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INDUCTION AND PREVENTION OF PATTERNED NEURODEGENERATION BY AMYLOID PRECURSOR PROTEIN

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INDUCTION AND PREVENTION OF PATTERNED NEURODEGENERATION BY AMYLOID PRECURSOR PROTEIN

by

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Dedication

I would like to dedicate this thesis to mother. She has supported me through many difficult times, even when it was not so easy for herself. As anyone knows, the work that goes into a thesis is not menial. It pales in comparison to what my mother deals with each day, but I want her to know that all of this work has been for her.

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Alzheimer disease is characterized by the initial degeneration of a subset of cholinergic neurons. This pattern of degeneration can be triggered by overexpression of the amyloid precursor protein (*APP*) gene in humans. Interestingly, *APP* is widely expressed; it is therefore unclear why only certain cholinergic neurons are vulnerable to degeneration. We show that widespread expression of the human *APP* gene in the nematode *Caenorhabditis elegans* also induces age-dependent apoptotic degeneration of select cholinergic neurons. Identical results were obtained by overexpressing the orthologous worm gene *apl-1*. The pattern of neurodegeneration matched the cell-autonomous accumulation of APP protein in vulnerable neurons and could be activated cell-non-autonomously by distinct portions of APP. Vulnerability to APP accumulation and degeneration depended inversely on the level of ASK1/p38MAPK innate-immune signaling in cholinergic neurons. Lastly, we identify a compound P7C3 that blocks entrance to apoptosis caused by APP or immunodeficiency. Our results suggest that immunosenescence sculpts the cellular pattern of neurodegeneration by APP.

Table of Contents

List of Tables

List of Figures

Chapter 1: General Introduction

Why Study Alzheimer's Disease?

Alzheimer's disease is the most common form of dementia which leads to eventual death in all cases. It was first described in 1906, and is characterized as a terminal progressive degenerative disorder. Today, an estimated 5.4 million Americans suffer from Alzheimer's disease (AD) (2012 Alz Facts and Figures); it is currently ranked as the sixth leading cause of death in the United States. By 2030, a projected 66 million people worldwide will be living with dementia—a statistic set to rise to 115 million by 2050 (Alzheimer's Disease International Consortium, 2009). For such a prevalent disease, the precise ethology of AD remains unclear. There has been a large amount of time, money, and resources devoted to elucidating this mystery; but developing an effective treatment in the absence of understanding the true cause of the disease continues to be a major challenge. As the elderly population battling this debilitating disease increases, it has left not only a burden on the patients themselves, but also a tremendous emotional burden on society. The cost of care for a patient with AD is approximately $57,000 per year in the United States (Burns and Llife, 2009).

It is, therefore, critically important to uncover the root cause of Alzheimer's disease, not just the inevitable consequences, and to develop sufficient treatment and prevention strategies. It has been difficult for researchers overcome the time and financial limitations of mouse models while still preserving disease relevance to the

human condition. For example, many mouse models take 6 months to a year before showing any disease pathology, with many models only showing a small subset of phenotypes associated with Alzheimer's disease. This thesis sought to develop a physiologically relevant model of AD without these limitations and has successfully done so in the model organism *Caenorhabditis elegans*.

What Exactly is Alzheimer's Disease?

AD can be divided into early-onset (younger than 65) and late-onset (older than 65); both forms have a genetic component. Early-onset AD (also known as familial Alzheimer's Disease-FAD) accounts for 5-10% of all disease cases. Early-onset AD generally becomes apparent in an individual's mid-50's and 60's. Based on genealogical and genetic studies, FAD is inherited as an autosomal dominant mutation in one of a handful of key genes; Amyloid Precursor Protein (APP), Presenilin 1(PSEN1), and Presenilin 2 (PSEN2) (Bertram and Tanzi, 2008). Late-onset AD is considered to be a sporadic version, although there are a number of risk factors associated this form of disease. The genetic and environmental causes of the sporadic form of the disease are very poorly understood. Even though early-onset and familial AD account for only a small percentage of all cases, researchers have strived to understand the familial form in the hopes that it can help to elucidate some of the mystery behind the sporadic version of the disease.

Two histological hallmarks of this disease are confirmed post-mortem, marked by the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles. Until recently, this post-mortem affirmation made it difficult to diagnosis individuals exhibiting early signs of the disease. The molecular mechanism(s) still have yet to be uncovered; therefore, it is yet unknown if the presence of plaques and tangles themselves serve a causative role in the pathology or a consequence of unidentified upstream culprits. Mature plaques contain cleavage fragments of the Amyloid Precursor Protein (APP), mainly Aβ42 and Aβ40 (Iwatsubo et al, 1994). These plaques are surrounded by activated microglia and astrocytes as well as inflammation-related proteins that may have negative consequences on the nearby cells (Eikenlenboom et al., 2002). Neurofibrillary tangles are composed of hyperphosphorylated forms of microtubule-associated protein tau (MAPT). These fibers are normally associated with maintaining cellular structure and transport via microtubules, but become unstable in tangle formation. This results in a loss of cellular transport ability throughout the cells (Iqbal, 2005).

A controversy exists as to which (or whether) these hallmarks might have a causative role in disease pathology. Extensive research has been done looking into both APP and tau; the major consensus is that APP pathology is upstream of tau. Using the model *C. elegans*, it is possible to address both APP and tau questions. Strong orthologs exists for both mammalian genes in the worm. This thesis has focused on the contribution of APP to Alzheimer's disease in a developed AD model. One future direction of the project will be to assess the contributions of tau, as well as the potential

interaction between APP and tau in this *in vivo* system that allows rapid genetic maniuplations.

New Model for AD Research

Some cases of AD are clearly linked to the amyloid precursor protein (APP). Individuals who carry an additional wild-type copy of the *APP* gene or have rare mutations in *APP* develop early-onset AD (Bertram and Tanzi, 2008). For example, individuals with Down syndrome carry an addition copy of APP due to a triplication of the 21st chromosome (which is where APP lies). This then leads to the inevitable result of early-onset AD in DS patients (Ness et al., 2012). Gain-of-function mutations in APP have also been shown to give rise to AD (Goate et al., 1991). Conversely, a distinct APP mutation has recently been linked to lower incidence of AD in an Icelandic population (Jonsson et al., 2012). Despite the certainty about the involvement of APP in AD, it remains unclear how a single extra wild-type copy of APP contributes to AD and how potential pharmacological intervention may prevent this neurodegeneration. This idea was the central theme for research conducted in this thesis. The goal was to generate an AD model in *C. elegans* that expressed this additional wild-type copy of APP (or the worm ortholog *apl-1*) to see if we could observe any physiologically relevant phenotypes associated with human AD. The speed and low-cost to which this can be done is a very attractive alternative to generating new AD mouse models. *C. elegans* also allows us to address questions that are not possible in other model systems.

Mouse Models of AD

Multiple labs have developed transgenic mouse and invertebrate models of AD in which the *APP* gene is overexpressed (Ting et al., 2007; Calhoun et al., 1999; Sturchler-Pierrat et al., 1997; Ghosal et al., 2009). These models include both wild-type and mutated versions of the gene, as well as cleavage products of the mature APP protein. The models have proven to be important tools to study the progression of APP-induced neurodegeneration and to discover potential methods to slow or prevent damage. For instance, in mice, overexpression of either APP or the Aβ peptide in mice recapitulates the formation of plaques and neurodegeneration of cholinergic neurons in adult-stage animals mirroring human AD (Small and Duff, 2008). However, recent studies have found that medical treatments eliminating Aβ-rich plaques do not prevent the progression of neurodegeneration and death in humans (Howlet and Richardson, 2009).

This raises the question of whether additional portions of APP, other than Aβ, might be more relevant to AD. In addition, these models recapitulate some, but not all, of the pathological symptoms associated with AD. (Comprehensive Review- Chin, 2011) Unfortunately, the process of strain generation is expensive and slow in the mouse, severely limiting the number of hypotheses that can be tested in a timely fashion. It often takes 6 months or more to begin to observe any of the traditional characteristics of AD, as well as many months more to analyze the progression of pathology in this system. As the number of baby boomers continue to edge closer to the late-onset age for AD, an expedited approach is become ever more valuable.

C. elegans as a model system for Alzheimer's

C. elegans as a model organism offers several advantages to study Alzheimer's disease causes and consequences in the context of a whole animal. It has a short life span (3 days to reach adulthood), making aging studies possible in very short time frames. Forward and reverse genetics are extremely facile in the worm, as is molecular manipulation of single genes and production of transgenic animals. This allows researchers to easily test several gene hypotheses in parallel or in combination. Every cell in the tiny (1mm) worm is identified, including the 302 neurons which compose its nervous system. Furthermore, individual cells can be examined by microscopy in the living, transparent worm. Hundreds of promoters have been characterized that allow the expression of genes in subpopulations of cells, enabling the marking of individual cells so they can be identified with fluorescence. The ability to track individual cells *in vivo* throughout the life span of the worm non-invasively represents a huge advantage not previously available. It is also possible to monitor hundreds of animals simultaneously, making large population studies in a variety of genetic backgrounds easily manageable. *C. elegans* has a number of discrete behaviors governed by distinct, and often independent, neural circuits. The roles of particular neurons in these behaviors can be discerned by mutagenesis as well as by single cell ablation with a laser. Approaches using *C. elegans* are tried and tested. Because of the conservation of genes and cellular mechanisms during evolution, studies in *C. elegans* were the first to identify several vital pathways, including those involved in cell death and necrosis (Putcha and Jonhson,

2004). *C. elegans* share 40% homology, 66% orthology, of genes with humans. Therefore, conclusions drawn from specific genes in this facile and expatiated system can lead to valuable conclusions relevant to their human gene counterparts.

Despite these advantages, previous attempts to produce a model of AD in *C. elegans* have been less productive, in large part due to the extreme phenotypes that result from the overexpression of multiple copies of *APP*. For example, in contrast to the mouse model, multi-copy overexpression of the *APP* ortholog, *apl-1*, led to a collection of extreme phenotypes including partial lethality, arrested development, and extensive vacuolization (Hornsten et al., 2007). I have also confirmed these findings by overexpressing multiple copies of the *apl-1* gene and the *APP* gene with a pan-neuronal promoter. Neurodegeneration could not be easily discerned in these animals. Utilizing the MOSCI technique (Frokjaer-Jensen et al., 2008), which allows the insertion of a single gene into the *C. elegans* genome, it is now possible to determine if an extra single copy of the *apl-1* or *APP* results in phenotypes that better reflect the human AD condition. If so, it will then be possible to discern the molecular mechanism(s) by which these pathological phenotypes arise.

An additional advantage of using a *C. elegans* disease model is the ability to conduct high-throughput drug screening in a short timeframe. It is possible to screen through hundreds, even thousands, of potential drug candidates in just a few months. Positive hits can then be immediately tested in murine models of AD to determine if the beneficial effects are transferable to a mammalian system. Mouse researchers have to be very selective about the drugs they test, based on the constraints of their system

mentioned above. If we can streamline the candidate drug target process for them, it could expedite the timeframe to which a therapeutic drug could make it to the aging AD population in desperate need of a cure.

This thesis proposed using the *C. elegans* system to investigate how *apl-1* and *APP* overexpression lead to degeneration of specific neuron subclasses. We will also test genetic and pharmacological strategies to prevent selective neuron loss. This research has resulted in a model for which an additional wild-type copy of APP can lead to the neurodegeneration of a specific subset of neurons; the same neurons lost first in the human pathology. Though this model cannot recapitulate certain aspects of the disease, including higher-order learning and specific brain region analysis, it does allow us to discern the molecular mechanism by which a select subset of neurons degenerate in this disease pathology. Results obtained from this study can then yield powerful insights to potential parallels in the mammalian system not previously available in such a timely fashion. This research has also lead to the identification of novel compounds that can prevent APP-induced neurodegeneration, as well as their potential location and mechanism of action.

It would have taken vast amounts of time, money, and resources to conduct a fraction of this research in a mouse model; some experiments can only be done in a simper system like *C. elegans*. This model can continue to be used in the future to explore other pathological hallmarks of AD not elucidated here; some of which include plaque formation, tau aggregation, and the spreading of neurodegeneration to other cell types in an aging animal.

APP and Alzheimer's Disease

APP is a single transmembrane-spanning protein with a large extracellular domain and a smaller intracellular domain (Figure 1; LaFerla, 2002). It is expressed in many tissues, but in high prevalence at neuronal synapses. The primary function of APP has yet to be determined, but roles have been supported in neuron growth, survival, and post injury repair (Priller et al., 2006; Turner et al., 2003). This is based on increased expression levels during neuronal differentiation and after neuronal injury occurs. Roles in cell signaling and adhesion have also been proposed, but the research is still limited (Zheng and Koo, 2006). Structural analysis of the APP protein reveals a few potential function s (not mutually exclusive): as a receptor at the cell surface, fragments may be released at the cell surface that can act as signaling molecules, or the intracellular domain can be cleaved and influence transcription of downstream targets. Because of the genetic link between APP and AD, much research has been put into how this protein is processed. As a result, the cleavage process of APP into its many parts is much more understood than the actual endogenous function of these cleavage products or the pathogenic roles they may have.

Mature APP undergoes a sequential cleavage by 3 or more proteases during post-translational processing which lead to several cleavage products. (Figure 1). Initially, an α-secretase or β-secretase cleaves the extracellular domain from the membrane-anchored carboxy-terminal fragment. After initial cleavage, a secondary δ-secretase cleaves within the membrane-spanning domain. The major δ-secretases in humans are (PSEN1 and PSEN2 (mentioned earlier). This generates an Aβ fragment and a released intracellular-

portion. The α-cleavage pathway is the main pathway used under normal physiological conditions, while the proposed amyloidgenic pathway involves the β cleavage products. Studies have shown that APPα acts as a neurotrophic and neuroprotective protein in the

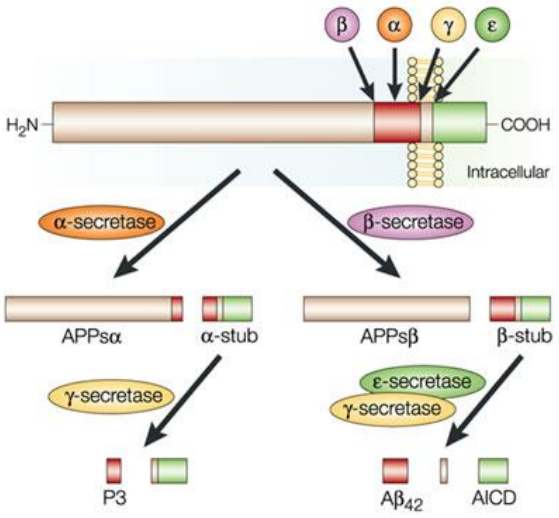


Figure 1. Proteolysis of APP. APP is a single transmembrane protein that undergoes several proteolytic cleavages to produce multiple products. α-secretase cleavage is thought to be the non-amyloidgenic pathway, while the β-secretase cleavage produces potentially disease causing fragments. Illustration adapted from LaFerla, 2002.

human brain (Meziane et al., 1998; Mattson, 1997). Increased Aβ loads in patients with AD are accompanied by high levels of the secreted cleavage product of β-secretase (sAPPβ) and the intracellular domain cleavage product (AICD). One of the major modes of AD thinking is that the β cleavage process is somehow preferentially chosen in the disease state, leading to more toxic Aβ cleavage products (mainly Aβ42). This is the main line of thinking behind the Amyloid Cascade Hypothesis, one competing theory for

the root cause of AD. Using our model of AD in *C. elegans*, we are able to address the contributions of different portions or cleavage products of APP to disease pathology. It was also possible to combine a subset of cleavage products *in vivo* to assess any potential synergistic effects. This is not easily accomplished in mouse models.

Competing Theories in the Field

Denoted as the Amyloid Cascade Hypothesis, amyloid deposition is thought to be the initiating step to which the remaining observed characteristics are a consequence. Downstream consequences include inflammation, tau hyperphosphorylation, and eventually neurodegeneration (Hardy, 2006). Much of the drug development has been based on this view, yet none to date have proved to be effective in improving the care of these patients. The goal of many of these drugs was to eliminate or neutralize plaques. This adds to the questions of whether the plaques themselves are destructive themselves or a consequence of upstream targets.

An alternative hypothesis has emerged around the microtubule-associated protein Tau. This view holds that tau abnormalities initiate the disease cascade (Mudher et al., 2002). Phosphorylated tau is a necessary physiological function to promote effective cellular transport along microtubules in the cell. Pathology results when the tau becomes hyperphosphorylated, pairing with other tau fibers in the cell—producing intracellular neurofibrillary tangles (Goedert et al., 1991). These tangles, in turn, lead to the disintegration of transport along microtubules in the cell, potentially leading to apoptosis. Deletion of tau was also shown to prevent many of the AD phenotypes in APP transgenic

mice (LeRoy et al., 2012). The Tau Hypothesis lost momentum when a number of mouse models can exhibit AD like symptoms in the absence of tangles (Kitazawa et al., 2012; Lalonde et al., 2012). Currently, most scientists agree that tau pathology is secondary to amyloid pathology.

Technological advances in genome sequencing have yielded the identification of more than ten risk genes for late-onset disease (Hollingworth et al., 2011; Lambert et al., 2009; Seshadri et al., 2010). APOE4 remains the largest risk factor, but TREM2 has been getting a lot of attention recently. TREM2 is a receptor located on myeloid cells that are triggered during an immune response. It is believed that a mutation in TREM2 prevents white blood cells from controlling the amount of Aβ present, conferring a 3-5 times increased risk of contracting late-onset AD (Jonsson et al., 2013). Identification of these additional risk factors has led to the investigation of other components contributing to the disease pathway; including lipid metabolism, the immune system, and synaptic functioning mechanisms.

