BIOLOGY OF COGNITIVE AGING: MODEL SYSTEMS, TECHNOLOGIES AND BEYOND

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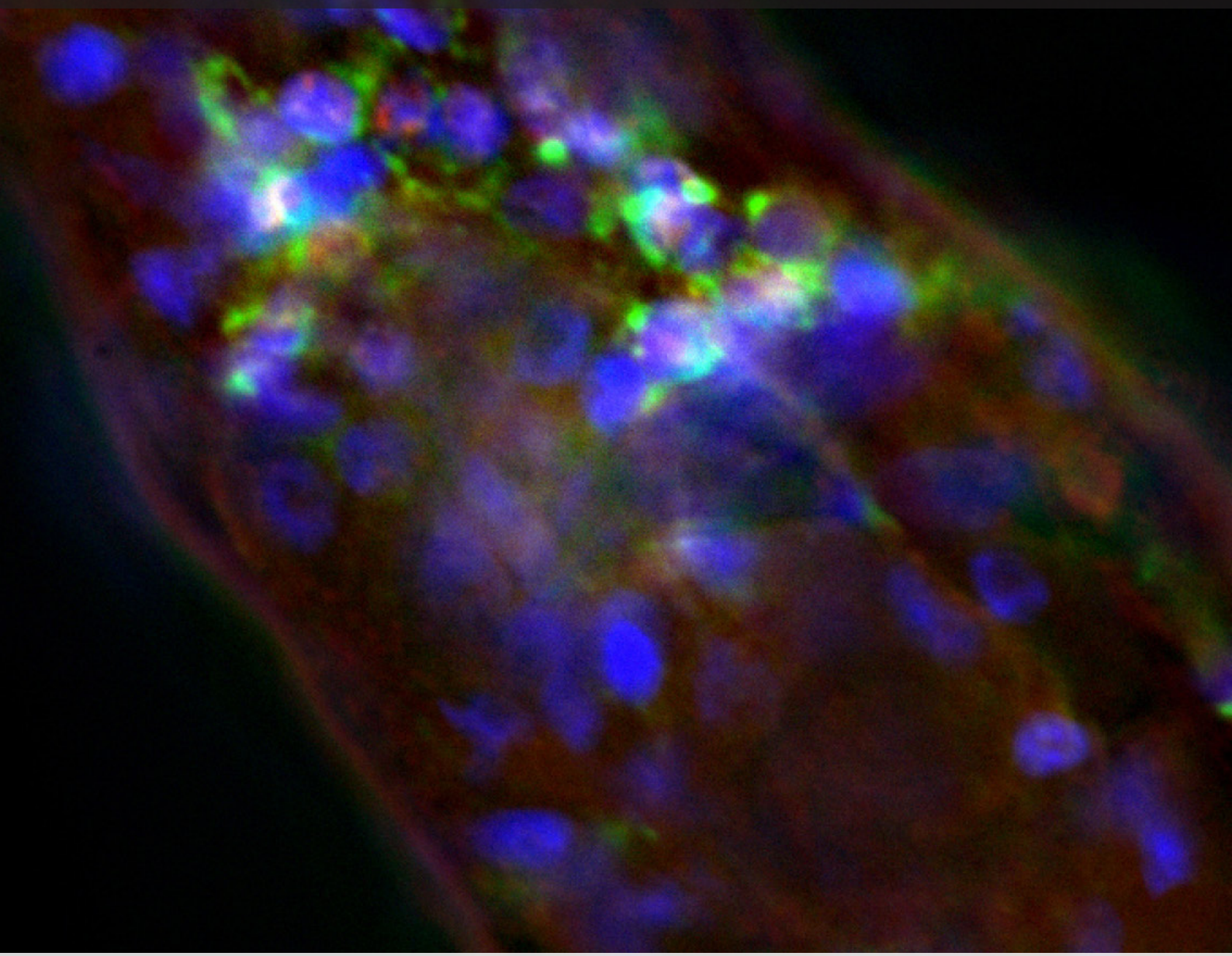

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BIOLOGY OF COGNITIVE AGING: MODEL SYSTEMS, TECHNOLOGIES AND BEYOND

Topic Editor:
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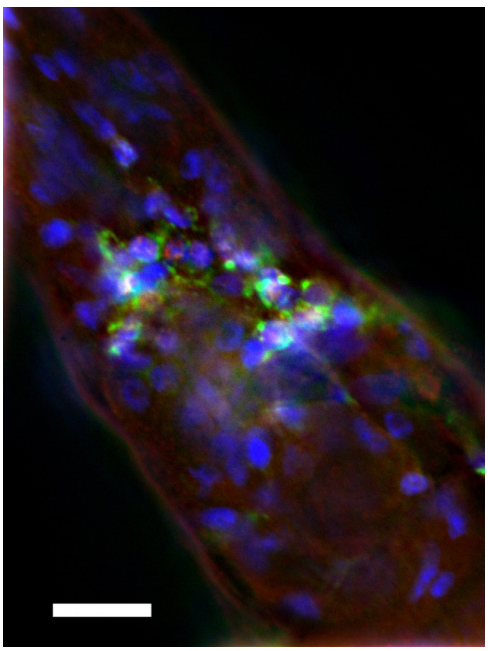

Alzheimer's worm overexpressing beta-amyloid in the neurons.
Figure taken from: Machino K, Link CD, Wang S, Murakami H and Murakami S (2014) A semi-automated motion-tracking analysis of locomotion speed in the C. elegans transgenics overexpressing beta-amyloid in neurons. Front. Genet. 5:202. doi: 10.3389/fgene.2014.00202

Welcome!

We, humans, tend to experience forgetfulness when we get old. The forgetfulness may become more serious memory impairment, dementia. Presumably, we have known it for a long time, but we still do not know the mechanism behind. A normal part of forgetfulness is called age-related memory impairment (AMI), which is considered the first step towards mild cognitive impairment (MCI; transition state) and dementia (disease state). The majority of dementia is attributable to Alzheimer's disease (AD). Progression to dementia occurs at a high rate in patients with AMI. This eBook covers exciting but yet challenging field of cognitive aging.

AMI is specific to neural tissues of the brain and is considered to be segmental aging. It happens not only to humans but also to a variety of species. Learning and memory are vulnerable to aging in a wide variety of model species, including worms, fruit flies, insects, snails, fishes, and rodents. Aging specifically reduces the ability to learn new information but leaves "old" memories and procedural memory intact. A comparative approach including the use of model systems seems to facilitate understanding of the molecular mechanisms that lead to AMI and AD. We advocate research on model systems.

