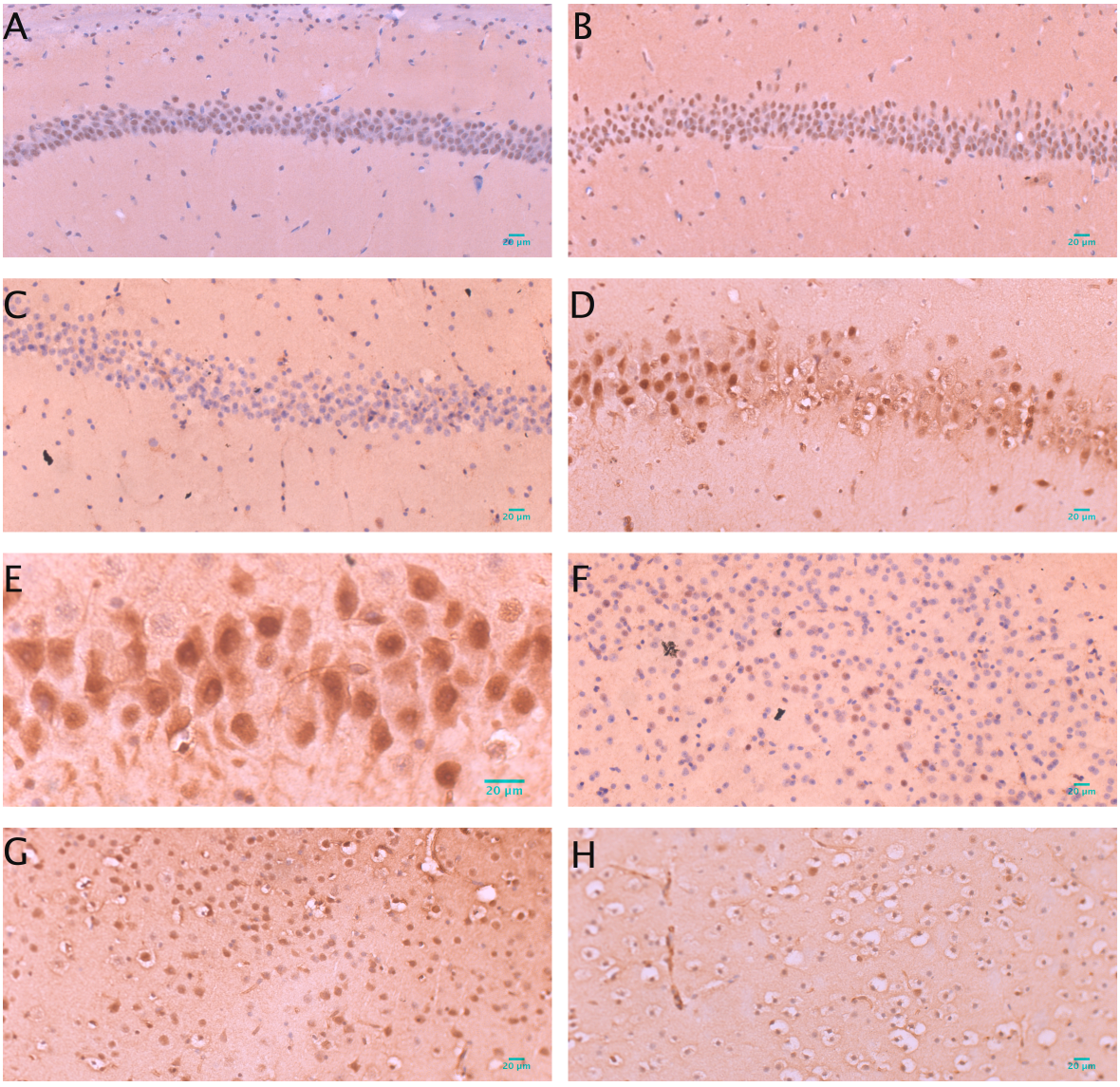


Figure 6 | LSD1 localization appears abnormal in a HemiHet mouse CA1 region. (A) Immunostained LSD1 appears within CA1 nuclei in an *Lsd1*$^{\Delta/+}$ control mouse. **(B)** Immunostained LSD1 is localized to nuclei in the CA1 of an attenuated-tauopathy Tg^{P301S} hemizygous control mouse sacrificed at 7 months of age. **(C)** There is an apparent lack of immunostained LSD1 in the CA1 region of a terminal 6.4-month-old Tg^{P301S} hemizygous mouse