Molecular Basis for Cell Death in AD

There is extensive neurodegeneration in the progression of AD. The process by which these vulnerable cells die is poorly understood. There are two main modes to which a cell can execute its demise: the process of necrosis or apoptosis. Necrosis is the type of cellular death normally associated with trauma, infections or toxins. Not easily controlled, cell death products are often released into the extracellular space and can result in harm to the surrounding cells (Proskuryakov et al., 2003). Alternatively,

apoptosis is thought to be a much more regulated "programmed" cell death process. Apoptosis follows a number of characteristic steps that allow dead cells to be engulfed before any harmful products can be released (Alberts et al., 2008). This type of cell death is also a normal part of development in many organisms.

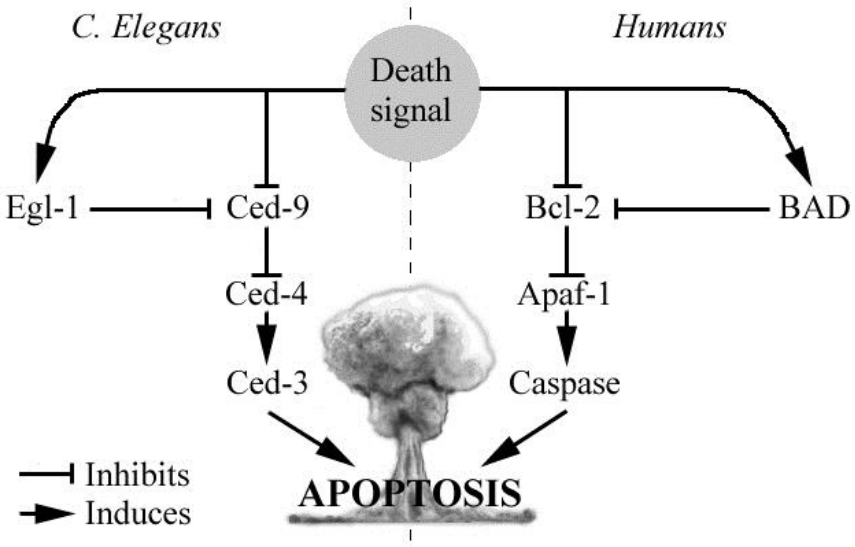


Figure 2. Conservation of Apoptotic Machinery across Species. A comparison between *C. elegans* and mammals shows conservation of key genes throughout evolution. Illustration adapted from herkules.oulu.fi.

Both of these pathways have key gene players involved in the execution of their processes, a few of which have been assessed in mouse models of AD. Caspase-3 is a protease that plays a central role in the execution phase of apoptosis (Figure 2). Acting as an executioner, it can trigger cell death based on intrinsic or extrinsic cell signals. It has been argued that there are activated caspases and apoptosis-like events in Alzheimer brains (Su et al., 2001). This has been further supported by an enrichment of caspase-3

found in a synaptic fraction of Alzheimer brains (Louneva, 2008; Ribe et al., 2008). Conversely, treatment of a mouse model of AD with caspase inhibitors resulted in improved synaptic dysfunction, assessed by contextual fear conditioning (D'Amelio et al., 2011).

These studies have only been able to correlate caspase activation and presence to AD pathology; there is still no direct evidence that the neurons in AD are dying through the process of apoptosis. *C. elegans* has a very clear ortholog of mammalian caspase-3, denoted *ced-3,* in addition to other molecules conserved in apoptosis. The Nobel Prize was awarded in 2002 to *C. elegans* researchers for their work on apoptosis and discoveries that paralleled in the mammalian system, further validating this model as physiologically relevant. Using our *C. elegans* model of AD, we have been able to definitively show for the first time in any AD mutant model that APP can trigger cell death through apoptosis in *vivo*.

Role of Innate Immune System in AD

Aging is the leading risk factor for Alzheimer's disease. Aging is associated with increased mortality from infection in evolutionarily diverse species (Shanley et al., 2009). These observations have been attributed to an age-dependent decline in immune function, also known as immunosenescence. It has been proposed that the immune system efficiency declines with aging, making these neurons more vulnerable to insults. Could this be a "tipping point" for effective neurodegeneration in AD brains? Immune activation and inflammation within the central nervous system is a classical feature of

neurodegenerative diseases. It remains controversial as to whether immune activation is contributing to the neuronal damage or attempting to prevent damage in AD. One hypothesis is that Aβ plaques and tangle stimulate a chronic inflammatory reaction to clear debris, but this chronic activation can eventually erode surrounding tissues (Town et al., 2005).

As part of the innate immune system, microglia act to defend against pathogens and to clear injured neurons and debris. Engulfment of these apoptotic cells is essential to prevent an inflammatory response (Napoli and Neumann, 2009). It has also been shown, however, that microglia can promote inflammation, potentially exacerbating neurodegenerative diseases (Minghetti, 2005; Block et al., 2007). TREM2 is a gene expressed in microglia and neurons that has received a lot of attention; its role in neurons remains to be studied. Depletion of TREM2 amplifies inflammatory cytokine responses (Jonsson et al., 2012; Guerreiro et al., 2012), while treatment with TREM-2 expressing cells can reduce inflammation and improve disease pathology in a mouse model (Takahashi, 2010). Together, these results demonstrate a critical role for immune signaling and inflammation in degenerative pathology.

Clinical pharmaceutical trials aimed at modulating the immune system in AD have largely focused on either dampening down central proinflammatory innate immunity axis or manipulating adaptive immunity to facilitate the removal of centrally deposited beta amyloid. One immune-modulatory strategy in AD was centered on a vaccination against Aβ in an attempt to reduce plaque burden. The hope was that a decreased plaque load would result in cognitive recovery, or at least halt further decline.

The initial studies in mouse models demonstrated decreased protein aggregation and improved cognitive signs (Yamada et al., 2009). This approach in human clinical trials proved disappointing. Antibodies did reduce plaque aggregation in patients but did not result in improved cognitive function. (Holmes et al., 2008). It is unclear if treatment of patients before plaque deposition would have had positive effects. Collectively, current research demonstrates that alterations in the immune system can contribute to the disease pathology of AD. If the molecular basis of increased APP can be elucidated, it might lead to the development of novel and effective therapeutic targets.

Most of the genes involved in the innate immune signaling cascade are conserved in *C. elegans*. This allows us to address the possible roles of many of the immunity genes in a timely manner using our model of AD. We can address the consequences of increasing or decreasing single immunity genes *in vivo*, as well as manipulating many pathway components simultaneously. The speed of this model lends itself to quick epigenetic analysis, pinpointing exactly where overexpression of APP and the immune system come together and contribute to disease pathology. We have shown that boosting a conserved innate immune signaling axis can lower APP-induced neurodegeneration in or model of AD, while lowering immune signaling can promote degeneration even in a wild-type background.

Cell-type Specific Degeneration

It was originally thought that AD was induced by reduced synthesis of the neurotransmitter acetylcholine in the brain; this is the oldest known hypothesis of AD

causation. It is known that the cholinergic neurons are the first do die in the disease progression of AD, though it is not known why these cells are particularly vulnerable. Selective vulnerability is not exclusive to AD; many other neurodegenerative diseases target a specific subset of neurons first ranging from ALS to Parkinson's disease (Boillee et al., 2006; Davie, 2008; Estrada et al., 2008). The model of AD proposed in this research demonstrates selective degeneration of a subset of cholinergic neurons, similar to that in the human condition.

Most of the current AD drug therapies are aimed at increasing cholinergic neuron health and signaling. Four major acetylcholinesterase inhibitors are currently on the market—tacrine, rivastigmine, galantamine, and donezeile (Pohanka, 2011). Unfortunately, none of these drugs have been shown effective in delaying the onset of the diseases. If we can delineate what makes cholinergic neurons the initial target of this disease, we can begin to develop preventative strategies instead of ineffective coping strategies. Using *C. elegans* as a model can exacerbate this process and get valid answers in a fraction of the time it would take in mouse models.

Conclusion

This thesis will show demonstrate that a simple genetic system like C. elegans can be utilized to address large scientific questions. Here, we show that a single additional copy of APP is sufficient to induce a select pattern of degeneration during middle age, mimicking the human condition. We have also begun to elucidate the molecular mechanism by which this cellular death occurs. Finally, we also reveal novel compounds

that can alleviate select degeneration in this model, paving the way for future drug targets to be discovered.

Chapter 2: Induction and Prevention of Patterned Neurodegeneration in Alzheimer's Disease

Abstract

Alzheimer disease is characterized by the initial degeneration of a subset of cholinergic neurons. This pattern of degeneration can be triggered by overexpression of the amyloid precursor protein (*APP*) gene in humans. Interestingly, *APP* is widely expressed; it is therefore unclear why only certain cholinergic neurons are vulnerable to degeneration. We show that widespread expression of the human *APP* gene in the nematode *Caenorhabditis elegans* also induces age-dependent apoptotic degeneration of select cholinergic neurons. Identical results were obtained by overexpressing the orthologous worm gene *apl-1*. The pattern of neurodegeneration matched the cell-autonomous accumulation of APP protein in vulnerable neurons and could be activated cell non-autonomously by distinct portions of APP. Vulnerability to APP accumulation and degeneration depended inversely on the level of ASK1/p38MAPK innate-immune signaling in cholinergic neurons. Lastly, we identify a compound P7C3 that blocks entrance to apoptosis caused by APP or immunodeficiency. Our results suggest that immunosenescence sculpts the cellular pattern of neurodegeneration by APP.

Introduction

Alzheimer's disease (AD) is the most common cause of dementia and the sixth leading cause of death in the US, but the precise etiology of AD is unclear (Herbert et al.,

2003). Some cases of AD are clearly linked to the amyloid precursor protein (APP). For instance, rare mutations in *APP* have been linked to familial cases of early onset AD (Bertram et al., 2008; Small et al., 2008) and a distinct *APP* mutation has been recently connected to lower incidence of AD in people from Iceland (Jonsson et al., 2012). More commonly, individuals that carry additional wild-type copies of the *APP* gene, including those with Down syndrome, also develop AD (Zigman et al., 2007). Conversely, individuals with Down syndrome that reach advanced age without signs of dementia have been shown to be disomic for *APP* (Prasher et al., 1998). Consistent with these findings, overexpression of a wild-type (WT) copy of *APP* in mouse causes neurodegeneration (Simon et al., 2009), and a mouse model of Down syndrome that was made disomic for *APP* did not display AD-type symptoms (Salehi et al., 2006). *APP* encodes a transmembrane protein that is enzymatically cleaved into several peptides, including Aβ peptides (Zhang et al., 2012). Different variants of Aβ compose amyloid plaques found in the brains of AD patients and produce neurodegeneration and/or neural dysfunction in multiple model systems (Huang et al, 2012). Despite this certainty about the involvement of *APP* in AD, it remains uncertain how only a single extra wild-type copy of *APP* contributes to AD and how pharmacological treatments may prevent this neurodegeneration.

One of the most mysterious aspects of AD is that the first group of neurons to die includes a specific subset of cholinergic neurons important for memory (Davies et al., 1976). This initially limited pattern of degeneration is puzzling because the *APP* gene is

widely expressed throughout the brain (even in non-neural tissue (Neve et al., 1998)). Also perplexing, risk factors such as genetic allele status (e.g. *APOE* and *TREM2*) and medical history (e.g. chronic infection) increase the incidence as well as hasten the onset and progression of this pattern of degeneration without obviously targeting these cholinergic neurons (Kim et al., 2009; Honjo et al., 2009; Hamerman et al, 2006; Takahashi et al., 2007). While progress has been made on theories to explain the progressive spread of degeneration throughout the brain (e.g. trans-synaptic spread of hyperphosphorylated TAU protein (De Calignon et al., 2012; Liu et al., 2012) and nonfibril Aβ (Nath et al., 2012)), fewer theories have been advanced to explain why certain cholinergic neurons are the first to degenerate, and why they only begin to degenerate in middle age (Saxena and Caroni, 2011; Benilova et al., 2012; Gotz et al., 2009).

Experimental study of the initial pattern of degeneration in AD can be difficult in rodent models of AD and humans due to the ambiguity of onset. Salient behavioral phenotypes that correlate with initial degeneration are often absent and non-invasive methods to detect degeneration *in vivo* cannot provide insight until substantial portions of the brain have deteriorated (Ashe et al., 2010; Iqbal and Grundke-Iqbal, 2011). To overcome certain limitations of rodent models, some have turned to simpler *in vivo* models, including the genetic model nematode *Caenorhabditis elegans*, to study aspects of AD (Hornsten et al., 2007; Link et al., 2003). Studying the molecular origin of patterned neurodegeneration may be easier in *C. elegans* because it offers more rapid

genetic manipulation, a transparent body to visualize its completely described nervous system, and an easier way to link behavioral dysfunction to identified neurons. *C. elegans* models of AD have been limited, however, because until now they have not yet demonstrated neurodegeneration (Wentzell et al., 2010).

Here, we describe the first example of a *C. elegans* model of AD that displays age-dependent patterned cholinergic degeneration upon expression of a single copy of human *APP* gene. We propose a novel mechanism to explain how the widely-expressed APP protein destroys a select subset of cholinergic neurons in middle age through cell-autonomous selective APP accumulation, cell-non-autonomous signaling by distinct portions of APP, and decline in innate immunity. Additionally, we present the neuroprotective effects of a newly characterized compound, P7C3, which prevents APP-induced neurodegeneration by blocking entrance to apoptosis. By combining the use of pharmacologically active agents with genetic manipulation of the nematode, we have attempted to dissect the molecular mechanism of patterned neurodegeneration found in AD.

Materials and Methods

Strains: Nemotode (*C. elegans*; Bristol strain N2 as wild type) strains were grown at 20°C as described on 1.7% agar plates containing nematode growth medium and seeded with Escherichia coli strain OP50 (Brenner et al., 1974). Animals cultured on plates contaminated with fungi or other bacteria were excluded from this study. The genotypes

of mutant and transgenic strains generated for this study were confirmed through PCR and/or sequencing and are listed with other strains used in Table 1.

Number	Strain & **Shorthand**	Genotype
1	JPS6 ***SC_apl-1***	*vxSi1[Prab3::apl-1::unc-54UTR, Cb-unc-119 (+)] II; unc-119(ed3) III.*
2	JPS7 ***MC_apl-1***	*vxEx1[Prab3::apl-1::unc-54UTR, Cb-unc-119 (+)]; unc-119(ed3) III.*
3	JPS11	*vxSi1 II; vsIs13 IV; lin-15B(n765) X.*
4	JPS22 ***SC_con***	*vxSi50[Cb-unc-119 (+)] II.* (designated WT for this paper)
5	JPS26	*vsIs13 IV; egl-1(n4065) V; lin-15b(n765) X.*
6	JPS27	*vxSi1 II; unc-119(ed3) III; vsIs13 IV; egl-1(n4065) V; lin-15b(n765) X.*
7	JPS35 ***SC_apl-1:mCh***	*vxSi35[Prab3:apl-1:mCherry-unc-54-UTR,Cb-unc-119(+)] II; unc-119 (ed3) III.*
8	JPS37	*pha-1(e2123); mdIs162; vxSi1 II; unc-119(ed3) III.*
9	JPS38 ***SC_APP***	*vxSi38[Prab3:huAPP695:unc54UTR, Cb-unc119(+)] II, unc-119(ed3) III.*
10	JPS39 ***MC_APP***	*vxEx39[Prab3:huAPP695:unc54UTR, Cb-unc-119 (+)], unc-119(ed3)III.*
11	JPS40	*vxSi35 II; unc-119 III; vsIs13 IV; lin-15B(n765) X.*
12	JPS57	*ced-6(tm1826) III; vsIs 13IV; lin-15B(n765) X.*
13	JPS66	*ced-3(ok734), vsIs13 IV; lin-15B (n765) X.*
14	JPS67	*vsIs38 II; unc-119(ed3) III; vsIs13 IV; lin-15B(n765) X.*
15	JPS70	*vsIs13 IV; crt-1(ok948), lin-15B(n765) V.*
16	JPS71	*vsSi 1 II; ced-6(tm1826), unc-119(ed3) III; vsIs 13IV; lin-15B(n765) X.*
17	JPS75	*vxSi1 II; unc-119(ed3) III; vsIs13 IV; crt-1(ok948), lin-15B(n765) V.*
18	JPS98	*vsIs13 IV; lin-15B(n765) X; vxEx98[Prab-3::mCherry, Cbunc-119 (+)].*
19	JPS112 ***SC_APP IV***	*unc-119(ed3) III ; vxSi38 IV.*
20	JPS113	*unc-119(ed3) III; vsIs13, vxSi1 IV; lin-15b(n765) X.*
21	JPS114	*vxSi II; unc-119(ed3) III; vs48[unc-17::GFP]*
22	JPS115	*vsIs13 IV; egl-1(ok1418) V; lin-15b(n765) X.*
23	JPS116	*vxSi1 II; unc-119(ed3) III; vsIs13 IV; egl-1(ok1418) V; lin-15b(n765) X.*