This eBook also provides the first manuscript co-authored with an AD patient to create a feedback loop from patients incorporated into research. We also included a manuscript on the semi-automated system that was inspired by such a feedback. Those may place a nice flavor to this exciting series of comparative research on cognitive aging.

We hope you enjoy this eBook.

Warm regards,
Shin Murakami, Ph.D.

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Table of Contents

Section 7: Worm

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EDITORIAL
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Editorial: Biology of Cognitive Aging: Model Systems, Technologies, and Beyond

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Keywords: research outreach, Alzheimer's disease, Huntington disease, dementia, age-related memory impairment (AMI), age-related memory loss, model systems, patient care

The Editorial on the Research Topic

Biology of Cognitive Aging: Model Systems, Technologies, and Beyond

Age-related memory impairment (AMI), also called age-associated memory impairment (AAMI), describes the normal emergence and progression of memory problems associated with aging. It is one of diverse age-associated phenotypes found across a variety of organisms, including bees, fruit flies, nematodes, snails, rodents, and humans. The goal of this editorial is to provide an overview of our current understanding of AMI and its progression to more severe deficits related to advanced age and dementia. This research issue focuses on model systems and technologies relevant to cognitive aging and Alzheimer's disease, and the study of AMI using invertebrate disease models.

AMI can be viewed as a reduction in neuroplasticity, wherein the retention of existing memories is relatively well-preserved, compared with the ability to acquire new information. Aging specifically compromises mental processes underlying learning and some types of long-term memory, while leaving other types of long-term and procedural memories intact. AMI, however, may also include loss of existing memory, a condition termed age-related memory loss. In a simplified model, cognitive aging can be described to occur in the following sequence: AMI (normal state), mild cognitive impairment (MCI [transition state]), and then lastly, dementia (disease state). The neural mechanisms underlying the development of AMI into more advanced age-related disease remain unclear.

A wide variety of model systems and comparative analysis techniques show promise in guiding therapeutic strategies for elderly patients with memory impairments. Despite their potential, the use of disease models in this field of medicine has been discouraged. As stated by the Alzheimer's Association[1], "a limitation of these models is that they do not capture the full complexity of the human condition, which is problematic if one wants to use them to predict the success of specific therapeutic interventions in individuals with Alzheimer's disease (Accessed on Dec 2015)." This research issue thoroughly discusses both the advantages and limitations of disease models of cognitive aging.

A major aim of this editorial is to compile information about and identify connections between patients with dementia and other organisms, such as bees (Farooqui), fruit flies (Mukherjee et al.), nematodes (Alexander et al.; Gkikas et al.; Machino et al. ; Scerbak et al.; Therrien and Parker), snails (Hermann et al.), fish (Newman et al.), and rodents (Webster et al.; Way et al.); a manuscript co-authored by an early-stage patient with Alzheimer's disease (Murakami and Halperin) is also discussed. The work described herein includes rigorous reviews and original research articles on models of Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic lateral

[1]Alzheimer's association. *Areas of Focus for the 2014 International Research Grant Program*. Available online at: http://www.alz.org/research/downloads/2014_area_of_focus.pdf (Accessed on Dec 2015).

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sclerosis, and other neurodegenerative conditions. Alexander et al. comprehensively review neurodegenerative disease in the nematode, *Caenorhabditis elegans* (*C. elegans*), while Webster et al. address the significance of mouse models to our understanding of human Alzheimer's disease. Gkikas et al. discuss pathways of longevity that potentially restore memory function during aging in *C. elegans*. Machino et al. propose the use of a semi-automated motion-tracking system as a platform for genetic and pharmacological screening, which was inspired by feedback from an early-stage patient with Alzheimer's disease (Murakami and Halperin). The study of AMI and dementia also utilizes other non-mammalian models, such as the freshwater snail (Hermann et al.) and a zebra fish model of Alzheimer's disease (Newman et al.), among others.

Animal models enable functional analysis of AMI and dementia and provide useful information for minimizing the risks for humans. Thus, it would not be beneficial to underestimate the role of model systems in medicine. Additionally, research using such systems would be better positioned to advance future therapies if patient feedback is incorporated. To this end, Murakami and Halperin have proposed soliciting feedback from patients in an effort to align basic research with clinical practice, patient care, and the patient experience. Such feedback emphasizes the importance of existing comorbidities and potential interaction/association between co-occurring diseases. This approach is consistent with the "mid-life crisis theory," in which earlier deleterious events are associated with aging and later age-related diseases (Murakami et al., 2011; Murakami, 2013).

I thank all of the authors for their contributions. I would especially like to thank my friend and collaborator, Dr. Alexander "Sandy" Halperin, who, despite difficult personal circumstances, continues to provide valuable insight from a patient's point of view.

AUTHOR CONTRIBUTIONS

SM conceived and wrote this editorial.

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OPINION ARTICLE
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Alzheimer's patient feedback to complement research using model systems for cognitive aging and dementia

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Keywords: Alzheimer's disease (AD), age-related memory impairment (AMI), age-associated memory impairment (AAMI), *C. elegans*, cognitive impairment, dementia, outreach, stigma

This opinion article was co-authored with an early-stage Alzheimer's patient to assemble essential ideas regarding potential outreach initiatives and to describe the nature of research to and from model systems. Additionally, we hoped to strengthen basic medical research and patient-centered care through feedback from patients affected with Alzheimer's disease (AD). Recent studies in model systems have shed light on genetic and epigenetic mechanisms of neurodegenerative diseases, including AD, Parkinson's related dementia, dementia with Lewy bodies, and frontotemporal dementia, among others. However, most studies have focused on establishing a solid knowledge base and may have missed important aspects of communications between patients with cognitive impairment and healthcare professionals. In addition, scientists are often out of the loop from such communication, which requires multiple steps before it becomes available to researchers and may be a source of unexpected bias. Thus, we propose to include scientists, especially those studying early-stage patients, in these communications so that patient input can be directly integrated into research protocols. In this manner, we hope to make a significant contribution by integrating basic medical sciences and patient-centered care.