(D) A 3.8-month-old HemiHet mouse exhibits weakly immunostained LSD1 within condensed CA1 nuclei and also abnormal cytosolic staining. **(E)** A 40X higher magnification of the HemiHet mouse's CA1 nuclei shows immunostained cytosolic LSD1 appearing around nuclear LSD1. **(F)** The terminal 6.4-month-old Tg^{P301S} hemizygous mouse exhibits immunostained LSD1 in some neocortical neurons, but not others. This indicates a positive staining result despite the lack of immunostained LSD1 within the CA1 region. **(G)** LSD1 within the HemiHet mouse's neocortex appears normal. **(H)** LSD1 immunostaining in the HemiHet mouse brain appears in condensed subcortical nuclei enclosed by empty spaces.

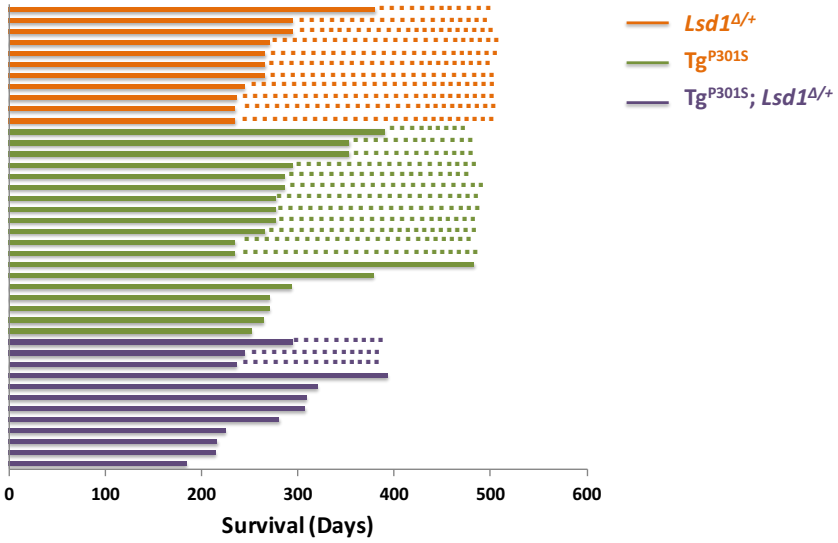


Figure 7 | F2 generation Tg^{P301S} mice heterozygous for *Lsd1* exhibit decreased lifespan despite attenuation of tauopathy phenotype. This survival graph depicts the lifespan of each individual F2 generation HemiHet mouse (purple) compared to Tg^{P301S} (red) and $Lsd1^{\Delta/+}$ controls, all descended from the same F1 Tg^{P301S} founder that survived to 15.9 months. Only mice from litters comprised of both HemiHet mice and Tg^{P301S} controls are included. Mice euthanized before reaching terminal paralysis are not included in this graph. Dotted lines indicate that mice are still alive at the time of this report.