Table 1. Strains Generated and Utilized in This Study

24	JPS 126 ***SC_apl-1 IV***	*Unc-119(ed3) III; vxSi1 IV.*
25	JPS127	*unc-119(ed3) III; vsIs13, vxSi38 IV; lin-15b(n765) X.*
26	JPS128 ***SC_APP:mCh***	*VxSi128[Prab3::huAPP695::mcherry:unc54UTR, Cbunc-119(+)] II; unc-119(ed3) III; vsIs13 IV; lin-15b(n765) X.*
24	JPS126 ***SC_apl-1 IV***	*unc-119(ed3) III ; vxSi1 IV.*
27	JPS145	*vxSi [Papl-1:apl-1:unc54UTR, Cbunc-119 (+)] II; unc-119(ed3) III; vsIs13 IV; lin-15b(n765) X.*
28	JPS146 ***SC_Papl-1:apl-1***	*vxSi [Papl-1:apl-1:unc54UTR, Cbunc-119 (+)] II; unc-119(ed3) III.*
29	JPS150	*vxSi128 II; unc-119(ed3) III; vsIs13 IV; egl-1(ok1418) V; lin15b(n765) X.*
30	JPS151 ***Prab3::mCh***	*vxEx151[Prab3::mCherry]; vxSi1 II; unc-119(ed3) III; vsIs13 IV; lin-15b(n765) X.*
31	JPS152	*vxEx151[Prab3::mCherry]; vxSi38 II; unc-119(ed3) III; vsIs13 IV; lin-15b(n765) X.*
32	JPS166	*VxSi1 II, juIOs76 II; unc-71(ju156), unc-119(ed3) III.*
33	JPS167	*vxSi1 II; unc-119(ed3) III; ced-3(ok734), vsIs13 IV; lin-15B (n765) X.*
34	JPS175 ***SC_APPC59***	*vxSi175[Prab3::huAPPC59::unc-54UTR, Cb-unc-119 (+)] II; unc-119(ed3) III; vsIs13 IV; lin-15b(n765) X.*
35	JPS176 ***SC_APPN636***	*vxSi176[Prab3::huAPPN636::unc-54UTR, Cb-unc-119 (+)] II; unc-119(ed3) III.*
36	JPS178 ***SC_mCh***	*vxSi178[Prab3::mCherry::unc-54UTR, Cb-unc-119 (+)] II; unc-119(ed3) III.*
37	JPS188 ***Papl-1::apl-1***	*vxEx188[Papl-1::apl-1]; vxSi1 II; unc-119(ed3) III; vsIs13 IV; lin-15b(n765) X.*
38	JPS 203	*unc-119(ed3) III; vxSi 203[Prab3:huAPP695C59:unc-54UTR, cb-unc-119(+)] IV.*
39	JPS 204	*unc-119(ed3) III; vxSi203,vsIs13 IV; lin-15b(n765) X.*
40	JPS 205	*vxSi176 II; unc-119(ed3); vxSi203, vsIs13 IV; lin-15b(n765) X.*
41	JPS 206	*vxSi176 II; unc-119(ed3) III; vsIs13 IV; egl-1(n1418), lin-15b(n765) X.*
42	JSP 211	*nsy-1(ok593) II; vsIs13 IV; lin-15b(n765) X.*
43	JPS 212	*nsy-1(ok593), vxSi II; vsIs13 IV; lin-15b(n765) X.*
44	JPS 227	*nsy-1(eg681) II; vsIs13 IV; lin-15b(n765) X.*
45	JPS 228	*nsy-1(eg681), vxSi128 II; vsIs13 IV; lin-15b(n765) X.*
46	JPS 229	*nsy-1(ok593), vxSi II; vsIs13 IV; egl-1(n4065), lin-15b(n765) X.*
47	JPS 230	*acEx102; pmk-1(km25), vsIs13 IV; lin-15b(n765) X.*
48	JPS 231	*vsIs13 IV; sek-1(km4), lin-15b(n765) X.*
49	JPS 232	*tir-1(qd4) III; vsIs13 IV; lin-15b(n765) X.*

Table 1. Strains Generated and Utilized in This Study (cont.)

50	JPS 291	*vxSi291[Punc-30::huAPP695::mCherry::unc-54UTR, Cb-unc-19(+)] II; unc-119(ed3)III.*
51	JPS304	*vxSi304[Ptph-1::huAPP695::mCherry::unc-54UTR, Cb-unc-19(+)] II; unc-119(ed3)III.*
52	JPS305	*vxSi291II; unc-119(ed3) III; vsIs13 IV; lin-15b(n765) X.*
53	JPS306	*vxSi304II; unc-119(ed3) III; vsIs13 IV; lin-15b(n765) X.*
54	JPS307	*vxEx307[Punc-17::nsy-1(+), PCFJ90]; nsy-1(ok593) II; vsIs13 IV; lin-15b(n765) X.*
55	JPS308	*vxEx308[Prab3::huAPP695L10stop::unc-54UTR, PCFJ90]; vsIs13 IV; lin-15b(n765) X.*
56	JPS309	*VxSi128 II; unc-119(ed3) III; unc-43(e408),vsIs13 IV; lin-15b(n765) X.*
57	JPS310	*VxSi128 II; unc-119(ed3) III; unc-43(n498),vsIs13 IV; lin-15b(n765) X.*
58	JPS452	*VxEx452[Punc-17::nsy-1(+), PCFJ90]; VxSi291 II; unc-119(ed3) III; VsIs13 IV; lin-15b(n765) X.*
59	JPS453	*VxEx453[Punc-17::nsy-1(+), PCFJ90]; VxSi128 II; unc-119(ed3) III; VsIs13 IV; lin-15b(n765) X.*
60	JPS454	*VxSi454[Prab3:huAPPC59:mcherry,unc-54UTR, cb-unc-119(+)] II; unc-119(ed3) III.*
61	JPS455	*VxSi454 II; unc-119(ed3) III; VsIs13 IV; lin-15b(n765) X.*
62	JPS456	*VxSi456[Prab3:huAPPN636:mcherry,unc-54UTR, cb-unc-119(+)] II; unc-119(ed3) III.*
63	JPS457	*VxSi456 II; unc-119(ed3) III; VsIs13 IV; lin-15b(n765) X.*
64	LX929	*vsIs48[unc-17::GFP]*
65	EG1285	*lin-15b(n765) X; oxIs12 [unc-47p::GFP + lin-15(+)]*
66	OH7235	*zdIs13[tph-1::GFP]*
67	LX959	*vsIs13 IV; lin-15b(n765) X.*
68	MT2236	*egl-1(n4065)*
69	RB1021	*crt-1(ok948)*
70	RB1305	*egl-1(1418)*
71	RB885	*ced-3(ok734)*
72	FX01826	*ced-6(tm1826)*
73	CZ1931	*juIs76 II; unc-71(ju156) III.*
74	EG4322	*ttTi5605 II; unc-119(ed3) III.*
75	EG5003	*unc-119(ed3) III; cxTi10882 IV.*
76	KU25	*pmk-1(km25) IV; acEx102.*
77	KU4	*sek-1(km4*
78	ZD101	*tir-1(qd4)*
79	VC90	*nsy-1(ok593)*
80	BZ981	*nsy-1(eg691)*
81	CB408	*unc-43(e408)*
82	MT1092	*unc-43(n498)*

Table 1. Strains Generated and Utilized in This Study (cont.)

Transgenesis: Transgenic animals with a single copy of pan-neuronal *apl-1, huAPP695* full length, *huAPP695N363* extracellular/transmembrane region, or *huAPP695C59* intracellular region were generated through the MOSSCI technique as previously described (Frokjaer-Jensen et al., 2008). Briefly, we used Gateway technology (Invitrogen) to construct a vector with the *apl-1*(genomic) or *huAPP695*(cDNA) gene driven by the pan-neuronal promoter (*Prab-3*) and an *unc-54* UTR adjacent to the *Cb_unc-119(+)* positive selection marker. Transgenes were flanked by Mos1 transposon insertion sites complementary to a specific region on the 2^{nd}(ttTi5605) or 4^{th}(cxTi10882) chromosome. *unc-119* mutant worms with specific Mos1 insertions sites were then injected with DNA. Selection of mobile, non-fluorescent progeny led us to identify single-copy insertion animals. Full insertion of the *apl-1* or *APP* was confirmed by PCR and sequencing across the full transposon insertion site. Strains were then termed *SC_apl-1*, *SC_APP, SC_APPC59* and *SC_APPN636* to signify a single gene copy insertion. A control strain (*designated WT for this paper*) was also generated that contained only a single inserted copy of the *Cb_unc-119(+)* positive selection marker. Transgenic animals with *SC_apl-1* or *SC_APP* genes tagged with mCherry were generated as above with the mCherry cDNA sequence fused in frame to *APP* or *apl-1* before the *unc-54* UTR to tag the C-terminal of the protein product. An additional strain using MOSSCI to integrate a single copy of mCherry cDNA driven by *Prab-3* with *unc-54* 3' UTR was also generated (SC_*mCh*). The full sequence of the inserted *mCherry* gene was confirmed by sequencing of genomic DNA. Transgenic animals with multiple copies of pan-neuronal *apl-1* or *APP* were also generated through injection *(MC_APP*

and *MC_apl-1* respectively). These multi-copy strains displayed a high level of lethality and developmental defects consistent with a previous report (Hornsten et al., 2007). DNA (25-50ng) vectors described above were injected into *unc-119* mutant animals with fluorescent markers only. Formation of an extra-chromosomal array containing multiple gene copies was confirmed in mobile progeny that retained fluorescent markers. Expression level of unique sequence common to both *APP* and *apl-1* genes was determined using real time (RT)-PCR with primers forward AAGCAGTGCAAGACCCAT and reverse TCATCATCGTCCTCATCATCA.

Quantification of Neurodegeneration: All analyses were completed with the experimenter blind to genotype and drug treatment. Age-synchronized animals were immobilized on 2% agar pads containing 0.7 mM sodium azide. Neurons that had dimly-lit somas with missing/broken projections, abnormally shaped somas, or absent GFP labeling in the appropriate neuronal location were classified as degenerating. GABAergic neurons were visualized with *Punc-47p::gfp* (strain EG1285), VA and VB cholinergic neurons with *Punc-17::gfp* (LX929); serotonin neurons included NSM, ADF, AIM, RIH, HSN class members which were visualized with *Ptph-1::gfp* (strain OH7235). All animals were evaluated within 10 minutes of azide treatment. In cases where the worm strains were too defective in egg laying to reach day 3 or 5 adult stage, the animals were treated at L4-larval stage onward with the sterilization drug 5-fluoro-2'-deoxyuridne (FUDR, 0.12mM final) (Sigma). We found that this drug had no

effect on the progression of degeneration of VC neurons (e.g. compare untreated animals in Figure 2D to FUDR-treated animals in Figure 6B). The percentage of VC4 and VC5 neurons that succumbed to APP-induced degeneration was compared for different groups (strain, age, drug condition) using planned Fisher's exact tests (Zar et al., 1999).

Protein Localization Analysis: Day-1, -3, and -5 adult mCherry-tagged APP and APL- 1 strains were immobilized on 10% agarose pads containing microbeads (Fang-Yen et al., 2012). Animals expressing GFP/mCherry-tagged transgenes were observed under a Leica laser scanning confocal microscope (Leica TCS SP5 II) with a 63x oilimmersion objective (numerical aperture: 1.4). When two channels were used, images are acquired sequentially with the pinhole diameter set to 1.2 Airy units. Z-stacks were then taken at 40x magnification under red and green fluorescence separately, then pooled together to visualize co-localization with ImagePro Plus (Mediacybernetics). For comparison of fluorescence intensities in different areas of the worm, images were acquired under an identical exposure time, gain and pinhole diameter. Quantitative comparison of mCherry-tagged APP accumulation in VC neurons was performed by measuring average intensity of red mCherry signal versus green gfp signal in select areas of interest using ImagePro (Media Cybernetics) and compared with a *t*-test.

Laser Ablation of Neurons: Neurons were ablated as previously described (Fang- Yen et al., 2012). VC neurons were visualized using an integrated fluorescent strain, in which

VC neurons are labeled with GFP (strain LX959). All ablations were compared with shams of the same genetic background in which a laser was shot next to the animal.

Pharmacology: Drugs were freshly prepared in buffer (M9) solution and pipetted onto small seeded plates. These plates were allowed to dry at least 2 hours at room temperature before use. The final drug concentrations of solid media are: Dimebon, 50 μM (Tocris); P7C3, 50 μM (generous gift from Drs. Pieper, Ready and McKnight, UTSW). All other chemicals used in this study were at a concentration of 50μM unless specifically stated otherwise. Well-fed larval stage 4 animals were picked onto seeded plates containing .12mM FUDR with or without drug. Animals were then allowed to age at 20°C for ~20 (day 1 adult), ~68 (day 3 adult) and ~116 hours (day 5 adult) before analysis. Controls using buffer with no drug were performed on each strain for each assay, and each assay was repeated multiple (3-8) times. Data were then averaged and statistically compared with planned non-parametric X2 analyses (Zar, 1999).

Percent Lethality: The number of eggs laid by 10 first-day adult worms on bacterial plates (OP50) over 3 hours was counted, and surviving adult-stage progeny were then counted 72 hours later. Assays were repeated across 3 plates at least 3 times and percentages that failed to survive to adulthood were averaged.

Egg-retention Assay: Plates of non-starved adult worms were bleached to yield a synchronized population of eggs. Eggs were allowed to develop for ~55 hours (day 1

adult) and ~103 (day 3 adult) hours at 20°C. Animals were then individually dissolved in 1M NaOH in individual wells of a 96-well plate, and eggs retained within the dissolved adult animal were counted.

Swimming Kinematics: Worms were submerged in NGM liquid and recorded for 1 minute following a 2-minute acclimation period. Spine analysis software was then used to calculate and plot the midbody angle of the worm as previously described (Pierce-Shimomura et al., 2008). Head-bend frequency was determined based on the average time it took an animal to make 20 complete-cycle head bends. Defective swimming of single-copy *APP* or *apl-1* strains could not be simply explained by accumulated eggs restricting motion because animals sterilized with 5-fluoro-2'-deoxyuridne (0.12mM final) (Sigma) displayed the same defect.

Statistics: Unless indicated otherwise, averages are stated as mean ± SEM.

Results

Pan-neuronal overexpression of APP induces age-related degeneration of a specific subset of cholinergic neurons

Both human *APP* and nematode ortholog *apl-1* genes are widely expressed throughout the nervous system as well as in non-neuronal tissues (Bertram et al., 2008; Link et al., 2003). To focus on the nervous system, we generated strains that used a pan-

neuronal promoter (*Prab-3*) to express a single wild-type copy of human neuronal *APP*, or an extra single copy of *apl-1*, knocked into a specific locus on the second chromosome. These transgenic strains are designated *SC_APP* and *SC_apl-1* strains respectively. Both strains appeared normal in morphology and development from egg to young adult, and displayed an expected 2-fold higher expression of sequence common to both genes compared to wild type as determined by real-time PCR (Figure 1A).

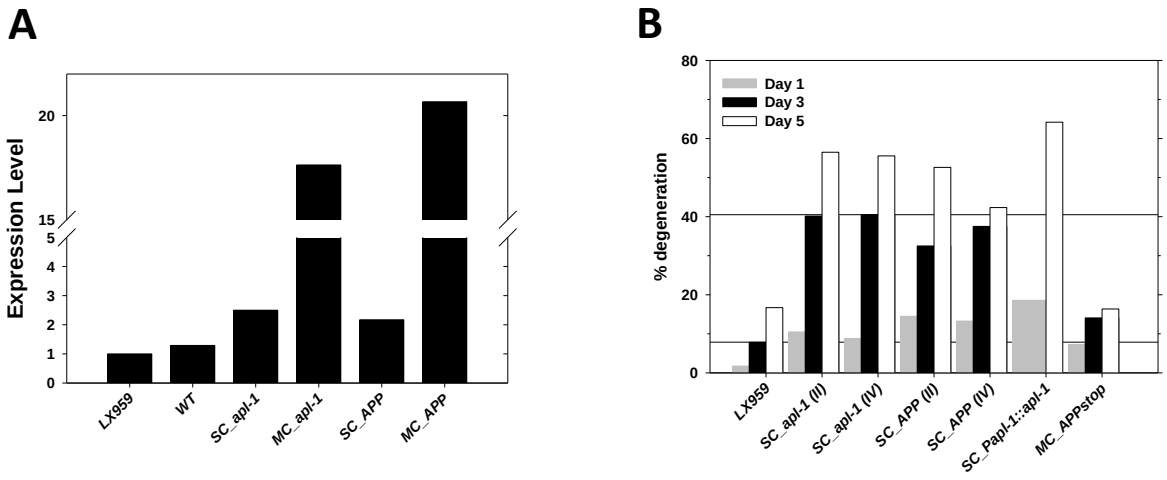
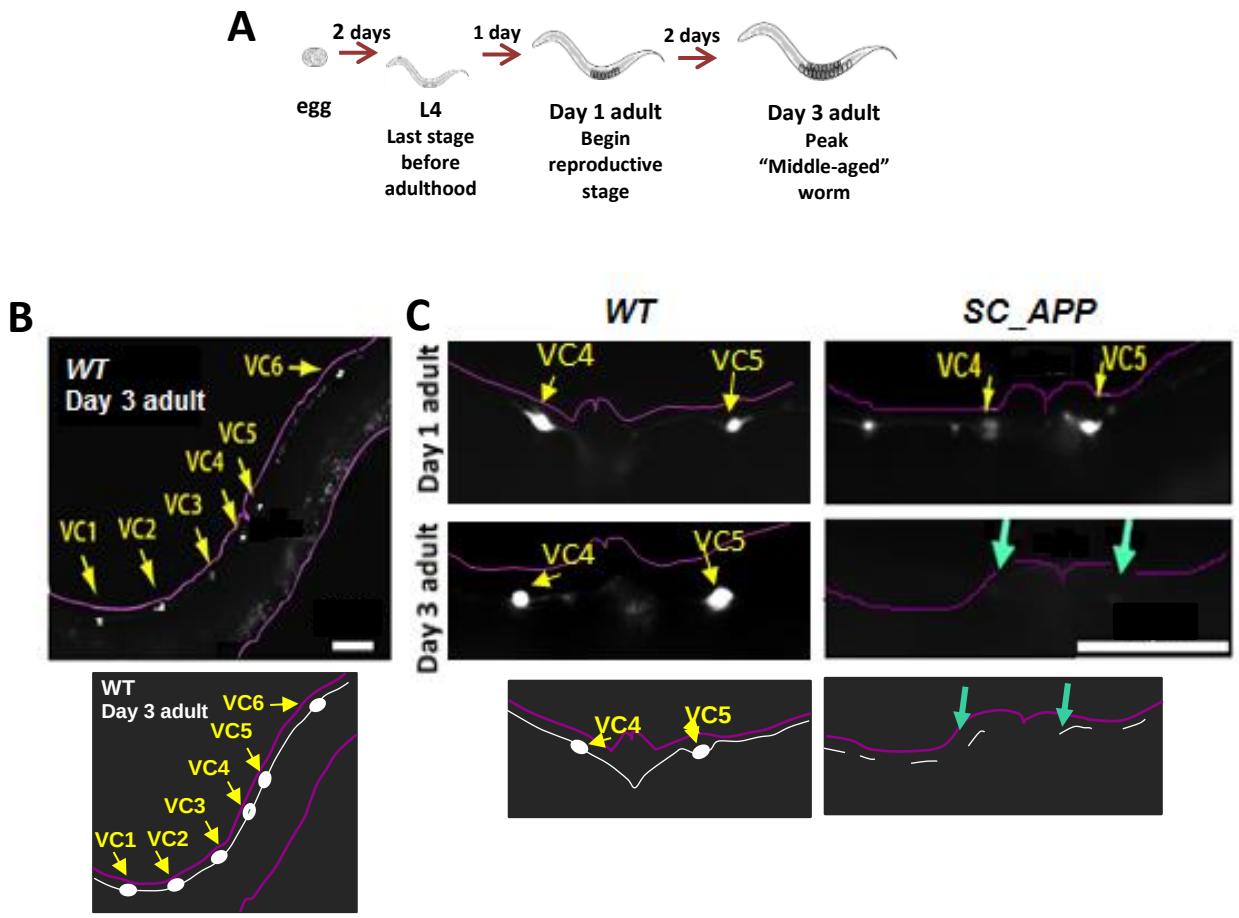