Stigma can be defined as an unfavorable way of assessing a particular person or population with a misunderstanding (a knowledge problem), which may eventually result in prejudice (an attitude problem) and discrimination (a behavior problem) (Batsch and Mittleman, 2012). It is surprising to learn about misconceptions surrounding dementia and those who have cognitive impairment similar to those in AD. In the World Alzheimer Report 2012, Batsch and Mittleman (2012) state:

> *"Symptoms of dementia are perceived differently... considering dementia as a normal part of ageing, mental illness, something metaphysical linked to supernatural or spiritual beliefs... It is very important that there is better public awareness and understanding to reduce the stigma associated with dementia."*

How can the basic sciences help to reduce the stigma and embarrassment that are often associated with dementia? The American Alzheimer's Association has initiated a vital program that invites patients, caregivers, and advocates to the National Alzheimer's Association conference to be held in the summer of 2014 (http://www.alz.org/). The Society of Neurosciences also has an outreach program (http://www.sfn.org/), though it focuses on exposing young students to neuroscience research. Although these resources provide opportunities for research education and interactions among early-stage patients, clinicians, and caregivers, there are still limited opportunities to facilitate effective communications with scientists.

OUTREACH TO AND FROM BASIC MEDICAL RESEARCHERS TO PATIENTS

THREE KEY FACTORS

In this section, we discuss the following key factors that may help build specific strategies, initiatives, and programs: (1) better education using clear verbal and written communication with early-stage patients with cognitive impairments, (2) better involvement of early-stage patients, using a precise protocol to record their feedbacks, and (3) participation of medical students, scientific researchers, and healthcare providers, before or early in their studies, to understand what patients are experiencing, therefore allowing research to better target the problems that these patients express.

First, although talking to patients is often not part of scientific studies, it can be highly informative for scientists to be part of the communication between patients with cognitive impairment and healthcare professionals. We believe it is important that scientists carefully listen to early- and more advanced stage patients. Some patients can explicitly explain their experiences and feelings with regard to their memory impairment. Thus, researchers and healthcare professionals can better understand the thoughts of those who are impaired. Early-stage patients have enough cognition to clearly explain their individual situations, which may differ from patient to patient. Generally, they have the capability to verbalize much of their moment to moment activities during the day, in terms of what

Abbreviations: AD, Alzheimer's disease; AMI, age-related memory impairment.

they can and cannot do. Here are some examples:

1) A patient stated, "I have problems with writing e-mails or any letters, but I can write with greater ease when I am listening to music." The conversation with the patient was spontaneous and routine, but his ability to write while listening to music delivered an important message to one of the authors; the effects of music can be studied in the laboratories of model systems. Music is known to reduce anxiety and is dependent on ovarian steroid hormones, including progesterone, in female mice (Chikahisa et al., 2007). Music is also known to modulate brain-derived neurotrophic factor, which is essential for neuron survival and function (Li et al., 2010). In addition, the effects of music may be studied using "omics" analysis in model systems with auditory functions, including fish and rodents.
2) Another conversation mentioned that some patients with AD had trouble with falling, which inspired Machino et al. (2014) to revisit locomotion analysis of a *C. elegans* strain overexpressing beta amyloid (referred to as AD strain). This AD strain has been reported to show relatively normal locomotion (Dosanjh et al., 2010). However, the study used a manual assay and may have missed locomotion deficits. Using a semi-automated assay with better accuracy, they found that the AD strain shows reduced locomotion speed (Machino et al., 2014). Importantly, motor activities are not always defective in AD but are abnormal (Webster et al., 2014). Thus, studies with model systems could possibly identify how behavioral disturbances may occur in AD.
3) AD patients are advised to exercise, which may improve the quality of life or even delay functional declines (Press and Alexander, 2014). Exercise can be easily integrated into studies of locomotion and motor activity in model systems of AD (Dao et al., 2013; Intlekofer and Cotman, 2013).
4) Some patients experienced worsening symptoms in the late afternoon and evening. This symptom, called "sundawning," is easily noticed when communicating with patients. The mechanism is not well understood but could be studied in mice (Bedrosian and Nelson, 2013).

It is essential to study sensitivity to AD and degeneration of neurons. Having relaxed and detailed conversations with early-stage patients could provide a number of potential approaches. Such communication would otherwise remain vague and unnoticed unless patient feedback is documented and easily accessible to scientists. Integration of research with patient feedback should be informative. Thus, a potential strategy could include: (1) opportunities to have routine communication, (2) documentation of potentially important information, and (3) incorporation of the information into research protocols. To integrate feedback into studies of model systems requires an additional step. As challenging as it may seem, it is our opinion that such feedback will eventually provide specific approaches to the study of AD by focusing on AD-resistant functions, as discussed below.

Second, communication with early-stage patients could better emphasize research that is oriented to the needs of the patients. A benefit of communication with early-stage patients is the ability to differentiate between what scientists seek (i.e., an understanding of mechanisms) and what patients need (i.e., treatment and an improved quality of life). Current research emphasizes the mechanisms causing AD and develops solutions to prevent AD. In contrast, patients need to have proper examinations, diagnoses, treatment plans, and a variety of lifestyle changes during the disease process, which unfortunately at this time has no cure. It is evident that there is a distinct gap between the needs of each patient and what some research may be providing—therapies that may be partially effective in terms of prevention and/or treatment. For this reason, scientists should reach out to patients to acquire feedback necessary to aid other researchers and healthcare providers in developing patient-centered care and research. Each patient is clearly different but, collectively, the information obtained may be highly beneficial.

Third, to close the knowledge gaps that likely exist, medical students, research scientists, and healthcare providers must have the best information from each patient. Grants and scientific meetings will also benefit from input by AD patients. To make these activities most effective for patient treatment, it is essential to have students spend more time with patients in a variety of outreach settings. In addition to the activities previously discussed (Batsch and Mittleman, 2012), there are pilot approaches being initiated. These include an international Alzheimer's association conference that will be held in the summer of 2014 and an outreach program where students can communicate their research to patients (Biotech Academy Touro Internship Program). Because there is presently no such process regarding outreach from model systems to patients (PubMed search using two key words, "model system" and "outreach"; accessed on March 28, 2014), this is most likely the first article that describes an outreach from model systems to patients.

OUTREACH FROM MODEL SYSTEMS TO KNOWLEDGE

TWO TYPES OF RESISTANCE TO AD

Among the many approaches in studying AD, we will review an approach that utilizes neurons and nerve function resistant to AD. Although a number of studies have focused on the disease, it is important to understand the well-preserved memory functions in dementia patients. The objective of this approach is to complement, activate, or preserve declining cognitive functions that are relatively resistant to dementia. They include cognitive exercises (Woods et al., 2012), music, and emotions (Baird and Samson, 2009; Groussard et al., 2013; Moore, 2013; Ueda et al., 2013). Physical exercise may also be included in this category. Implicit memory for music, including the ability to play instruments (procedural memory), is well-preserved in the middle to late stages of AD, whereas explicit memory is sensitive to AD in the early stages (Baird and Samson, 2009; Groussard et al., 2013). In addition, music may be beneficial in controlling emotion and memory (Moore, 2013; Ueda et al., 2013).