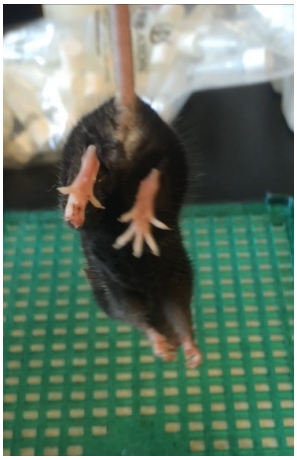
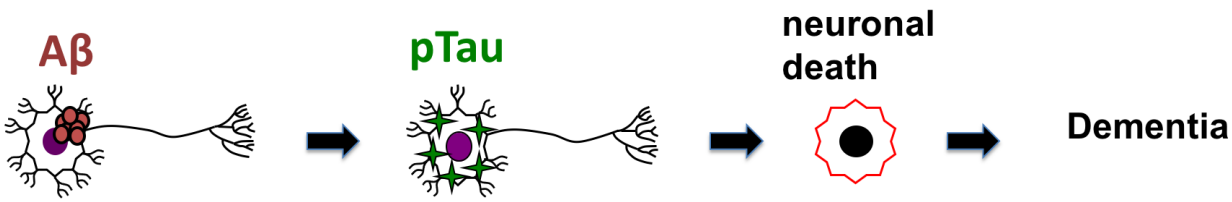
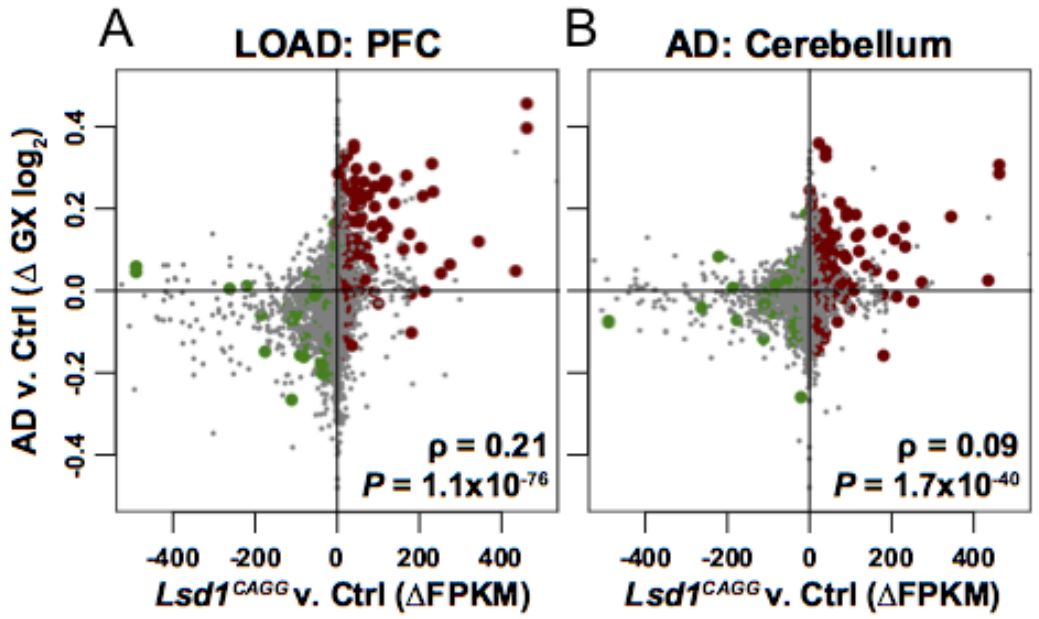


Figure 8 | Attenuated-tauopathy HemiHet mice exhibit a hindlimb weakness similar to Tg^{P301S} controls. Unlike the F1 generation HemiHet mice that died suddenly, the F2 generation HemiHet mice displaying an attenuated mutant tau phenotype first exhibited signs of hindlimb clasping (pictured) before progressing to severe paralysis.