Figure 1. Quantification of *APP* and *apl-1* expression. (A) mRNA expression levels of sequence shared with *apl-1* and APP are about 2-fold higher in *SC_* overexpression strains compared to WT. Strains that express multiple copies (*MC*) of *APP* or *apl-1* show even higher levels of expression. Primers used were common to both *APP* and *apl-1* sequences (see methods). (B) Quantification of VC 4&5 degeneration shows indistinguishable pattern of degeneration for *SC_apl-1* and *SC_APP* strains generated on different chromosomes (II and IV) under control of pan-neuronal promoter (Prab-3) or using endogenous promoter Papl-1 (n>124 neurons, 62 animals per bar). All day-3 and day-5 data are significantly higher than a "wild-type" strain (LX959) that expresses GFP in VC neurons ($P<0.001$). Horizontal lines for comparison with day-3 adult WT and *SC_apl-1* data.

Figure 2. APP induces age-related degeneration of a specific subset of cholinergic neurons in *C. elegans*. (A) Time course to middle age. (B) All six VC neurons visualized with GFP with animal outlined in pink. (C) Same individuals on adult days 1 and 3. VC4&5 neurons selectively degenerate in a strain that expresses a single copy of *APP* (*SC_APP*). Remnant GFP, green arrows. Cartoon depictions for day 3 adult below. (D) Quantification of percentage degeneration of different neuronal classes. VC4&5 selectively degenerated in *SC_apl-1* and *SC_APP* strains. Planned Fisher's exact tests compare *vs* same age WT where * $P<0.003$, ** $P<0.00001$, $n>124$ neurons (62 animals) per bar.


A
2 days
1 day
2 days
egg
L4
Last stage
before
adulthood
Day 1 adult
Begin
reproductive
stage
Day 3 adult
Peak
"Middle-aged"
worm
B
WT
Day 3 adult
VC6
VC5
VC4
VC3
VC1
VC2
WT
Day 3 adult
VC6
VC5
VC4
VC3
VC1
VC2
C
WT
SC_APP
Day 1 adult
Day 3 adult
VC4
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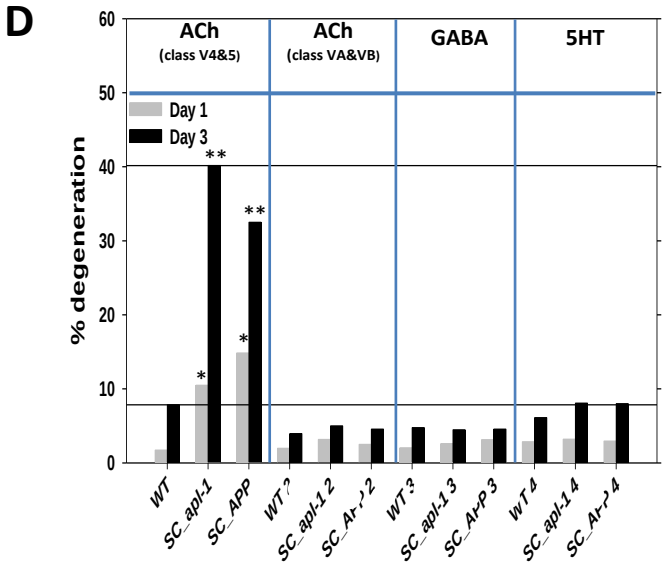
D
ACh
(class V4&5)
ACh
(class VA&VB)
GABA
5HT
Day 1
Day 3
% degeneration
60
50
40
30
20
10
0
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WT
SC_apl-1
SC_APP
WT
SC_apl-1 2
SC_Ar-P2
WT
SC_apl-1 3
SC_Ar-P 3
WT
SC_apl-1 4
SC_Ar-P 4

We noticed, however, that *SC_APP* and *SC_apl-1* worms displayed an abnormal hinging motion at the midbody during swimming on the third day of adulthood. This corresponds to post-reproductive peak and thus is considered "middle age" for *C. elegans* (Figure 2A) (Tartar et al., 2010). Coincident with this phenotype, we observed one or more small vacuoles at the midbody that were suggestive of neurodegeneration (Figure 4A, inset shows Nomarski image of dying neuron identified by position as cholinergic neuron VC5). This prompted us to investigate the health of neurons more easily by crossing in fluorescent reporters to label distinct classes of neurons. We discovered that the VC-class cholinergic (ACh) neurons were often absent or dying in the *SC_APP* and *SC_apl-1* strains in middle-aged adults. Out of the six VC neurons, the VC4 and VC5 neurons (VC4&5) degenerated most reliably (Figure 2BC; Figure 3).

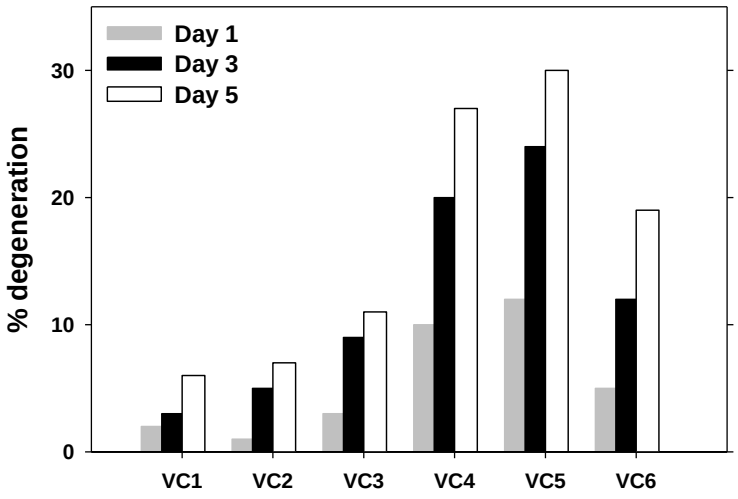


Figure 3. Incidence of degeneration of VC cholinergic neurons. Although all six of the VC-class cholinergic neurons show age-related progression of degeneration, neurons VC4 and VC5 show the highest incidence of degeneration. n>124 neurons, 62 animals per bar.

Thus, for convenience, we confined further analyses of VC-class neurons to these two neurons. Compared to a control "wild-type" strain (see methods), our AD model strains had a significant percentage of VC4&5 neurons that degenerated by the first-day (~10%) and third-day (30–40%) of adulthood (Figure 2D). After inspecting individual neurons from different classes, including those that are adjacent to the VC neurons and others throughout the ventral nerve cord appeared healthy in appearance in our AD model strains (Figure 2D). These included all 25 GABAergic neurons in the worm nervous system, as well as the 24 VA- and VB-class cholinergic neurons in the nerve cord. Likewise, nine serotoninergic (5HT) neurons appeared normal in our AD models (Figure 2D). Thus, after direct inspection of 64 of the 302 neurons that compose the *C. elegans* nervous system (White et al., 1986), we find that the six VC-class cholinergic neurons are particularly vulnerable to APP-induced degeneration.

Human AD is associated with an age-related decline in memory and cognition behaviors that depends on proper function of a vulnerable subset of cholinergic neurons (Davies et al., 1976; Bowen et al., 1976; Perry et al., 1977). Although *C. elegans* does not possess sophisticated forms of memory comparable to humans, we found that our AD models displayed a decline in simple behaviors with age. We focused on two behaviors: egg laying and swimming, which depend, in part, on the VC neurons and many other neurons (Zhang et al., 2008; Vidal-Gadea et al., 2011). *SC_APP* and *SC_apl-1* strains retained a normal number of eggs in first-day adults, but retained significantly more eggs than wild-type and a control strain (designated WT for this paper, see methods) in third-

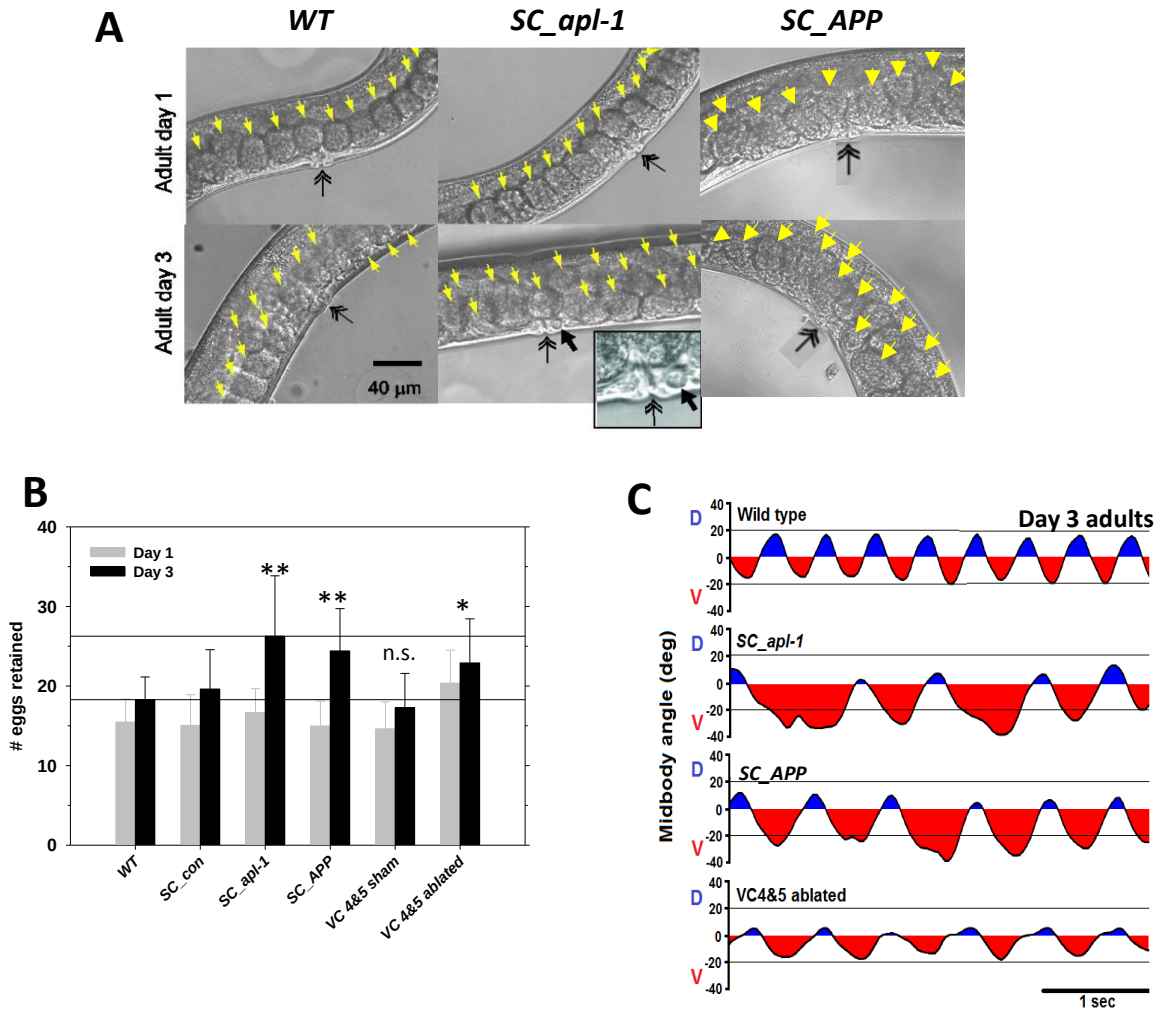


Figure 4. APP induces age-related decline of behaviors that depend on specific cholinergic neurons that degenerate. (A,B) Overexpression strains retain significantly more eggs in middle age compared to WT. (A) Normarski photomicrographs of eggs (yellow arrows) retained in the midbody of adults. Solid arrow indicated vacuole indicative of neurodegeneration with Nomarski optics; double arrow indicates vulva. Inset shows magnified view. Quantification of egg retention (B) and midbody curvature time course (C). Deficits are recapitulated with laser ablation of VC4&5 neurons. For statistical comparisons of egg retention, n>48 animals per bar, planned *t*-tests *vs* same age WT (or ablated *vs* sham) where **, $P<0.001$ and *, $P<0.05$. Error bars, s.e.m.

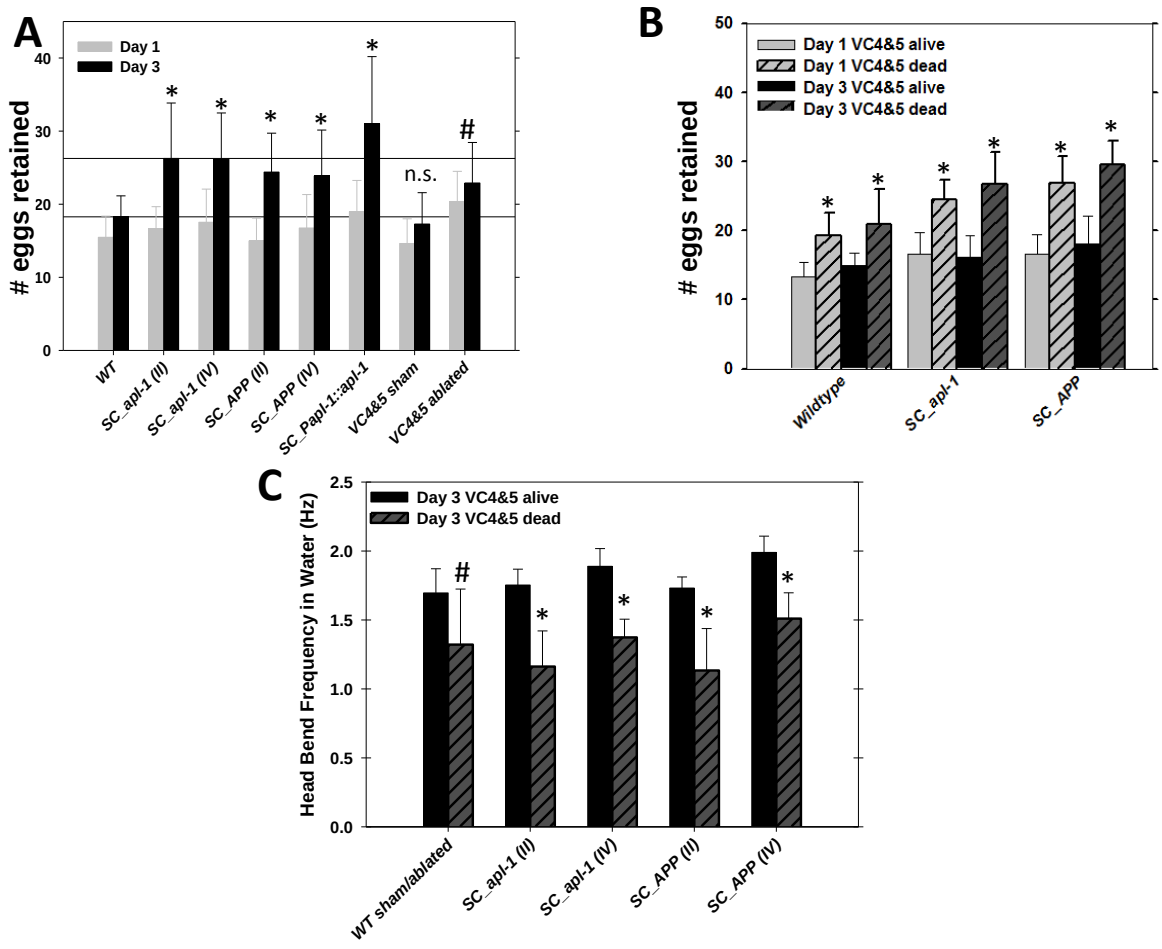


Figure 5. APP overexpression produces same effects regardless of chromosomal insertion site and promoter. (A,B) Egg-retention defects were Supplemental Text new 2 similar in *SC_apl-1* and *SC_APP* strains generated on different chromosomes (II or IV) with pan-neural promoter (Prab-3), using the endogenous *apl-1* promoter, or after laser ablation of VC4&5 neurons in a LX959 background (data same as in Figure 2) (n>48 per bar). Asterisks denote significant difference from WT in (A) and from same condition dead *vs* alive.(B). (C) Deficits in head-bend frequency of *SC_apl-1* and *SC_APP* swimming correlates with VC4&5 death, irrespective of chromosome integration site (n>20 per bar). For all panels, statistically compared with planned *t*-tests *vs* same age WT where *, P<0.01, expect for ablated animals which were compared to same age sham animals where #, P<0.05.

day adults (yellow arrows in Figure 4A,B; Figure 5A). Likewise, our AD models exhibited defective swimming in third-day but not first-day adults. Frequencies of head-bending were slightly decreased (Figure 5C) and bends at the midbody were even slower and biased towards the ventral side (Figure 4C). The pattern of degeneration and unique behavioral defects could be not explained by a non-specific effect of the chromosomal integration site because integration of these genes into a different locus yielded identical results (Figure 1B; Figure 5A,C). Nor could it be explained by the particular *Prab-3* pan-neuronal promoter because we also observed the same pattern of degeneration and behavioral defects when using the endogenous *apl-1* promoter that expresses throughout the nervous system and additional tissues (Figure 1B; 5A,C).