Despite the past belief that implicit memory is well-preserved, it is now accepted that certain types of implicit memory, including "old memory" and

memory of well-learned training, are well-preserved during normal aging (Johnson et al., 2002; Howard and Howard, 2013; Murakami, 2013; Ward et al., 2013). Implicit memory includes associative learning and memory, which is well-conserved in model systems including nematodes (*C. elegans*) (Murakami, 2007, 2013; Stein and Murphy, 2012; Chen et al., 2013; Gkikas et al., 2014), flies (Yamazaki et al., 2010), bees (Behrends et al., 2007; Farooqui, 2014), snails (Hermann et al., 2007), rodents (Villarreal et al., 2004; Sharma et al., 2010; Woodruff-Pak et al., 2010), and humans (Labar et al., 2004) (summarized in an editorial, to be published). Nematodes show relatively well-preserved associative memory through food conditioning but show impairment in associative memory through conditioning to a lack of food (Murakami, 2007; Stein and Murphy, 2012; Chen et al., 2013; Murakami, 2013; Gkikas et al., 2014). In this regard, memory for survival and prosperity is presumably important for natural selection, which has been involved in the evolution of animal species.

CONCLUDING REMARKS

Aging brings vulnerability that occurs unexpectedly in middle life and is described as the "middle-life crisis theory" (Murakami et al., 2011; Murakami, 2013). This theory states that age-related changes are mostly reversible in the early phase and eventually transition to a more advanced and irreversible phase around the middle of the lifespan. The early phase of age-related memory impairment appears reversible and can be stored, for example, in bees (Baker et al., 2012) and *C. elegans* (Murakami et al., 2008; Stein and Murphy, 2012). According to this theory, the early phase involves a shift from one state to another rather than damage to biological functions. By contrast, dementia presumably involves mixed functions in neurons, including damaged (irreversible), less damaged/affected (reversible), and normal (well-preserved) functions. Understanding altered nerve functions as well as the interactions among functions should provide strategies for developing treatment options for dementia. Information resulting from research in model systems could lead to possible treatments that may stimulate or complement declining cognitive functions in dementia patients. Collaboration among scientists, healthcare professionals, and caregivers and listening to feedback from patients with early-stage AD and other types of dementia can hopefully lead to improvements in model systems and basic medical research.

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Using mice to model Alzheimer's dementia: an overview of the clinical disease and the preclinical behavioral changes in 10 mouse models

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The goal of this review is to discuss how behavioral tests in mice relate to the pathological and neuropsychological features seen in human Alzheimer's disease (AD), and present a comprehensive analysis of the temporal progression of behavioral impairments in commonly used AD mouse models that contain mutations in amyloid precursor protein (APP). We begin with a brief overview of the neuropathological changes seen in the AD brain and an outline of some of the clinical neuropsychological assessments used to measure cognitive deficits associated with the disease. This is followed by a critical assessment of behavioral tasks that are used in AD mice to model the cognitive changes seen in the human disease. Behavioral tests discussed include spatial memory tests [Morris water maze (MWM), radial arm water maze (RAWM), Barnes maze], associative learning tasks (passive avoidance, fear conditioning), alternation tasks (Y-Maze/T-Maze), recognition memory tasks (Novel Object Recognition), attentional tasks (3 and 5 choice serial reaction time), set-shifting tasks, and reversal learning tasks. We discuss the strengths and weaknesses of each of these behavioral tasks, and how they may correlate with clinical assessments in humans. Finally, the temporal progression of both cognitive and non-cognitive deficits in 10 AD mouse models (PDAPP, TG2576, APP23, TgCRND8, J20, APP/PS1, TG2576 + PS1 (M146L), APP/PS1 KI, 5×FAD, and 3×Tg-AD) are discussed in detail. Mouse models of AD and the behavioral tasks used in conjunction with those models are immensely important in contributing to our knowledge of disease progression and are a useful tool to study AD pathophysiology and the resulting cognitive deficits. However, investigators need to be aware of the potential weaknesses of the available preclinical models in terms of their ability to model cognitive changes observed in human AD. It is our hope that this review will assist investigators in selecting an appropriate mouse model, and accompanying behavioral paradigms to investigate different aspects of AD pathology and disease progression.

Keywords: Alzheimer's disease, mouse models, neuropsychological assessment, behavior, cognition, APP mice, APP/PS1 mice, 3×TG-AD mice

INTRODUCTION

Alzheimer's disease (AD) is characterized by a progressive decline in cognitive function, usually starting with memory complaints and eventually progressing to involve multiple cognitive, neuropsychological and behavioral domains. The definitive diagnosis of AD comes from postmortem analysis of the neuropathological changes in the brain. Analyses of both clinical and pathological features, i.e., clinicopathological correlation studies, have provided important insights into how the pathology correlates with cognitive status. Complementing these studies in humans has been the development of preclinical model systems of AD pathology. These preclinical animal models, especially mouse models, have been extremely useful to test mechanistic hypotheses about AD pathophysiology and to predict outcomes from pharmacological interventions. However, no animal model recapitulates the entirety of AD in humans, and therefore it is important to understand both the utility and limitations of particular animal models. With this in mind, we will present an overview of the neuropathological changes seen in the AD patient population as individuals transition from normal cognitive aging to dementia, review the clinical neuropsychological assessments used in the AD field, review the mouse behavioral tasks commonly used in preclinical testing and discuss how they relate to these clinical neuropsychological assessments, and outline the temporal progression of cognitive and non-cognitive deficits seen in the commonly used mouse models of AD.