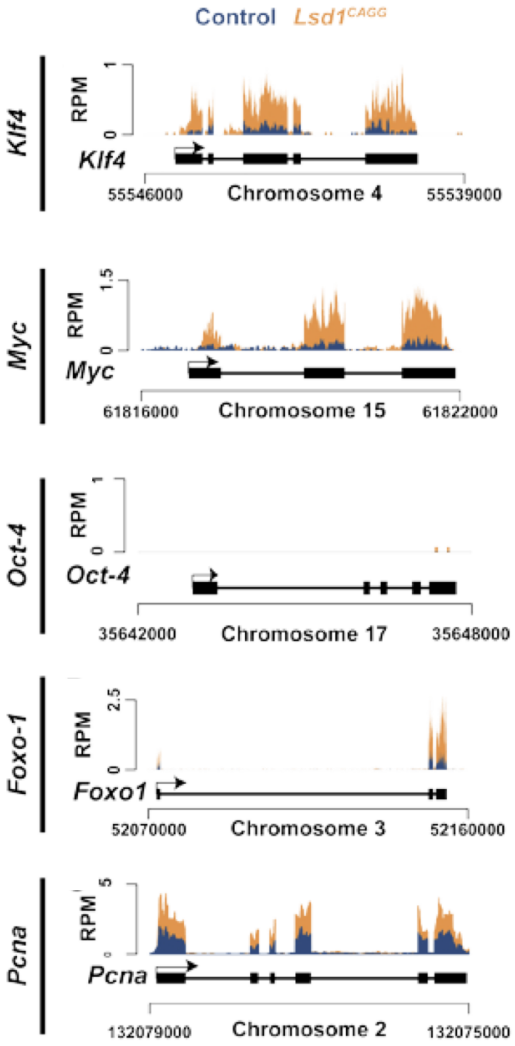


Supplementary Figure 1 | Illustration of the amyloid-beta hypothesis of Alzheimer's disease. The aberrant accumulation of amyloid-beta stresses the brain and results in pathological aggregation of tau that leads to neurodegeneration.



Supplementary Figure 2 | Expression changes in Lsd1$^{\Delta/\Delta}$ mice correlate with changes in AD. Genome-wide expression changes are depicted as correlation scatter plots where difference between *Lsd1*^{CAGG} and control FPKM value of each gene is plotted on the x-axis and the $\log_2$ expression difference of each gene from **(A)** LOAD prefrontal cortex and **(B)** LOAD cerebellum is plotted on the y-axis. Differently expressed genes from *Lsd1*^{CAGG} hippocampus are shown in red (upregulated) and green (downregulated). All other genes are shown in grey. Genes that are positively correlated plot in quadrants 1 and 3, and genes that are not correlated fall into quadrants 2 and 4. *P*-values and ρ Pearson correlation coeffiecient are given. Human cerebellar tissue was used as a negative control in RNAseq comparison because the cerebellum does not exhibit neurodegeneration in Alzheimer's disease.

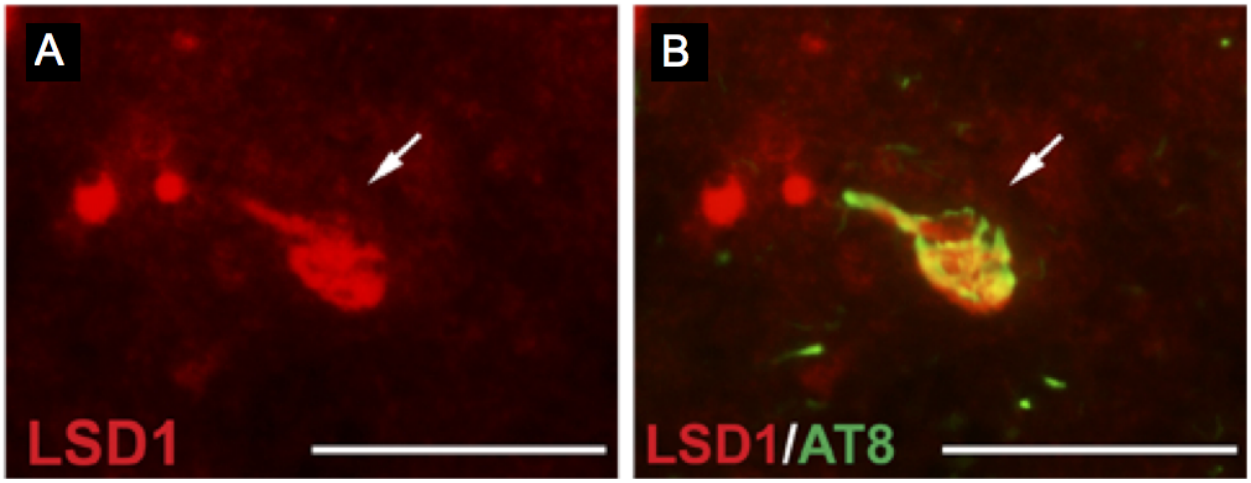
Modified from "LSD1 inhibition links protein aggregates to neuronal cell death in Alzheimer's and Frontotemporal Dementia," by Christopher MA, Myrick DA, Barwick BG, Levey AI, Katz DJ. (Submitted 2016). Modified with permission.