Further experiments with laser ablation revealed that the age-related decline in behaviors could be primarily explained by the death of the VC4 and VC5 cholinergic neurons. Ablation of VC4&5 in a WT background caused retention of a modest but significant number of eggs in first-day adults, and even more eggs in third-day adults (Figure 4B; Figure 5A). Another striking effect of *APP* and *apl-1* overexpression is ventral-hinged swimming where over 80% of the single swim-cycle lags on the ventral side. Ablation of VC4&5 neurons recapitulated this unique phenotype and decreased head-bend frequency during swimming (Figure 4C; Figure 5C). Behavioral defects appeared to be due to the death of VC neurons because there was a perfect correlation between individuals with behavioral defects and those with degeneration of VC4&5 neurons in WT and AD model strains (Figure 5B,C).

Distinct portions of APP induce identical pattern of neurodegeneration

The *C. elegans* APL-1 protein is highly homologous to human APP in several regions, especially in the intracellular portion (Figure 6A). The remaining portion with transmembrane and extracellular regions is less well conserved, and notably lacks homology with Aβ (Daigle et al., 1993). This suggested to us that the intracellular portion may be more important to produce neurodegeneration in *C. elegans*. We tested if pan-neuronal expression of a single copy of only the conserved intracellular portion of APP (C59) produced neurodegeneration with a new transgenic strain *SC_APPC59*. We found that it was sufficient to recapitulate the same pattern of degeneration and behavioral defects observed in the *SC_APP* strain that expressed full-length human APP (Figure 6B-D). We next tested whether a single copy of the remaining combined extracellular and transmembrane portion (N636) produced degeneration with an additional transgenic strain *SC_APPN636*. This strain also showed VC4&5 neurodegeneration and associated VC4&5-dependent behavioral defects (Figure 6B,C). Together, these additional transgenic strains demonstrate that distinct portions of wild-type human APP protein surprisingly lead to an identical pattern of cholinergic degeneration in *C. elegans*.

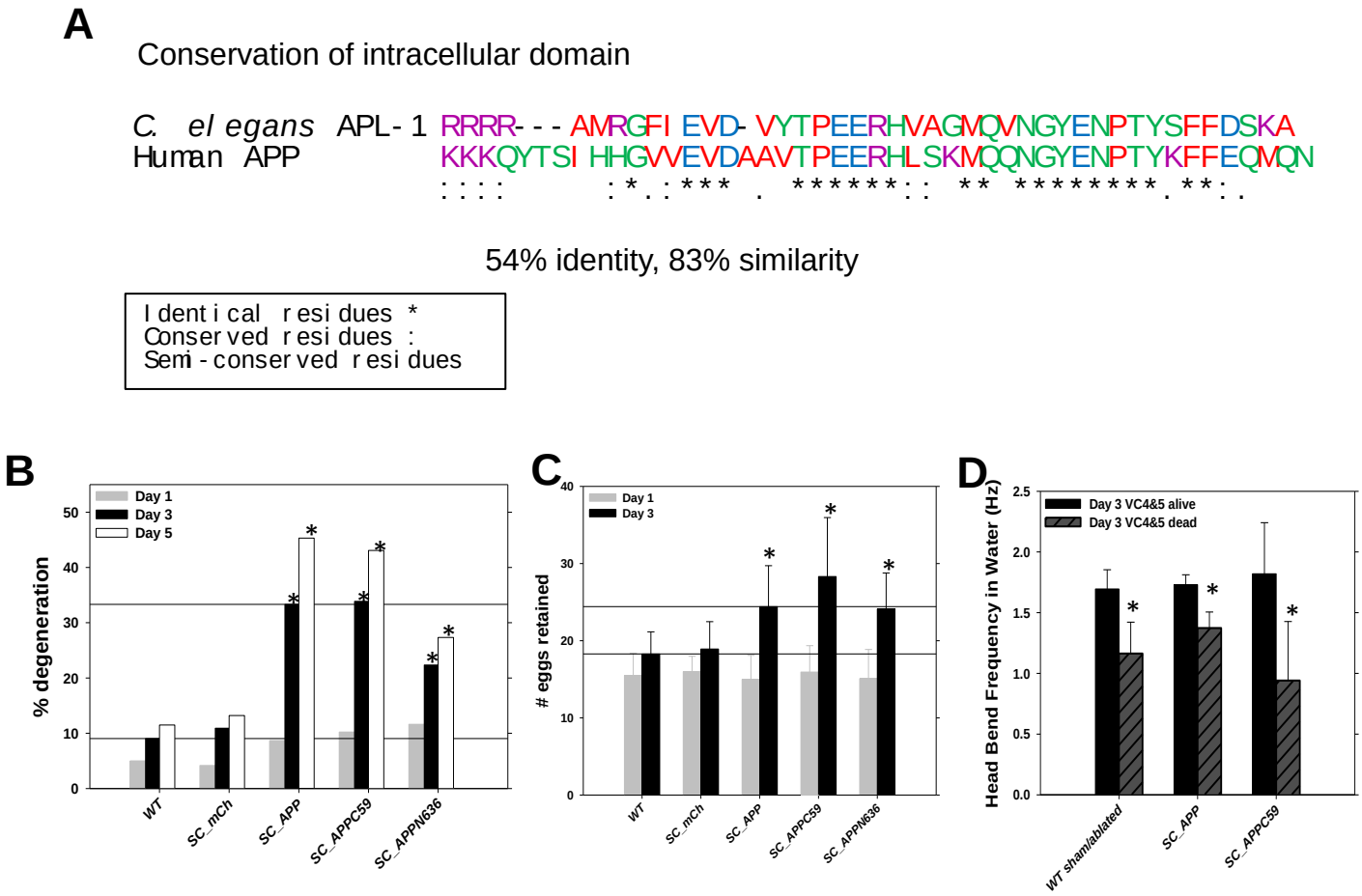


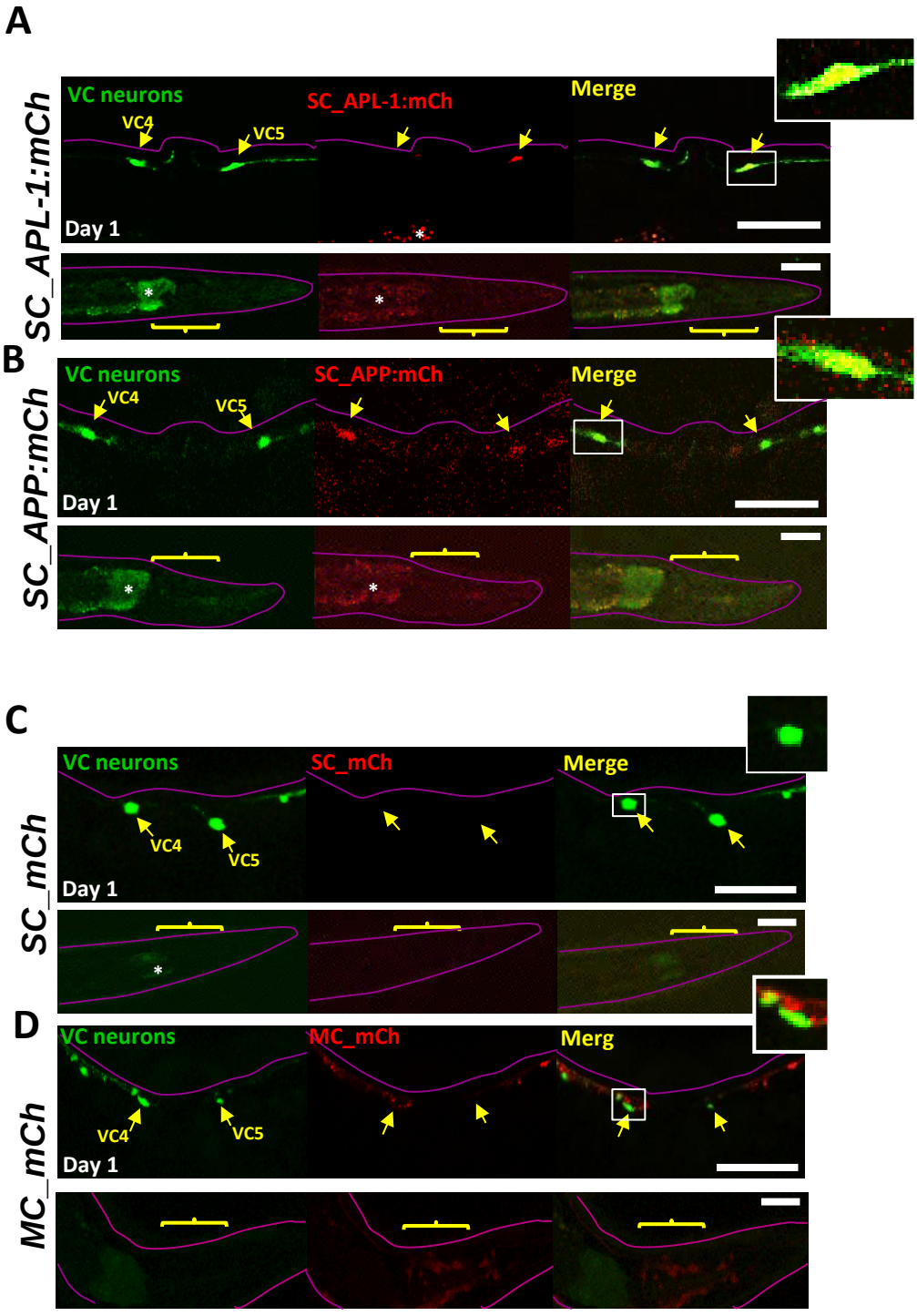
Figure 6. Distinct portions of APP are sufficient to induce identical pattern of cholinergic degeneration. (A) ClustalW alignment shows high conservation of intracellular domain of human APP with the equivalent portion of *C. elegans* APL-1 protein. (B) Quantification of percent VC4&5 neurodegeneration (n>124 neurons, 62 animals per bar). Single-copy overexpression of either the intracellular portion of APP (*SC_APPC59*) or transmembrane and extracellular portion (*SC_APPN636*) is sufficient to induce neurodegeneration and behavioral decline, similar to that seen in the *SC_APP* strain. Planned Fisher's exact tests comparing same age WT where * P<0.003, ** P<0.00001, n>124 neurons (62 animals) per bar. (C,D) Behavioral decline in egg retention (n>48 per bar) (C) and swim head-bend frequency (n>20 per bar) (D) in strains that express different portions of APP. For panels C and D, statistically compared with planned *t*-tests *vs* same age WT where *, P<0.01.

Pan-neuronally overexpressed APP accumulates inside select cholinergic neurons preceding degeneration in middle age

The widespread expression of APP in the nervous system of humans belies the selective vulnerability of specific cholinergic neurons in AD (Davies et al., 1976; Perry et al., 1977). Similarly, it was unclear why pan-neuronal expression of *APP* or *apl-1* would lead to selective degeneration of the VC cholinergic neurons. To visualize patterns of APP expression non-invasively in *C. elegans*, we constructed new transgenic strains with a single copy of C-terminal mCherry-tagged APP or APL-1 (strains *SC_APP:mCh* and *SC_apl-1:mCh*). Despite being expressed by a standard pan-neuronal promoter (*Prab-3*), just as in the *SC_APP* and *SC_apl-1* strains above, we only detected mCherry fluorescence in the six VC-class cholinergic neurons with confocal microscopy (Figure 7). The mCherry signal became apparent in first-day adults, and increased up until individual VC neurons died. In rare individuals that reached advanced age, mCherry expression was visible in all VC neurons, but not elsewhere (Figure 8).

We next generated a series of transgenic strains as controls to test whether accumulation could be due to a number of unexpected scenarios. One possibility is that the mCherry protein itself tends to accumulate in VC neurons. To test this idea, we generated a strain that expressed a single copy of mCherry with the same pan-neuronal promoter (strain designated *SC_mCh*); however, we failed to detect any fluorescence as might be normally be expected with such a low gene dose (Figure 9A). In addition, the specificity of mCherry-tagged protein accumulation in VC neurons could not be

Figure 7. Pan-neuronally expressed APP accumulates in specific cholinergic neurons that degenerate in middle age. (A,B) Confocal stack images of mCherry-tagged APL-1 and APP localization in the midbody and head of *SC_apl-1* (A) and *SC_APP* (B) strains. mCherry-tagged protein co-localizes in VC4&5 neurons starting on first day of adulthood but is undetectable in other areas including head. Brackets highlight area with hundreds of head neurons that lack noticeable mCherry signal. Asterisks, gut autoflourescence. Scale bars, 40 mm.


A
SC_APL-1:mCh
VC neurons
SC_APL-1:mCh
Merge
VC4
VC5
Day 1
B
SC_APP:mCh
VC neurons
SC_APP:mCh
Merge
VC4
VC5
Day 1
C
SC_mCh
VC neurons
SC_mCh
Merge
VC4
VC5
Day 1
D
MC_mCh
VC neurons
MC_mCh
Merg
VC4
VC5
Day 1

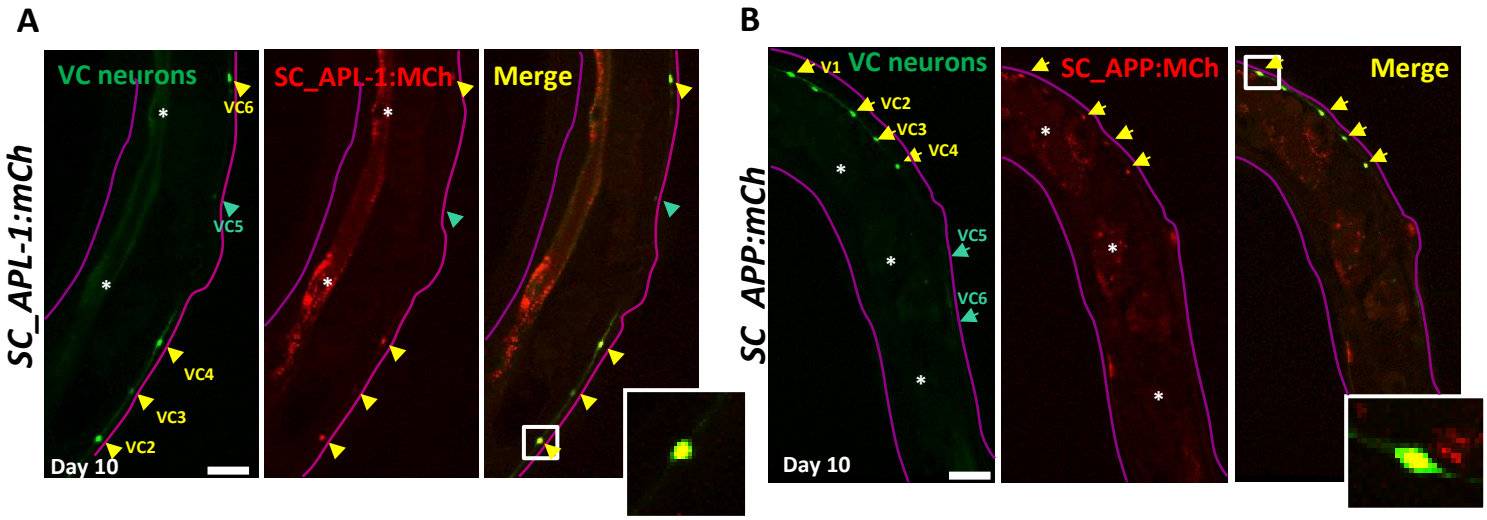


Figure 8. Localization of APP in VC-class cholinergic neurons. (A,B) In rare animals that reach advanced age (day 10 adults) mCherry-tagged APL-1 (A) and APP (B) both localize to VC neurons, in addition to surviving VC4&5 neurons, in day 10 animals. Green arrows, location of dead neurons; asterisks, gut autoflourescence; scale bars, 40 µm.

attributed to an artifact of the *Prab-3* promoter because we confirmed that a multiple-copy extrachromosomal array with a *Prab-3::mCherry* gene expressed mCherry throughout the nervous system from embryonic development onward in WT and our AD model backgrounds (Figure 9B,C). Alternatively, VC neurons might be expected to accumulate APP and APL-1 protein if these neurons did not normally express *apl-1*. However, consistent with a previous report (Hornsten et al., 2007), we found that the endogenous *apl-1* promoter expressed mCherry in the VC neurons and throughout the nervous system with a multiple-copy extrachromosomal array in WT and our AD model backgrounds (Figure 9D,E). Lastly, we ruled out that the APP mRNA might lead to degeneration because a transgenic strain that overexpressed a version of APP with an early stop codon showed no degeneration (Figure 1B). Thus, we tentatively concluded

that the selective accumulation of APP or APL-1 protein in these neurons may be a cause of the selective degeneration of VC cholinergic neurons in *C. elegans*.