OVERVIEW OF NEUROPATHOLOGICAL CHANGES IN AD

In 2012, new consensus guidelines for neuropathologic evaluation of AD were adopted (Hyman et al., 2012; Montine et al., 2012). The AD neuropathologic change is now ranked on three parameters (Amyloid, Braak, CERAD) to obtain an "ABC" score: histopathologic assessments of beta-amyloid (Aβ)-containing amyloid plaques (A), Braak staging of neurofibrillary tangles (B), and scoring of neuritic amyloid plaques (C). Standard approaches for the workup of cases, preferred staining methods, reporting of results and clinicopathological correlations are also recommended. Unlike the prior AD neuropathologic criteria (Hyman and Trojanowski, 1997) that required a history of dementia, the current guidelines recognize that AD neuropathologic changes can be present in the brain in the apparent absence of cognitive impairment. The updated guidelines thus emphasize the continuum of neuropathologic changes that underlie AD. For a disease process that is known to occur over a decade or more (Blennow and Zetterberg, 2013; Rosen and Zetterberg, 2013; Rosen et al., 2013), and encompasses the age range where people are likely to die of other causes, it is inevitable that many people will die in a prodromal or "preclinical" stage of AD. This consideration points to the complexity that clinicopathological studies face when examining AD pathological contributions to cognitive deficits (Nelson et al., 2009b).

There have been numerous clinicopathological studies attempting to correlate amyloid plaques with the cognitive deficits seen in AD (Blessed et al., 1968; Tomlinson et al., 1970; Duyckaerts et al., 1990, 1998; Berg et al., 1998; Gold et al., 2000; Mungas et al., 2001; Tiraboschi et al., 2002, 2004; Guillozet et al., 2003; Kraybill et al., 2005; Holtzer et al., 2006; Markesbery et al., 2006; Nelson et al., 2007a, 2009a; Beach et al., 2009; Sabbagh et al., 2010; Robinson et al., 2011). Apparent inconsistencies in the conclusions of these studies are due to differences in study cohorts, methodology used to classify plaque subcategories, plaque-counting techniques, and metrics used to assess cognitive deficits. Nevertheless, several important concepts have emerged pertaining to plaque pathology and cognition. First, the strongest correlation between amyloid plaques and cognition is in the early stages of the disease and this association weakens as NFTs and gross neocortical neurodegeneration become more widespread (Thal et al., 2002; Nelson et al., 2009b, 2012). As the disease progresses into the later stages, there is little evidence to support a continued contribution by amyloid plaques to the late-stage AD cognitive decline (Nelson et al., 2009b, 2012). Second, it appears that density of neuritic plaques correlates more strongly with the cognitive deficits than do "diffuse" amyloid plaques (Mckee et al., 1991; Crystal et al., 1993; Tiraboschi et al., 2004; Nelson et al., 2007a; Braak et al., 2011).

In contrast to the literature concerning amyloid plaques, a large number of studies have arrived at a common finding, namely, there is a strong link between neocortical NFTs and cognitive decline (Tomlinson et al., 1970; Duyckaerts et al., 1990, 1997, 1998; Mckee et al., 1991; Arriagada et al., 1992; Bierer et al., 1995; Davis et al., 1995; Dickson et al., 1995; Nagy et al., 1995, 1999; Cummings et al., 1996; Berg et al., 1998; Grober et al., 1999; Sabbagh et al., 1999; Gold et al., 2000; Mungas et al., 2001; Riley et al., 2002; Silver et al., 2002; Tiraboschi et al., 2002; Guillozet et al., 2003; Bennett et al., 2004; Kraybill et al., 2005; Holtzer et al., 2006; Markesbery et al., 2006; Koepsell et al., 2008; Whitwell et al., 2008; Beach et al., 2009; Brayne et al., 2009; Giannakopoulos et al., 2009; Sabbagh et al., 2010; Robinson et al., 2011). It should be noted that outside of frontotemporal lobar degeneration (FTLD), one does not see widespread cortical NFTs without abundant plaque pathology. In the earliest stages of AD (Braak stage I-II), NFTs are restricted to the entorhinal cortex (Braak and Del Tredici, 2011; Braak et al., 2011). NFTs spread to the limbic and medial temporal lobe (Braak stage III-IV), and this stage correlates with early AD symptoms related to memory (Schmitt et al., 2000; Riley et al., 2011). During the late stages (Braak stage V–VI), NFTs increase in number and manifest in neocortical areas responsible for higher cognitive domains such as executive function, visuospatial capacities, and speech in synchrony with observed AD-related cognitive deficits in these respective cognitive domains. Not all AD cases fall within the standard NFT distribution described by the Braak staging (Hof et al., 1997; Abner et al., 2011; Murray et al., 2011). Some cases classified as "high level" of AD neuropathological changes may show subtle or undetectable cognitive impairment, yet all cases with quantifiably "end stage" neocortical NFT pathology show cognitive impairment (Dickson et al., 1995; Berlau et al., 2007; Nelson et al., 2009a, 2012; Abner et al., 2011; Santacruz et al., 2011; Jicha et al., 2012). In sum, the correlations noted in human material support the hypothesis that plaques and tangles correlate with cognitive status. The data also support the "Amyloid Cascade Hypothesis"(Karran et al., 2011), a deceptively complex hypothesis which posits that beta-amyloid/plaque pathology kindles widespread tau/NFT pathology, with the tau/NFT pathology constituting the more direct cause of the cell loss and synapse elimination underlying clinical disease (Nelson et al., 2009b, 2012).

COGNITIVE NEUROPSYCHOLOGICAL ASSESSMENTS USED IN THE AD FIELD

Neuropsychological assessment is the most reliable means to clinically evaluate the cognitive deficits seen in humans. Many neuropsychological tests have been developed which are highly sensitive to the cognitive behavioral symptoms seen in AD, and these tests are extensively used as clinical diagnostic tools (Schmitt, 1994) as well as to track the progression of the disease (Flicker et al., 1984; Morris et al., 1989; Storandt and Hill, 1989; Storandt, 1991; Welsh et al., 1991, 1992; Locascio et al., 1995; Albert, 1996; Storandt et al., 1998; Schmitt et al., 2000; Salmon and Bondi, 2009). Current neuropsychological assessments (from the National Institute on Aging workgroups on diagnostic guidelines for AD) aim to detect disruptions in cognitive domains such as episodic memory, semantic memory, working memory, and attention, as well as dysfunction in language, praxis, and executive functioning (Flicker et al., 1984; Baddeley et al., 1986, 1991, 2001; Huff et al., 1987; Knopman and Ryberg, 1989; Hodges et al., 1992; Parasuraman and Nestor, 1993; Hodges and Patterson, 1995; Perry and Hodges, 1999; Salmon et al., 1999; Perry et al., 2000; Backman et al., 2001; Lambon Ralph et al., 2003). In the following section, we will cover several of the most common neuropsychological tests used clinically to assess the mental status

and memory disruptions in AD (for more in depth reviews see Perry and Hodges, 1999; Budson and Price, 2005; Bondi et al., 2008; Weintraub et al., 2012). **Table 1** provides an overview of four mental status examinations and two brief memory tests commonly used clinically.