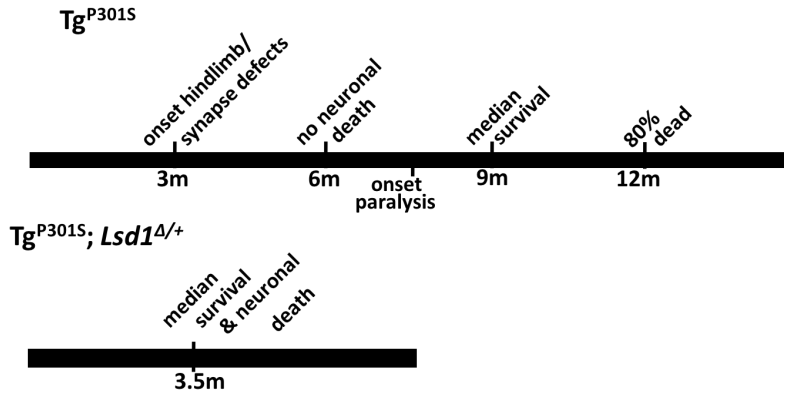
Supplementary Figure 3 | Ectopic expression of stem cell and cell cycle genes in *Lsd1*$^{\Delta/\Delta}$ mice.

RNA-seq reads from tamoxifen injected *Cre* minus control (blue) and *Lsd1*^{CAGG} mutant (orange) showing expression of genes *Klf4,* C-*Myc , Oct-4, Foxo-1,* and *PCNA*.

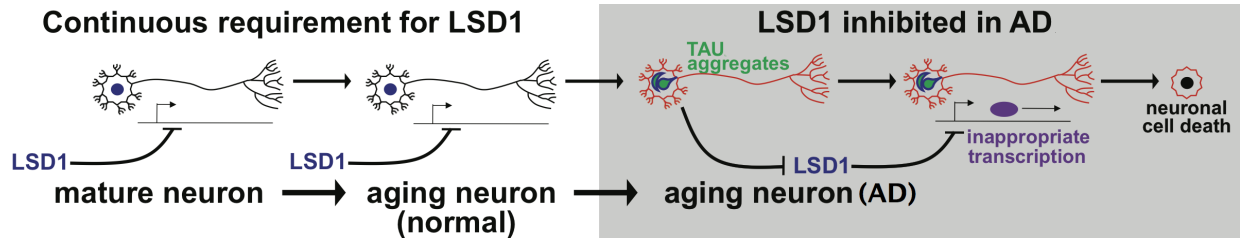
Modified from "LSD1 inhibition links protein aggregates to neuronal cell death in Alzheimer's and Frontotemporal Dementia," by Christopher MA, Myrick DA, Barwick BG, Levey AI, Katz DJ. (Submitted 2016). Modified with permission.



Supplementary Figure 4 | LSD1 colocalizes with hyperphosphorylated tau in human AD. (A) Immunofluorescence with Ab for LSD1 (red) in human AD tissue. **(B)** Immunofluorescence with Ab for LSD1 (red) shows colocalization with Ab AT8-labeled hyperphosphorylated tau (green). Modified from "LSD1 inhibition links protein aggregates to neuronal cell death in Alzheimer's and Frontotemporal Dementia," by Christopher MA, Myrick DA, Barwick BG, Levey AI, Katz DJ. (Submitted 2016). Modified with permission.



Supplementary Figure 5 | Illustration of experimental prediction. A hypothetical survival timeline of Tg^{P301S} mice compared to Tg^{P301S}; $Lsd1^{\Delta/+}$ mice. This illustration shows that experimental mice might die before controls if reduction of LSD1 exacerbates the mutant P301S phenotype.



Supplementary Figure 6 | Proposed Alzheimer's disease model includes inhibition of LSD1 by pathological Tau. LSD1 is continuously required in adult neurons to repress inappropriate transcription and maintain epigenetic identify. Alzheimer's disease, LSD1 is inhibited by perinuclear pathological Tau, and this results in neuronal cell death.

Modified from "LSD1 inhibition links protein aggregates to neuronal cell death in Alzheimer's and Frontotemporal Dementia," by Christopher MA, Myrick DA, Barwick BG, Levey AI, Katz DJ. (Submitted 2016). Modified with permission.

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