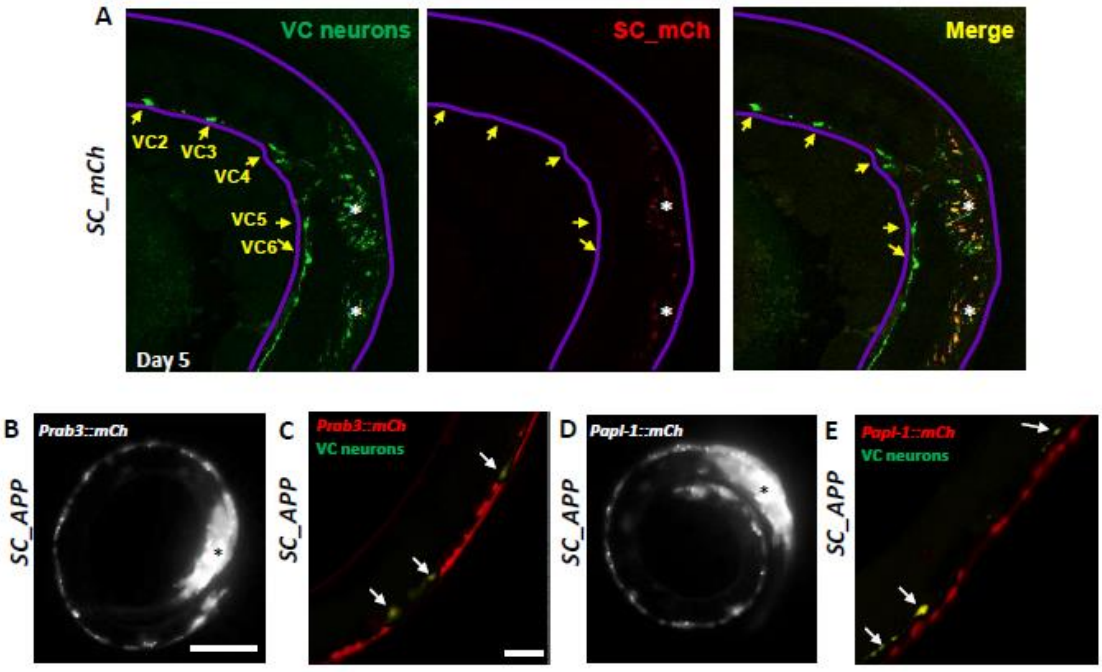
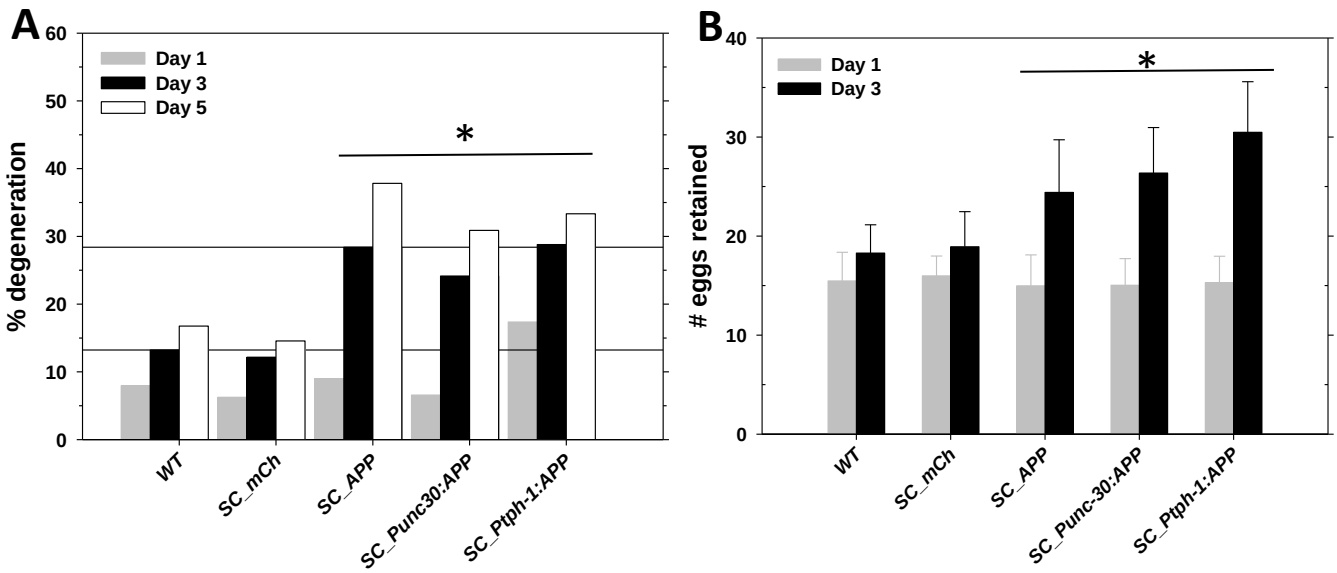
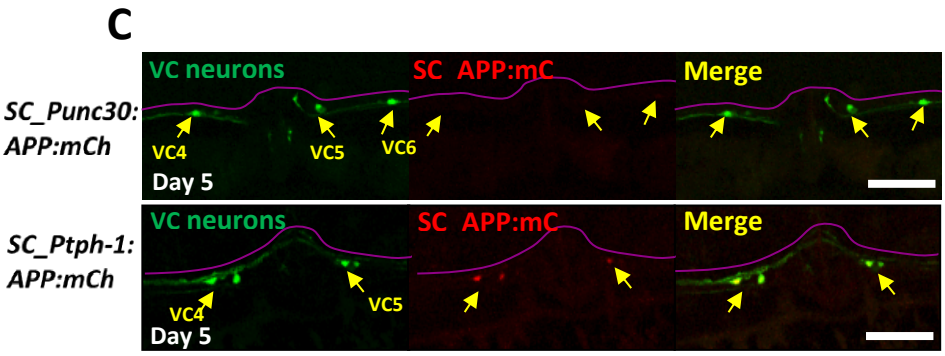


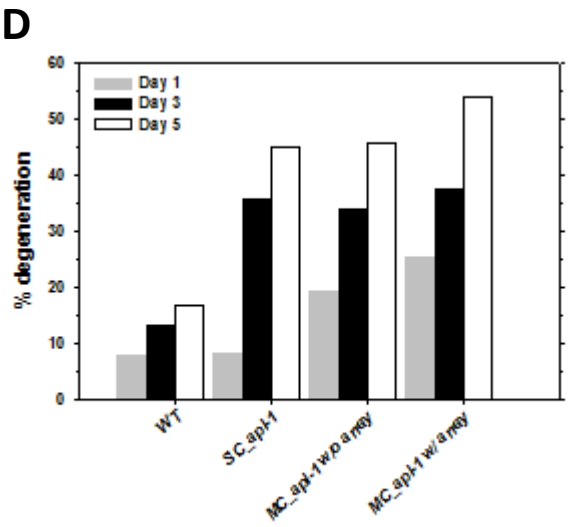
Figure 9. Confirmation that Prab-3 is a pan-neuronal promoter and that Papl-1 expresses in VC neurons. (A) A single copy Prab-3::mCherry knocked into the genome is found expressed in neurons throughout the ventral nerve cord, including VC neurons as indicated by double labeling (yellow) with GFP-specific VC neuron reporter (white arrows). (B,C) The pan-neuronal promoter Prab-3 expresses mCherry (displayed white) throughout nervous system in a S*C_APP* background (B) and also in VC neurons (yellow indicates overlap of red mCherry signal with green VC neurons) (C). (D,E) The endogenous promoter region (2kb) of *apl-1* expresses mCherry (displayed white) throughout nervous system in a S*C_APP* background (D) 3 and also in VC neurons (yellow indicates overlap of red mCherry signal with green VC neurons) (E). Worms are positioned in coiled posture to show entire nervous system and asterisks indicate numerous neurons in head in panels D and E. For panels A-D scale bars, 40 μm.

To test whether the pattern of neurodegeneration and APP accumulation depended on factors intrinsic and/or extrinsic to the VC neurons, we generated two additional transgenic strains. One strain used the Ptph-1 promoter to express a single copy of *APP* in VC4&5 neurons and a few other neurons, but not other VC neurons (strain SC_*Ptph-1:APP:mCh*). (Unfortunately, there is no VC-specific promoter) A second strain used the Punc-30 promoter to express a single copy of *APP* in all GABAergic neurons, including some in the nerve cord nearby the VC neurons (strain SC_*Punc-30:APP:mCh*). We found that VC4&5 neurons died in the SC_*Punc-30:APP:mCh* strain (Fig 10). This strongly suggests the existence of a cell-non autonomous route to patterned neurodegeneration. In addition, we found that VC4&5 neurons also died in the SC_*Ptph-1:APP:mCh* strain (Fig 10). Although this suggests the existence of a cell-autonomous route to patterned neurodegeneration, we cannot be conclusive because the Ptph-1 promoter expresses in a few neurons in addition to VC4&5 neurons. However, confocal imaging revealed that the SC_*Ptph-1:APP:mCh* strain showed accumulation of APP only in the VC4&5 neurons but not in other Ptph-1-expressing neurons, nor other VC neurons (Fig 10C). Imaging of the *SC_Punc-30:APP:mCh* strain revealed no detectable mCherry signal (Fig 10C). Taken together, our results show that the selective accumulation of overexpressed APP is a cell-autonomous, while the patterned degeneration is likely both cell-autonomous and cell-autonomous.

Figure 10. Different Expression Patterns of APP can Produce Similar Levels of Degeneration and Behavioral Deficits. (A) Quantification of percent VC4&5 neurodegeneration (n>124 neurons, 62 animals per bar). Expression of APP under a GABA promoter or 5HT promoter yields similar degeneration levels as with the pan-neuronal promoter Prab-3. (B) Behavioral decline in egg retention (n>48 per bar) in strains that express APP under different promoters. (C) Confocal stack images of mCherry-tagged APP localization in the midbody of *Punc30:APP:mCh* and *Ptph-1:APP:mCh.* Expression in 5HT neurons reveal accumulation only in VC neurons, while GABA expression revealed no detectable accumulation. Scale bars, 40 μm. (D) Quantification of degenration in animals expressing multiple copies of *apl-1*. Degeneration is not dependent upon expression within the VC neurons.


A
% degeneration
Day 1
Day 3
Day 5
*
WT
SC_mCh
SC_APP
SC_Punc30:APP
SC_Ptph-1:APP
B
eggs retained
Day 1
Day 3
*
WT
SC_mCh
SC_APP
SC_Punc-30:APP
SC_Ptph-1:APP


C
SC_Punc30:
APP:mCh
VC neurons
SC APP:mC
Merge
VC4
VC5
VC6
Day 5
SC_Ptph-1:
APP:mCh
VC neurons
SC APP:mC
Merge
VC4
VC5
Day 5


D
% degeneration
Day 1
Day 3
Day 5
WT
SC_apl-1
MC_apl-1 w/o amy
MC_apl-1 w/ amy

To gain insight into the cell-non-autonomous route to degeneration, we generated two additional transgenic strains that express a single copy of distinct portions of APP in the GABAergic neurons. Intriguingly, we observed that the VC4&5 neurons died in regardless of which portion was expressed in GABAergic neurons (Fig 11). This suggests the possibility of multiple extrinsic routes to patterned neurodegeneration.

Having established these new worm models of AD with age-related patterned cholinergic neurodegeneration, we then combined genetic and pharmacological approaches to determine how conserved cell death and immune signaling pathways influence this pattern of degeneration.

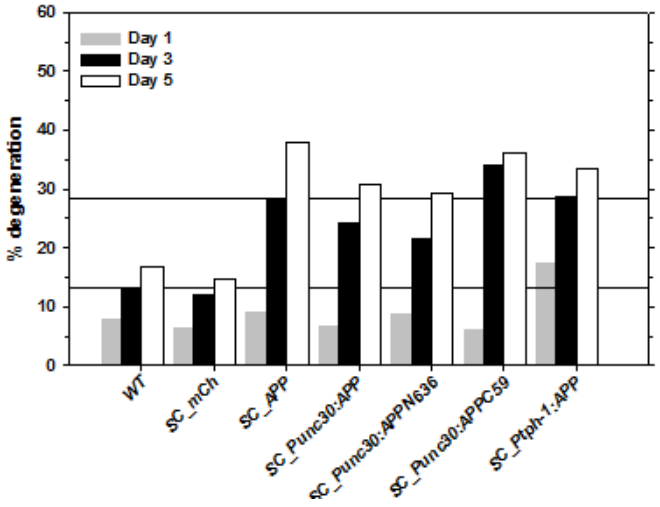


Figure 11. VC neurons are vulnerable to degeneration by cell-non-autonomous expression of disctinct portions of APP. Both intracellular and extracellular portions of APP expressed in GABA neurons is suffcient to induce degeration of VC neurons. (*WT, SC_mCh, SC_APP, SC_Punc30:APP, and SC_tph-1:APP* data same as in Figure 10).

Apoptotic signaling is required for APP-induced neurodegeneration

Many highly conserved components of necrotic, apoptotic, and phagocytic pathways that underlie cell death have been discovered in *C. elegans* (Putcha et al., 2004). Which ones are essential for APP-induced neurodegeneration? First, we ruled out a role for conventional necrosis because elimination of *crt-1*, the single homolog of the essential necrotic gene calreticulin (Park et al., 2001), had no effect on degeneration (Figure 12A). Next, we found an essential role for apoptosis with several experiments. Deletion of *egl-1*, a homolog of BH3 (Bcl-2 homology region 3) type activators of apoptosis in mammals, prevented degeneration (Asterisks denote significant decrease in degeneration in *egl-1;SC_apl-1* and *ced-6; SC_apl-1* strains versus *SC_apl-1* in Figure 12A). Additionally, deletion of *egl-1* partially rescued behavioral defects in *SC_APP* and *SC_APL-1* strains (Figure 12B,C). Conversely, activating apoptotic signaling with a gain-of-function mutation in *egl-1* did not exacerbate the incidence of degeneration (Figure 12A). Indeed, the *egl-1(gf)* mutant showed age-related degeneration of VC4&5 neurons even without *APP* (or *apl-1*) overexpression. Consistent with an essential role for apoptosis, deletion of *ced-3*, a homolog of the mammalian apoptotic *caspase-9/ICE*, similarly prevented degeneration (Figure 12A). Likewise, elimination of *ced-6*, the homolog of mammalian phagocytotic gene GULP, prevented degeneration and preserved function of VC4&5 neurons (Fig. 12A). This suggests that in our AD models CED-6 has a primary apoptotic role to act as a "killer" signal as found in other scenarios (Reddien et al., 2001; Hoeppner et al., 2001). Our results demonstrate that APP-induced

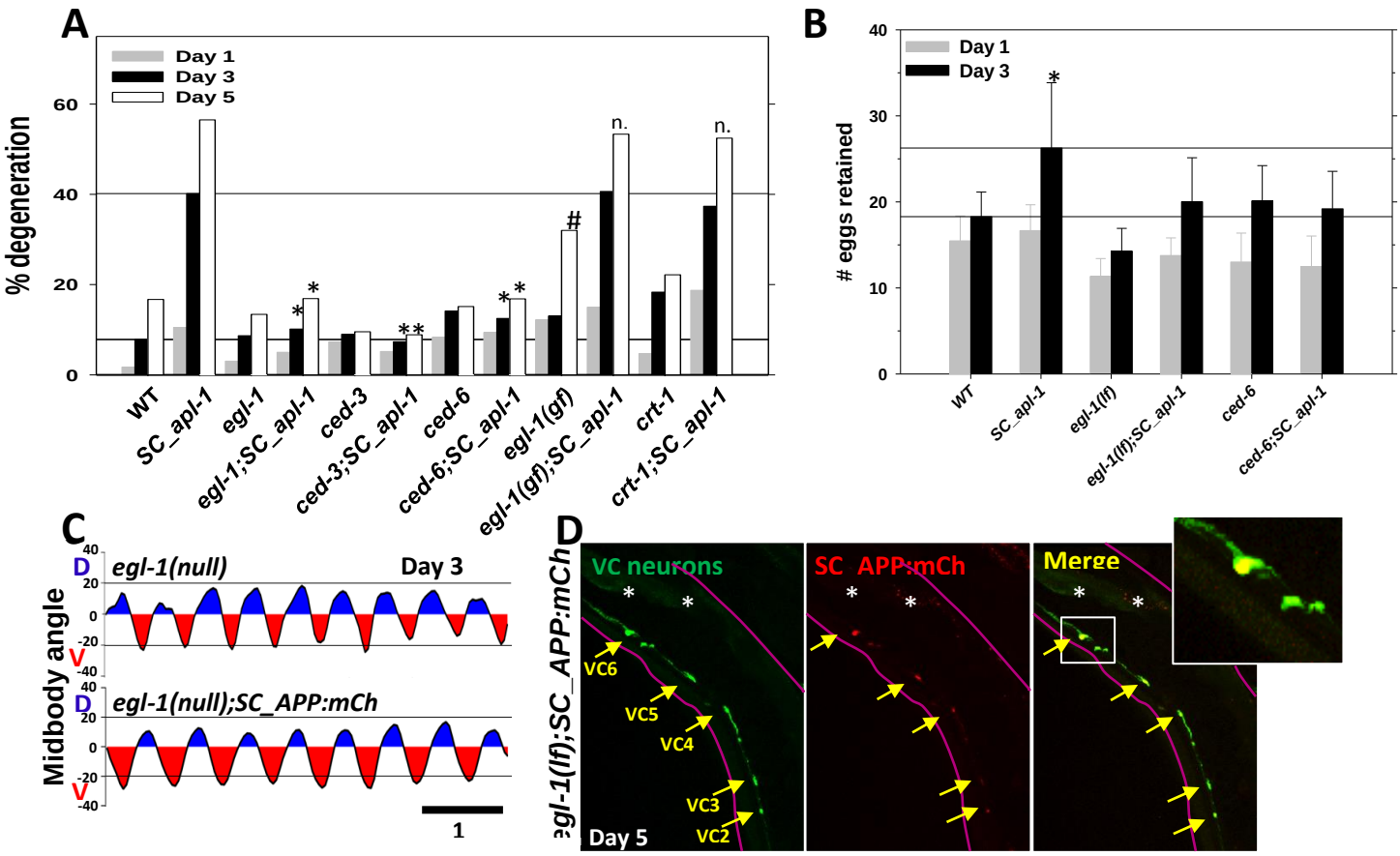


Figure 12. APP induces patterned neurodegeneration via an apoptotic pathway that **r**equires *egl-1, ced-3,* and *ced-6*. (A) Quantification of percent VC4&5 neurodegeneration in cell-death pathway mutants. Planned Fisher's exact tests where * represents P<0.00001 for comparing same age *SC_apl-1* and n.s., not significant. # represents P<0.0001 for comparing same age WT. n>124 neurons (62 animals) per bar. (B,C) Null mutation in *egl-1* preserved WT-like egg-laying and swimming behaviors in AD model strains. For statistical comparisons of egg retention, n>48 animals per bar, planned *t*-tests *vs* same age WT where **, *P*<0.05. Error bars, s.e.m. (D) mCherry-tagged APP still accumulates in VC neurons spared from degeneration in *egl-1(lf)* background, inset. Asterisks, gut autoflourescence. Scale bars, 40 µm.