MENTAL STATUS EXAMS

Mental status examinations assess multiple mental functions and cognitive abilities across multiple domains (see **Table 1**), and are generally more encompassing than specific verbal and visual memory tests (examples also seen in **Table 1**). Both categories of tests are clinically useful in assessing the cognitive progression of AD. Most mental status examinations assess mental functions and cognitive ability across a wide range of areas such as: language skills, arithmetic ability, visuospatial ability, attention, memory, and orientation to time and place.

The Mini-Mental Status Examination (MMSE) is one of the most commonly used neuropsychological screening tools for cognitive impairments seen in AD (Simard and Van Reekum, 1999; Snyderman and Rovner, 2009). The MMSE is a brief questionnaire that can both diagnose cognitive impairment and track the severity of this cognitive impairment throughout the pathogenesis of the disease. The MMSE covers multiple areas such as: attention, memory (semantic and episodic), orientation to time and place, and working memory. The scoring system ranges from 0 to 30 points. In general, a score of 27 or greater reflects normal cognition, a score of 19–24 represents mild impairment, a score of 10–18 represents moderate impairment, and a score below 9 indicates severe cognitive impairment. The MMSE has excellent reproducibility that lends itself well to longitudinal use in tracking the progression of the cognitive impairments associated with AD (Jacqmin-Gadda et al., 1997; Aevarsson and Skoog, 2000; Chatfield et al., 2007).

The Montreal Cognitive Assessment (MoCA) is a more recent mental status examination used for cognitive dysfunction seen in AD. This test battery takes approximately 10 min to administer and covers many similar cognitive domains to that of the MMSE. The total possible score is 30 points, with a score of 26 or above considered within the normal range. An example of a task included in the MoCA is the forward and backward digit span test. In the forward digit span test, a sequence of five numbers is read at a rate of one number per second and the test taker is required to repeat a set of numbers in exactly the same sequence as they were presented. In the backward digit span task, the test taker is required to repeat a three number sequence in the reverse order in which it was presented. One point is awarded for each of the digit span tests in which the test taker made no errors. The MoCA also includes a delayed recall memory test. Relatively near the beginning of the test, the examiner presents a short word list for the patient to remember. At the end of the test, the patient is again prompted to recall the word list and for each of the words correctly remembered one point is awarded.

Another commonly used test battery is that of the Short Blessed Test (SBT) which consists of a six-item test designed to identify the cognitive dysfunction seen in AD (Katzman et al., 1983). An appealing advantage of the SBT is the ease and speed with which it can be administered (often taking only a few minutes). In the SBT, errors are scored for incorrect answers and the scoring range falls between 0 and 28, with a score of 0–4 representing normal cognition, a score of 5–9 representing early impairment, and a score of 10 or more representing impaired cognition. Despite the simplicity and brevity of the SBT, the results that it produces have demonstrated excellent reliability (Fuld, 1978). Similarly, the SBT was the first mental status examination to be correlated with amyloid plaque burden at autopsy (Carpenter et al., 2011).

The Alzheimer's Disease Assessment Scale (ADAS) was specifically developed to measure the severity of symptoms commonly seen in AD (Rosen et al., 1984). Initially, it was developed in two parts (sub-scales): one for cognitive symptoms and one for non-cognitive symptoms. The cognitive sub-scale of the ADAS is commonly referred to as the ADAS-cog, and has become one of the most common neuropsychological tests used to assess AD progression. The scoring for the ADAS-cog ranges from 0 to 70, with a low score representing a cognitively intact person and a high score representing someone with cognitive impairment. Because the ADAS-cog has an excellent test-retest reliability and is considered to be one of the most sensitive scales for assessing cognitive changes related to AD (Emilien et al., 2004), this test is extensively used in AD clinical trials as an outcome measure of cognitive change (Schmitt and Wichems, 2006; Connor and Sabbagh, 2008).

MEMORY TESTS

Individual memory tests are generally shorter than mental status examinations and focus solely on assessing memory deficits. Both verbal and visual memory tests are commonly used clinically as stand alone tests or incorporated into a more comprehensive mental status examination. Examples of such memory tests are the Logical Memory Test I and II (LM-I and LM-II) and the Benton Visual Retention Test (BVRT) (Benton, 1992; Wechsler, 1997).

The LM-I and LM-II were originally developed as subtests to the Wechsler memory scale, but are commonly used as stand-alone memory tests. Both are verbal based memory tests that involve a short story read to the patient. In the LM-I, the patient answers immediate questions related to the narrative, whereas in the LM-II there is a delay between the presentation of the story and the questions. Thus, these memory tests are used to assess immediate recall (LM-I) and delayed recall (LM-II).

In the BVRT, the patient is shown 10 different visual designs, one at a time, and is then asked to reproduce each one from memory exactly as it appeared. While scoring the BVRT, errors of omissions, distortions, perseverations, rotations, misplacements, and size are all looked for and can give some insight into the progression of the disease. For example, if the patient has a high number of perseveration errors it is likely that the AD pathology has manifest itself in neocortical areas responsible for higher cognitive domains such as executive function, and visuospatial capacities (Braak Stage V–VI).

COGNITIVE NEUROPSYCHOLOGICAL ASSESSMENT SUMMARY

Each of the tests described above is aimed at assessing deficits in different cognitive domains. Each of these domains has been

Table 1 | Common neuropsychological assessment tasks seen clinically.

Task	Description	Cognitive domains	References
MENTAL STATUS EXAMS			
Mini-Mental State Examination (MMSE)	Nineteen item (30 points) test of general cognitive status	Working memory, attention, memory (semantic), praxis, etc.	Folstein et al., 1975
Montreal Cognitive Assessment (MoCA)	A rapid screening method to assess mild cognitive dysfunction	Working memory, memory (semantic and episodic), attention, visuospatial memory, etc.	Nasreddine et al., 2005
Short Blessed Test (SBT)	A short six item test measuring general cognitive status	Memory (semantic and episodic), working memory, and attention, etc.	Blessed et al., 1968
Alzheimer's Disease Assessment Scale (ADAS)	An 11 part test that measures cognitive dysfunction	Memory (semantic and episodic), and attention, etc.	Rosen et al., 1984
MEMORY TESTS			
Logical memory test I and II	A short story is presented to the patient and used to test immediate memory (test I) and delayed memory (test II)	Memory (episodic), verbal recall, etc.	Wechsler, 1997
Benton Visual Retention Test (BRVT)	Visual based test of general memory	Memory (episodic), and working memory etc.	Benton, 1992

shown to be impaired at some point in the spectrum of human AD. However, they are not uniformly affected throughout the course of the disease. Deficits in some domains occur relatively early, while deficits in others occur much later in the progression of the disease. **Figure 1A** depicts an overview of the time course of affected cognitive domains in human AD. It has become increasingly clear that identifying and targeting the cognitive deficits that occur early in the course of the disease is critical to producing the maximum impact of treatment on cognitive function and quality of life (Salmon et al., 2002). Thus, great efforts have been made to better understand the profile of cognitive deficits associated with early AD, and have resulted in earlier and more reliable clinical diagnosis (Bondi et al., 1995, 1999; Jacobson et al., 2002; Lange et al., 2002; Mickes et al., 2007).