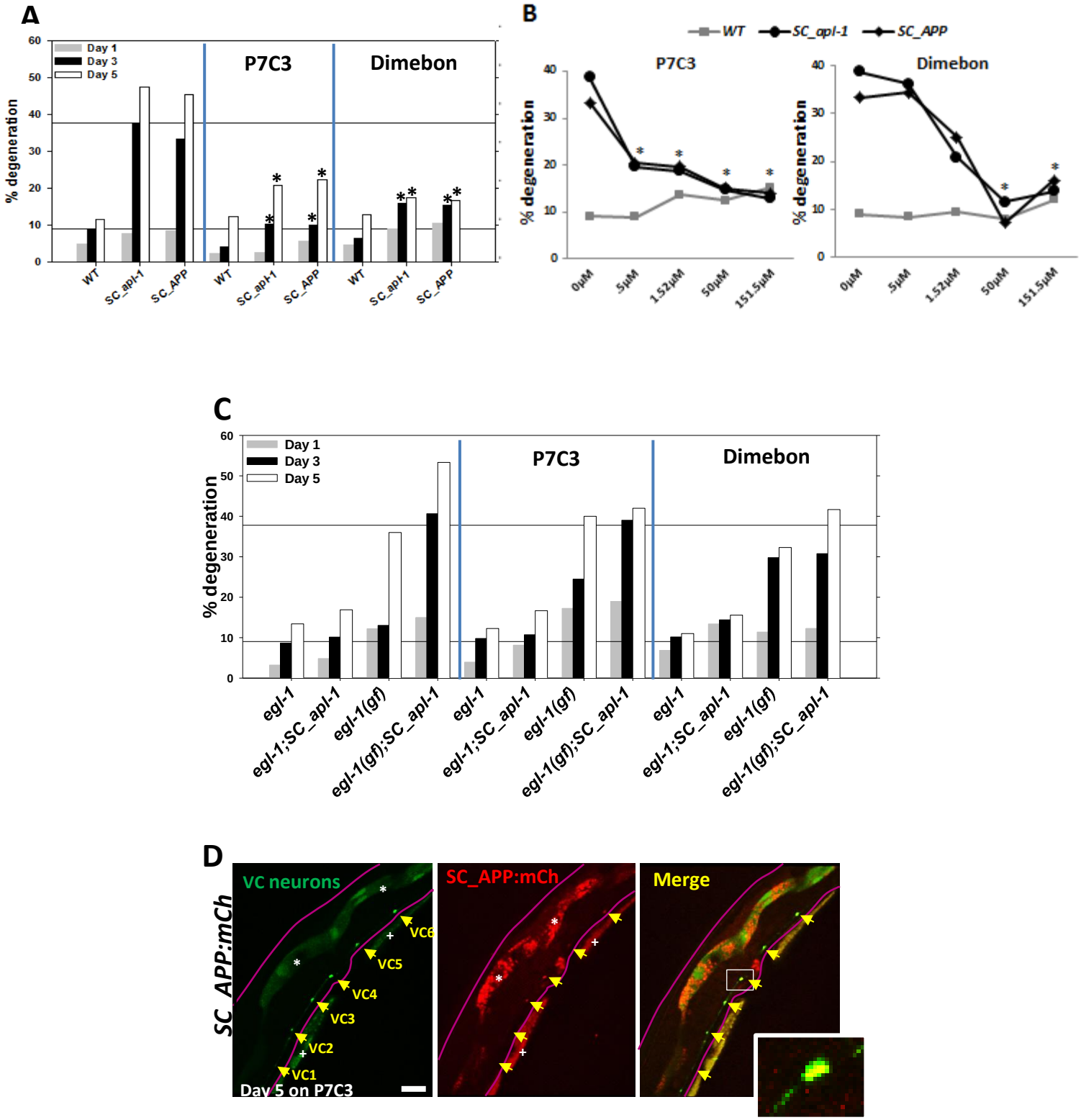
degeneration requires members of the conserved apoptotic pathway including EGL-1, CED-3, and CED-6.

If abnormal accumulation of APP (or APL-1) causes degeneration, might interference in apoptotic signaling prevent accumulation of APP (or APL-1)? Instead, we found that the level and pattern of mCherry-tagged APP (or APL-1) expression in an *egl-1(null)* background was indistinguishable from that of *SC_APP:mCh* and *SC_APL-1:mCh* strains (Figure 12D). Our results strongly suggest that apoptotic mutations prevent degeneration by blocking APP-triggered entrance into the apoptotic pathway.

P7C3 prevents APP-induced neurodegeneration while maintaining neural function

We next tested whether the new putative neuroprotective compound P7C3 could also prevent APP-induced degeneration. P7C3 was recently discovered in an unbiased screen for small molecules that increase the number of adult-born neurons in the hippocampus of mice, potentially by increasing their survival (preventing their death) (Pieper et al., 2010). The mechanistic basis for the neuroprotective effects of P7C3 remains unknown (Pieper et al., 2010). Animals were treated with P7C3 (50 μM) from L4-larval stage onward (Figure 2A). P7C3 treatment significantly prevented neurodegeneration induced by *APP* or *apl-1* (Figure 13A). Degeneration was more modestly prevented in the *SC_apl-1* strain but not in the *SC_APP* strain when animals were treated from day-1 of adulthood onward (Figure 14). This suggests that P7C3 may

Figure 13. P7C3 and Dimebon prevent APP-induced neurodegeneration by blocking entrance to apoptosis. (A) P7C3 and Dimebon both prevent degeneration of VC4&5 neurons. Planned Fisher's exact tests comparing untreated animals of same age and genotype where *, $P<0.00001$. (B) Dose-response curves. Planned Fisher's exact tests of treated *vs* untreated groups where *, $P<0.00001$. (C) Protective effects of drugs on neurodegeneration are not additive in an *egl-1(null)* background. Conversely, drugs cannot prevent degeneration induced by gain-of-function mutation in *egl-1*. (D) Confocal stack images show that mCherry-tagged APP still accumulates in neurons spared from degeneration with treatment of P7C3, inset. Asterisks, gut autoflourescence. Scale bars, 40 mm. For panels A-C all bars and data points represent $n>124$ neurons, 62 animals per bar.


A
Day 1
Day 3
Day 5
P7C3
Dimebon
% degeneration
WT
SC_apl-1
SC_APP
B
WT
SC_apl-1
SC_APP
P7C3
Dimebon
% degeneration
0μM
5μM
1.52μM
50μM
151.5μM
C
Day 1
Day 3
Day 5
P7C3
Dimebon
% degeneration
egl-1
egl-1;SC_apl-1
egl-1(gf)
egl-1(gf);SC_apl-1
D
SC_APP:mCh
VC neurons
SC_APP:mCh
Merge
VC6
VC5
VC4
VC3
VC2
VC1
Day 5 on P7C3

be ineffective once the degenerative process has initiated. The structure of P7C3 resembles Dimebon, a potential drug for AD (Bachurin et al., 2001; Doody et al., 2008; Steele et al., 2012). We found that both drugs prevented neurodegeneration; however, a dose-response analysis found that P7C3 was two-orders of magnitude more potent than Dimebon (Figure 13B). To determine whether P7C3 and Dimebon could prevent degeneration elicited by distinct portions of APP, we assayed the protective effects of each drug on our strains that overexpressed different portions of APP. Surprisingly, we found both drugs prevented degeneration for the *SC_APPC59* strain, but failed to provide protection for the *SC_APPN636* strain (Figure 14). Protective effects against degeneration for the *SC_APPC59* strain extended to protection of egg-laying and swimming behaviors that depend on the VC4&5 neurons (data not shown). These results suggest that when separated, different portions of human APP molecule may initiate apoptotic degeneration via different entrances, only one of which might be blocked by P7C3 and Dimebon.

Next, we used mCherry-tagged transgenes to determine whether the drugs might prevent degeneration by stopping accumulation of APP or APL-1. While drug-treated *SC_APP:mCh* or *SC_apl-1:mCh* individuals advanced in age without behavioral deficits (swimming and egg laying), mCherry-tagged protein accumulated specifically in the VC cholinergic neurons (Figure 13D). To narrow down the point at which these drugs act in the neurodegenerative pathway we tested how our AD models responded with different apoptotic mutations. P7C3 and Dimebon offered no further protection from degeneration

in an *egl-1(null)* mutant background (Figure 13C). Conversely, these drugs failed to prevent degeneration in *SC_APP* and *SC_apl-1* strains with a gain-of-function mutation in *egl-1* (Figure 13C). Taken together, these results suggest that the drugs act upstream of EGL-1 apoptotic signaling. Thus, P7C3 and Dimebon appear to prevent degeneration by blocking accumulated APP from triggering apoptotic signaling.

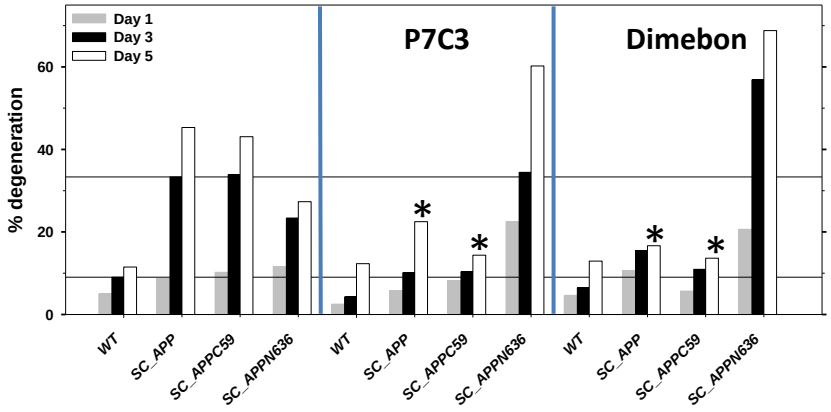


Figure 14. P7C3 and Dimebon can prevent degeneration induced by full-length AP and intracellular APP but not extracellular APP. Quantification of VC4&5 neurodegeneration (n>124 neurons, 62 animals per bar). Planned Fisher's exact tests compare *vs* same age and genotype where *, P<0.001.

Cell-specific pattern of degeneration and APP accumulation depend inversely on level of innate-immune signaling

Neurodegeneration in AD is accompanied by inflammation and the late onset of AD correlates with immunosenescence (Eikelenboom et al., 2012). AD researchers have found evidence for both harmful and protective roles for different aspects of the immune system in neurodegeneration (Rivest, 2009). Although *C. elegans* does not have an adaptive immune system, it shares core components of the innate immune system with mammals that show senescence in middle age adulthood (Pukkila-Worley and Ausubel,

2012; Youngman et al., 2011). We sought to explore linkage between innate immune signaling and neurodegeneration in our worm models of AD. We hypothesized that compromising immunity through genetic disruption of conserved innate-immune molecules might recapitulate the patterned neurodegeneration found in our AD models.

We first found that a loss-of-function mutation in the key conserved innate-immune gene, *nsy-1*, led to the same incidence of degeneration of the VC4&5 neurons, albeit precociously relative to our AD models (Fig 15A). *nsy-1* encodes the *C. elegans* ortholog of Apoptotic Signaling Kinase 1 (ASK1/MAP kinase kinase kinase) (Wes and Bargmann, 2001; Sagasti et al., 2001). Combining *nsy-1(lf)* with *SC_APP* did not hasten the onset nor increase the incidence of degeneration (Fig 15A) suggesting that the two act in the same genetic pathway for degeneration. Consistent with this idea, degeneration appeared to be primarily apoptotic because an *egl-1(lf)* mutation prevented degeneration in *nsy-1(lf);SC_APP* worms (Fig 7A). We also found that loss-of-function mutations in any of three additional conserved components of the ASK1/p38 MAPK innate-immune signaling axis led to identical levels of degeneration of VC4&5 neurons (*tir-1*, Toll-interleukin receptor domain containing receptor ortholog of human SARM; *sek-1*, MAP kinase kinase; and *mpk-1*, p38 MAP Kinase 1; Fig 15A). Cholinergic neurons other than VC-class neurons did not die in these innate immunity mutants (2% degeneration of VA

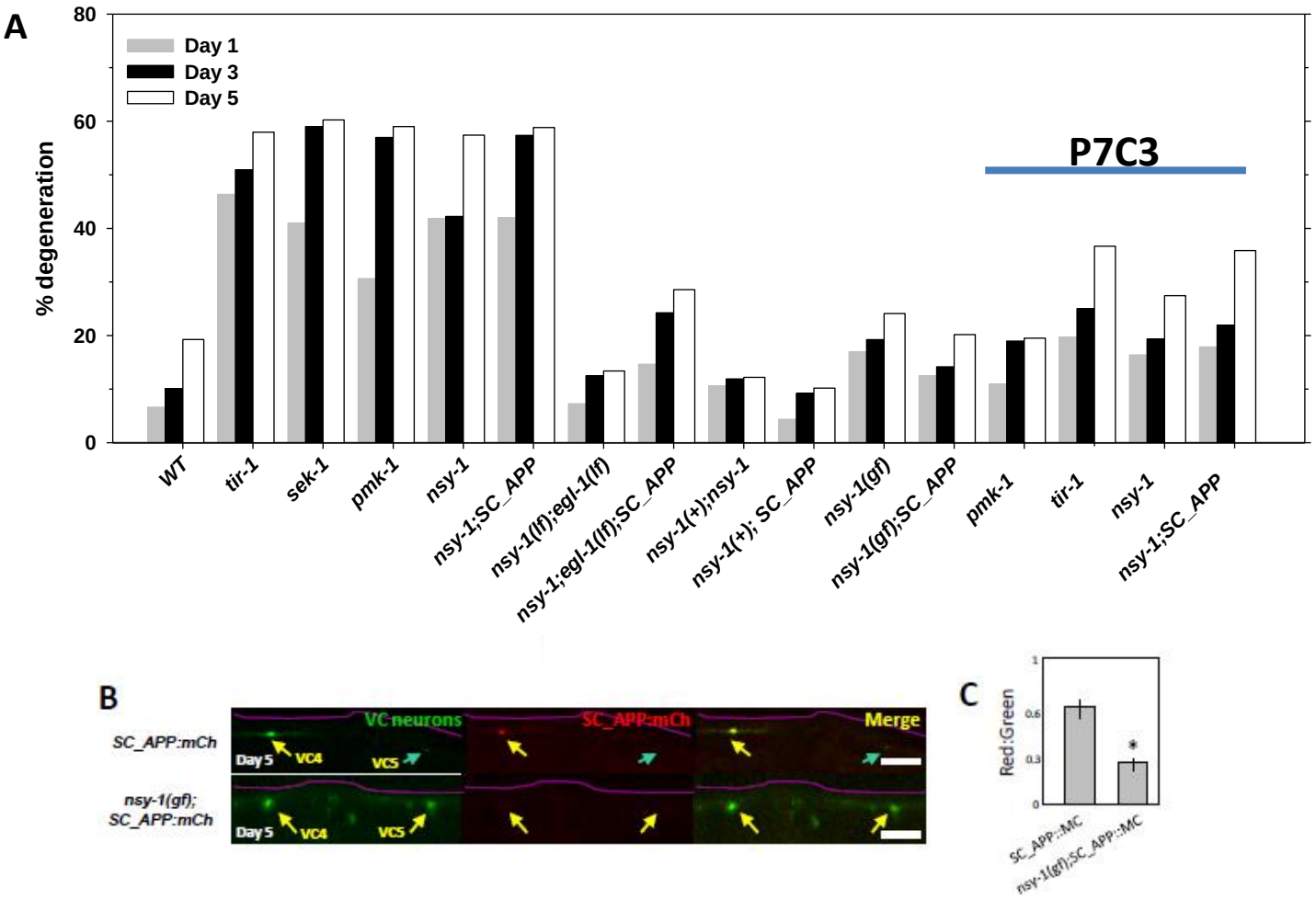


Figure 15. Vulnerability of patterned cholinergic neurodegeneration and APP accumulation depends inversely on level of innate immune signaling. (A) Quantification of VC4&5 neurodegeneration in innate-immune pathway mutants. Immunocompromised mutants display same pattern of VC4&5 degeneration as AD model worms. P7C3 prevents degeneration in *nsy-1(lf)* mutant backgrounds. Planned Fisher's exact tests comparing untreated animals of same age and genotype where *, P<0.00001 and n.s., not significant, n>124 neurons (62 animals) per bar. (B) Confocal stack images show that less mCherry-tagged APP accumulates in neurons spared from degeneration in *nsy-1(gf)* mutant background. Location of degenerated VC5 neuron indicated by blue arrow. (C) Quantification of intracellular accumulated mCherry-tagged APP by ratio of average red:green signals in VC4&5 neurons (*t*-test, n = 12 animals and neurons for each bar, P < 0.0005).

and VB neurons out of 100 animals investigated). These results suggest a role for innate-immunity in preserving VC neurons in the presence and absence of overexpressed APP.