Some of the earliest neuropathological changes in AD are in the hippocampus and entorhinal cortex, followed by changes in the medial temporal lobe. Consistent with this progression of pathology, the earliest detectable deficits in cognition are seen in medial temporal lobe-dependent episodic memory (Bondi et al., 1999; Collie and Maruff, 2000; Schmitt et al., 2000; Smith et al., 2007). These early deficits in episodic memory are followed closely by deficits in semantic memory, and both are developed before other deficits in cognitive domains such as attention, visuospatial memory, or executive function (Bondi et al., 2008). This suggests that cognitive functions such as episodic and semantic memory that depend heavily on the neural circuitry of the medial and lateral temporal lobes may be impaired earlier than cognitive abilities that depend on the circuitry of other brain regions. Further support for this idea comes from the time course of the frontal lobe dependent executive function deficits observed in patients. Slight deficits in executive functioning are first detectable near the end of the preclinical phase of AD but after the observed deficits in episodic and semantic memory (Storandt et al., 2006; Twamley et al., 2006). As the patient moves from the preclinical phase of AD into MCI, more cognitive domains begin to be affected. Most studies of MCI patients show consistent impairments in verbal recall (Larrieu et al., 2002; Tuokko et al., 2005; Kryscio et al., 2006) and a decline in general memory functioning (Tuokko et al., 2005; Bondi et al., 2008). Once the AD patient progresses past MCI and into dementia, general cognition continues to decline with deficits appearing in all respective cognitive domains (Huff et al., 1987; Locascio et al., 1995; Lambon Ralph et al., 2003; Mckhann et al., 2011).

The importance of neuropsychological testing cannot be overstated, as it is the only measure that provides information about a patient's current cognitive status and remains the most reliable means to clinically diagnose probable AD. Neuropsychological testing provides information on both general cognitive status and specific information on different cognitive domains affected in AD. Composite scores encompassing multiple neuropsychological tests are often used and can provide some of the most reliable assessments of global cognitive status relating to AD as well as serving as efficacy outcome measures in clinical trials (Bernick et al., 2012).

HOW COMMONLY USED PRECLINICAL MOUSE BEHAVIORAL TASKS RELATE TO THE CLINICAL NEUROPSYCHOLOGICAL ASSESSMENT TESTS IN HUMAN AD?

Ideally, preclinical rodent cognitive testing would assess identical cognitive domains to those examined through neuropsychological testing in human AD. Indeed, many rodent behavioral tasks have been specifically designed with this in mind, and while each task varies with respect to face, construct, and predictive validity, they all attempt to model different aspects of the cognition disrupted in AD and targeted by the human neuropsychological assessments listed in the previous section. Some cognitive domains disrupted in AD have been extensively modeled (reference memory, working memory and executive function), some less so (attention), and some nearly not at all (episodic memory). Reference memory, while not used clinically to describe human cognition, refers to learned knowledge for an aspect of a task that remains constant throughout the behavioral task and most closely correlates to human semantic memory. Working memory refers to a mental processing system used to hold transitory information for a limited time where it can be manipulated and operated on and used to guide behavior. Recognition

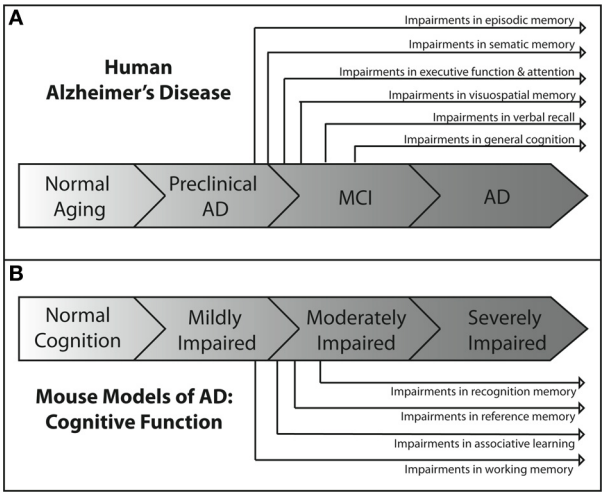

FIGURE 1 | Overview of the progression of cognitive deficits in human AD and in mouse models of AD. (A) In the human disease, the earliest AD-related cognitive deficits present themselves as episodic memory impairment during the late preclinical phase of the disease (Backman et al., 2005; Twamley et al., 2006; Bondi et al., 2008). Semantic memory deficits are the next to develop (Tuokko et al., 2005; Storandt et al., 2006), followed by impairments in executive functioning (Bondi et al., 2008), attention (Bondi et al., 2008), and visuospatial memory (Twamley et al., 2006) near the beginning of the MCI phase of the disease. As MCI progresses, deficits in verbal recall (Kryscio et al., 2006; Bondi et al., 2008) develop and impairments in general cognition (Bondi et al., 2008) become apparent. As the patient transitions into AD, all cognitive domains become affected. **(B)** The development of cognitive deficits in APP mouse models of AD shows similar patterns of progression. Consistently, the earliest observable impairments are in spatial working memory (Webster et al., 2013), as assessed through the use of water maze based tasks. These impairments are generally followed temporally by impairments in associative learning and reference memory, as assessed by maze alternation (Lalonde et al., 2012) and fear conditioning tasks (Kobayashi and Chen, 2005). Deficits in recognition memory usually present later in the spectrum of cognitive impairment than deficits in other domains (Eriksen and Janus, 2007; Hall and Roberson, 2012; Webster et al., 2013).

memory refers to the ability to recognize previously encountered events, objects, or individuals and is classified as part of long-term declarative memory. Other cognitive domains impaired in human AD such as those involving language (i.e., verbal acuity tasks and verbal recall tasks) simply cannot be modeled in rodent models. **Table 2** provides a short description of various behavioral tasks used to assess AD-like cognitive deficits in mice, and summarizes the respective cognitive domains measured by each task.