The ASK1/p38MAPK pathway was previously found to regulate calcium signaling in *C. elegans* neurons downstream of the highly conserved kinase CaMKII (Sagasti et al., 2001; Chuang et al., 2005; Bezprozvanny et al., 2008; Berridge et al., 2010). To determine whether CaMKII influenced degeneration, we assessed degeneration of VC4&5 neurons in the CaMKII knock-out mutant *unc-43(e408)*. We found that deletion of unc-43 had no effect on progression of degeneration (Sup Figure 9A). We thus conclude that the ASK1/p38MAPK pathway plays a role independent of its calcium signaling role in neurons to influence neurodegeneration.

If the ASK1/p38MAPK immune pathway is protective, then boosting innate immune signaling through gain-of-function mutation in the *nsy-1* gene might prevent degeneration in our AD model. We found that *nsy-1(gf)* conferred neuroprotective effects and maintained neuronal function of the VC neurons (Fig 15A). Protection by the *nsy-1(gf)* mutation may be achieved in part by reducing levels of APP protein because confocal imaging revealed that intracellular levels of mCherry-tagged APP in VC4&5 neurons were reduced by 60% (Fig 15B,C). Protection was also conferred by expressing a wild-type copy of *nsy-1* with the cholinergic promoter (Punc-17) in *nsy-1(lf)* and *SC_APP* backgrounds (Fig 15A). Intriguingly, we found that P7C3 prevented VC neurons from dying in *nsy-1(lf)* and *pmk-1(lf)* mutants (Fig 15A). This further refines

genetically where P7C3 must function to protect neurons: downstream of p38MAPK/PMK-1 and upstream of EGL-1.

Taken together, our results demonstrate that the conserved ASK1/p38MAPK innate immune axis is required to protect the VC-class cholinergic neurons cell-autonomously from accumulating APP and degenerating in adulthood (Fig 16). This strongly suggests that accumulated APP may selectively kill VC neurons in coordination with middle age immunosenescence in our AD models.

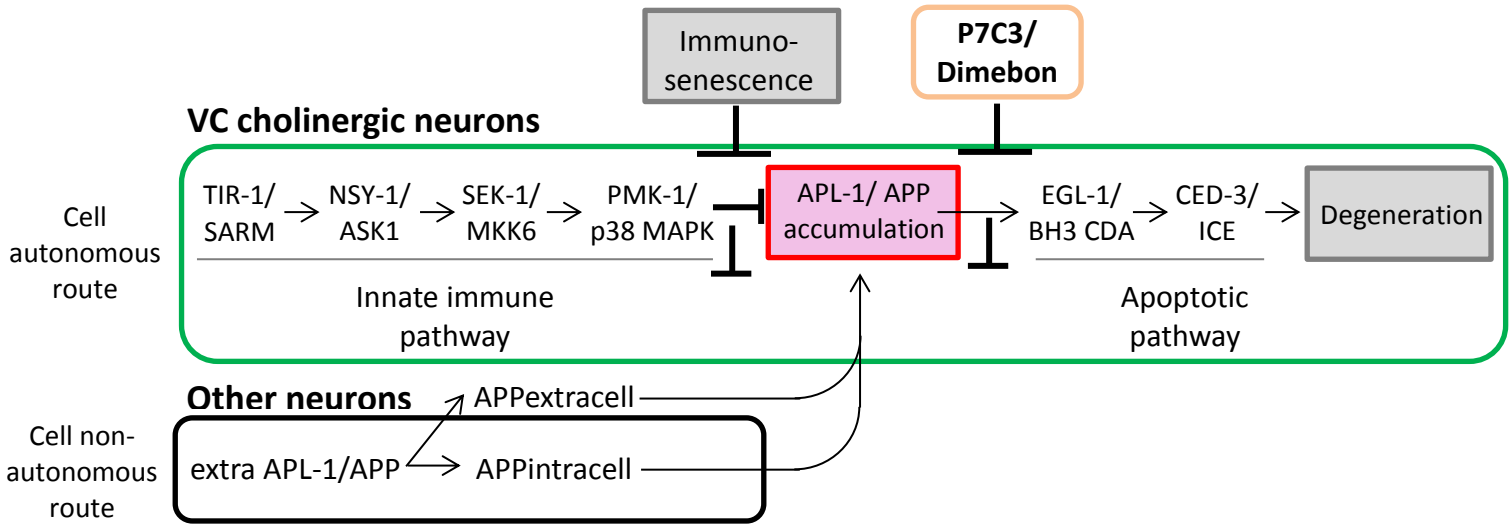


Figure 16. Working model of APP-induced patterned neurodegeneration. APL-1 or APP accumulates in VC neurons to a level that activates a conserved apoptotic pathway. The TIR-1/NSY-1/SEK-1/PMK-1 innate immune cascade inhibits accumulation of APL-1/APP until immunosenescence commences in middle age. The drugs P7C3 and Dimebon block entrance to apoptosis triggered by accumulated APP or APL-1 compromised immune signaling. This model cannot discern the contributions of cell-autonomous versus non-autonomous routes leading to accumulation; both have been shown to influence degeneration. Mammalian orthologs listed after *C. elegans* equivalent. Connections reflect order in pathway but not necessarily direct interactions in this abridged diagram.

Discussion

Many human neurodegenerative diseases, including AD, paradoxically present an initially limited pattern of degeneration caused by alteration of genes that are near-ubiquitously expressed throughout the brain (Gotz et al., 2009). Here we use *C. elegans* to provide a clear example of how widespread overexpression of a wild-type protein (APP or APL-1) can cause a specific pattern of neurodegeneration (VC-class cholinergic neurons) through selective accumulation in middle age. Accumulated APP triggers the death of cells by apoptosis coincident with decline of the neuroprotective ASK1/p38 MAPK innate immune pathway. To our knowledge, this study provides the first example of neurodegeneration in an AD-related *C. elegans* model, as well as, the first example of apoptosis of adult cells in *C. elegans* (excluding germ cells). In addition, although many *in vitro* and *in situ* studies have implicated apoptosis as the primary mediator of degeneration in mouse models of AD (e.g. Cotman et al., 2005), we believe this is the first *in vivo* evidence that deletion of apoptotic genes prevents APP-induced neurodegeneration.

The pattern of APP-induced neurodegeneration seems to be restricted to the VC-class cholinergic neurons because unique behavioral defects of the AD model strains could be accounted for by the death of these neurons alone. This pattern of neurodegeneration perfectly matches the exclusive pattern of mCherry-tagged APP and APL-1 protein accumulation in the VC neurons. Other neurons presumably expressed these proteins at levels undetectable by confocal imaging. This would be consistent with

how mCherry was undetectable in our additional control strain that expressed a single copy of the mCherry gene driven by a pan-neuronal promoter (Figure S5A). Thus, we conclude that the intracellular pattern of accumulated APP or APL-1 protein causes the pattern of neurodegeneration. Our finding that both worm APL-1 or human APP induced an identical pattern of neurodegeneration provides strong evidence that the molecular basis of this phenomenon is ancient and conserved.

Researchers have debated which portions of the APP molecule contribute the most to degeneration in AD (Benilova et al., 2012). In the most popular paradigm, abnormal proteolytic cleavage of the extracellular/transmembrane portion of APP contributes to neurodegeneration. The most toxic components are currently believed to be soluble variants of the Aβ peptide (Huang et al., 2012). In an alternate paradigm, overexpression of the intracellular portion of APP alone recapitulated common aspects of degeneration found in Aβ-mouse models (Ghosal et al., 2009). Our results with *C. elegans* support both paradigms, showing for the first time in a single study that distinct portions of human APP can induce the same cellular pattern of neurodegeneration. This surprising result suggests that future AD studies might do well to focus on why certain cholinergic neurons die irrespective of the APP-derived toxic peptide.

Consistent with the toxicity of different portions of APP, previous studies in *C. elegans* have found that overexpression of multiple copies of Aβ produces toxicity when expressed in muscle, and that overexpression of multiple copies of WT full-length

APP or *apl-1* causes partial lethality and behavioral dysfunction when expressed throughout the nervous system (Hornsten et al., 2007; Link et al., 2003; Ewald et al., 2012). The convenience of *C. elegans* transgenesis will enable further study of how different portions of APP induce patterned neurodegeneration at doses relevant to human disease.

It remains puzzling why only the VC neurons accumulate APP and APL-1 proteins. We consider several possibilities. First, these genes may be expressed at toxic levels due to certain cis-regulatory elements specific for the VC neurons. This seems unlikely, however, because we found the same results with the endogenous *apl-1* promoter and a conventional pan-neuronal promoter. In our control experiments, both promoters drove expression of mCherry in VC neurons as well as many other neurons from embryonic stage onward. Second, the *APP* and *apl-1* mRNA might be degraded efficiently in all cells except for the VC neurons. Degradation cannot be so efficient, however, because we found that both genes were expressed at levels two-fold higher as expected. Third, the APP and APL-1 proteins might be degraded efficiently in all cells expect for the VC neurons. This hypothesis is consistent with how mCherry-tagged versions of these proteins became visible in early adulthood and intensified into middle age until neurons died. Perhaps the VC neurons lack a proteolytic component critical for amyloidogenic proteins (Silva et al., 2011). Our finding that mCherry-tagged APP accumulated less after boosting innate immunity through *nsy-1(gf)* mutation fits with this theory. The innate immune system in mammals has been found to work with other cell

defense systems to promote proteasomal degradation of abnormal proteins and prevent apoptosis (Krüger and Kloetzel, 2012).

Another way to think about why VC neurons die and other neurons survive is to ask what is unique about VC neurons. The VC neurons represent only six of the ~124 cholinergic neurons in the *C. elegans* nervous system (J. Rand, personal communication; Duerr et al., 2008). They are distinct from other cholinergic neurons in that they arise post-embryonically, undergo synaptogenesis during the adult stage, and acquire their cholinergic fate independent of the conserved COE-type transcription factor UNC-3 (Sulston et al., 1983; Kratsios et al., 2011; Potts et al., 2009). In older WT animals, we also found that VC neurons show a higher basal level of degeneration relative to other cholinergic neurons (Figure 1D). This may be an apoptotic process because basal degeneration was slightly reduced in an *ICE/ced-3* mutant background (Figure 5A). The developmental lineage for VC neurons is also distinct from other cholinergic neurons. During development, cells that are orthologous to the VC neurons in the nerve cord naturally undergo apoptosis (Sulston et al., 1983). We speculate that the VC neurons may contain a factor(s) that delays their developmental apoptosis until old age, and that accumulation of APP or disruption of innate immune signaling may hasten this process. A similar process might eventually be found to explain patterned degeneration of cholinergic neurons in mammals.

Many of the traits of VC neurons described above are intriguingly reminiscent of the adult-born cholinergic neurons in the hippocampus in mammals. During adult

neurogenesis, a stem cell is thought to divide into four precursor cells (Li et al., 2009). Three of these usually undergo apoptosis, but the surviving cell differentiates into a neuron that participates in the processing of newly formed memories (Sierra et al., 2010; Li et al., 2009; Rodriquez et al., 2009). Like the VC neurons, these adult-born cholinergic neurons are selectively vulnerable to degeneration in AD models and natural aging (Rodriquez et al., 2009).

Although *C. elegans* lacks adaptive immunity, it shares an ancestral core cassette of innate immune molecules with mammals including orthologs of ASK1 and p38 MAPKinase1 (Pukkila-Worley and Ausubel, 2012; Matsuzawa et al., 2005). Our findings that weakening innate immunity hastened degeneration, while strengthening innate immunity prevented APP accumulation and neurodegeneration suggest that this conserved branch of innate immunity might play a similar role in preventing APP-induced degeneration in mammals. Boosting innate immunity through gain-of-function mutation in the worm ASK1 ortholog NSY-1 was previously shown to prevent degeneration of dopamine neurons after exposure to the toxins 6-hydroxydopamine and methamphetamine (Schreiber and McIntire, 2011). Although, hyperactivation of the ASK1/p38MAP kinase pathway is suspected of causing cell death in mammals (KO of ASK1 relieves indiscriminate killing of cells by astrocytes in a mouse model of MS (Guo et al., 2010)) and in worms (KO of the p38MAP kinase ortholog in *C. elegans* prevented excessive apoptotic death of germ cells by *Salmonella* (Aballay et al., 2003)), strategies to prevent neurodegeneration in AD by only modestly boosting specific branches of

innate immune signaling are currently being tested in rodent models (Rezai-Zadeh et al., 2011; Butchart and Holmes, 2012).

While it may be expected that the level of the innate immune signaling might dictate the temporal pattern of neurodegeneration, we unexpectedly found that it also dictated the spatial pattern of neurodegeneration. In either the presence or absence of overexpressed *APP* or *apl-1*, we found that the same VC neurons degenerated in *tir-1*, *nsy-1*, *sek-1* and *pmk-1* loss-of-function mutants at the same incidence via apoptosis. The identical pattern of limited neurodegeneration is intriguing because *apl-1* and innate immune genes are all widely expressed in *C. elegans*. We propose that the cellular pattern of APP-induced neurodegeneration may be explained by the VC neurons being more heavily dependent on innate immune signaling for the clearance of toxic proteins relative to other neurons. We suspect that the low level of degeneration of the VC neurons in WT as well as the degeneration in immunocompromised strains may reflect accumulation of endogenous APL-1 protein. Likewise, the timing of immunosenescence in *C. elegans*, which starts on third day of adulthood, may explain the onset of VC degeneration in WT. It will be interesting to determine whether the subset of cholinergic neurons that dies first in rodent models of AD also matches those vulnerable in immunocompromised mice. In line with this theory, recent reports found that repeated systemic challenge to the immune system caused a spatial pattern of neurodegeneration and behavioral symptoms that mimicked AD in mice (Meyer et al., 2008; Krstic et al., 2012).

We also demonstrated the ease in which drugs can be tested for *in vivo* neuroprotective effects with *C. elegans*. In less than one week, protective effects can be accessed by direct visualization of fluorescently labeled cholinergic neurons. Moreover, the functional integrity of these specific neurons can be assessed with simple behavioral assays. In contrast to most AD drugs in clinical trials that aim to reduce accumulation of APP and plaques, we show that P7C3 represents a novel drug class because it can prevent apoptotic degeneration and preserve neuronal function even in the face of APP accumulation. Because P7C3 and Dimebon show favorable pharmacological profiles in mice and humans respectively (Pieper et al.,2010; Doody et al., 2008), our results validate the use of *C. elegans* for the evaluation of potentially beneficial compounds in the treatment of AD and other neurodegenerative disorders with unprecedented speed and cost effectiveness.

P7C3 was found in a screen for small molecules that increase yield of adult-born hippocampal neurons in rodent models of aging and mental retardation (Pieper et al. 2010). They proposed that P7C3 may promote neurogenesis by preventing the apoptotic death of the precursor cells mentioned above. If so, the anti-apoptotic effects did not interfere with natural apoptosis during development because development of mice was normal with pre- or post-embryonic treatment with P7C3 (Pieper et al., 2010). Likewise, we found that P7C3 did not interfere with developmental apoptosis in *C. elegans* (data not shown). Considering both rodent and worm studies, these drugs appear to selectively

block a “pathological” or “adult” entrance to apoptosis – one conserved across diverse neurological disease and species separated by 1 billion years of evolution.

We also found neuroprotective effects for the structurally similar compound Dimebon. P7C3 and Dimebon both appear to block a pathological entrance to apoptosis triggered by accumulation of APP. Through genetic analysis, we narrowed the point of action of these two drugs to a position upstream of the conserved BH3-domain containing cell death activator EGL-1, and downstream of the conserved ASK1 innate immune signaling triple kinase NSY-1. Likewise, Dimebon has been shown to protect against degeneration and neuronal dysfunction in rodent models of AD, perhaps due to its ability to boost beneficial autophagy as demonstrated in cultured neurons (Steele et al., 2012). In humans, Dimebon originally showed promise in reducing AD symptoms, but had no beneficial effect in recent phase III clinical trials (Doody et al., 2008). With this new mechanistic understanding, future drug trials for AD may consider compounds based on the more potent P7C3 to fill the void left by Dimebon. Because P7C3 also generalized to prevent apoptotic neurodegeneration caused by interrupting innate immune signaling, we predict that P7C3 may further generalize to protect neurons in diverse degenerative disorders.

Age-related patterned neurodegeneration caused by a widely expressed protein is a paradoxical phenomenon observed in all major neurodegenerative disorders (Gotz et al., 2009). These findings of patterned neurodegeneration caused by APP in the well-

defined nervous system of *C. elegans* may yield general insight into mechanisms and medicine that translate to these other disorders.

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Chapter 1

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Symposium, New Orleans LA, USA, 2012; "Studying mechanism and medicine of Alzheimer's disease with worms." Center for Learning and Memory 6th Annual Retreat, Austin TX, USA, 2012; Crisp A., Pierce-Shimomura J. Induction and prevention of patterned neurodegeneration in Alzheimer's Disease (in submission).

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