MODELING WORKING MEMORY

Working memory is perhaps one of the most well modeled aspects of the memory deficits in AD. Clinically, many of the neuropsychological tests that assess working memory rely heavily on the use of verbal tasks (Kaplan et al., 1978; Benedict et al., 1998; Spreen and Strauss, 1998; Delis et al., 2000), employing language as a core construct and thus are not feasible to model using mice. Instead, spatial based working memory tasks are heavily employed in murine working memory testing and likely are more depictive of the visuospatial working memory tasks used clinically (Benton, 1992; Benedict and Groninger, 1994).

The most widely used paradigms for working memory in mice are maze type tasks which require spatial working memory to solve. The earliest variants of these are the T-maze and Y-maze alternation tasks, which are relatively simple tests consisting of three arms with a single intersection. These tasks rely on the natural exploratory behavior (tendency to choose an alternative arm over an arm which has been previously explored) of rodents and are considered the most rudimentary tasks to assess spatial working memory (Dudchenko, 2004). A more complex maze type task used to test murine spatial working memory is that of the Radial Arm Maze (RAM), consisting of several arms (usually 6–8) radiating outwards from a central platform (Olton and Samuelson, 1976; Olton et al., 1979). In the RAM, the animal is started in the center area and then some of the arms or all of the arms can be baited with a food reward. Depending on the baiting paradigm, unimpaired rats and mice will quickly learn where the food reward is and which arms have previously been visited, and will avoid re-entering a previously entered arm. Perhaps the most widely employed spatial working memory task is that of the Morris Water Maze (MWM) (Morris et al., 1982). The MWM consists of a large open pool with a hidden (submerged) escape platform located somewhere within the pool. Animals must learn where the platform is, remember the platform's location, and then use spatial cues on subsequent trials to navigate back to the hidden platform. Large numbers of AD mouse models have been tested in the MWM and most show AD related cognitive deficits (Webster et al., 2013). Other common tasks of murine spatial working memory are the Radial Arm Water Maze (RAWM) and the Barnes Maze (Barnes, 1979; Diamond et al., 1999; Alamed et al., 2006).

It is important to note that while many of the previously described behavioral tasks are considered tasks of working memory they can also be modified to test reference memory depending on the testing protocol used. Similarly, not all models of murine working memory are spatial working memory tasks. For example, there exist versions of both the RAM and RAWM that are considered non-spatial working memory tasks (Olton and Feustle, 1981; Crusio et al., 1993; Hyde et al., 2000). Other examples of non-spatial working memory tasks are operant tasks such as the Delayed Match to Sample (DMTS), Delayed Non-Match to Sample (DNMTS), and Delayed Stimulus Discrimination Task (DSDT) (Dudchenko, 2004; Buccafusco et al., 2008). In these non-spatial working memory tasks the animal is required to remember a stimulus (over a delay period) that is paired with a particular type of response (generally a lever press or a nose poke) and a correct response is rewarded. In many of the non-spatial operant working memory tasks each animal can perform many trials per day and thus can serve as its own control. This lends itself nicely to pharmacological based studies assessing potential therapeutic compounds for the treatment of the cognitive deficits seen in AD (Buccafusco et al., 2008).

MODELING EXECUTIVE FUNCTION

Executive function refers to a broad range of higher cognitive processes such as: reasoning, planning, cognitive flexibility,

Table 2 | Commonly used mouse behavioral tasks.

Task	Description	Cognitive domains	References
Morris Water Maze (MWM)	Widely used behavioral task where mice are placed in a circular pool and must find a hidden escape platform	Reference memory and working memory	Morris et al., 1982
Radial Arm Maze (RAM)	The maze usually consists of 6–8 arms radiating from a round central space. Various arms are baited with a food reward.	Reference memory and working memory	Olton and Samuelson, 1976
Radial Arm Water Maze (RAWM)	A submerged version of the RAM where the food reward is replaced by an escape platform.	Reference memory and working memory	Diamond et al., 1999
Barnes maze	Consists of a circular platform with holes around the circumference and an escape box	Reference memory and working memory	Barnes, 1979
T-Maze/Y-Maze alternation	A three arm maze which forces the animal to choose between two arms	Reference memory and working memory	Blodgett and Mccutchan, 1947; Glickman and Jensen, 1961
Novel Object Recognition (NOR)	A two trial memory task which uses the animal's innate exploratory behavior to assess memory	Recognition memory	Ennaceur and Delacour, 1988
Contextual and cued fear conditioning	The animal learns to predict an aversive stimulus based on an associated context/cue	Reference memory (associative learning/memory)	Fanselow, 1980; Curzon et al., 2009
Passive avoidance	An avoidance task where the animal must refrain from entering a chamber where an aversive stimulus was previously administered	Reference memory (associative learning/memory)	Van Der Poel, 1967
Active avoidance	A fear-motivated associative avoidance test where an animal must actively avoid an aversive stimulus	Reference memory and working memory (associative learning/memory)	Vanderwolf, 1964
Delayed Matching (non-matching) to Position/Sample (DMTP/DMTS)	The animal receives a sample stimulus and then after a short delay is required to choose the correct corresponding response	Working memory	Dunnett, 1993; Robinson and Crawley, 1993
Multiple-Choice Serial Reaction Time Task (CSRTT)	The animal must attend to several spatial locations (usually 3–5), observe a corresponding stimulus, and then correctly respond	Attention, impulsivity, and executive function	Carli et al., 1983
Attentional set-shifting tasks	The animal must shift back and forth between changing rules to successfully obtain a reward	Executive function and cognitive flexibility	Birrell and Brown, 2000
Reversal learning	Adjustment to changes in reward contingency	Executive function and working memory	Butter, 1969; Bussey et al., 1997
What-Where-Which Task (WWWhich)	The animal must associate an object (What) with its location (Where) in a specific visuospatial context (Which) to form an integrated memory	Recognition memory and episodic-like memory	Davis et al., 2013a,b

sequencing, response inhibition, and abstract concept formation. The current mouse models of executive function most closely replicate the human aspects of cognitive flexibility and response inhibition in executive function. Attentional set-shifting tasks are one of the main behavioral tasks used to assess executive function in the mouse. In many ways, set-shifting tasks are similar to the Wisconsin Card Sorting Task in that they form the gold standard for assessing executive function (Drewe, 1974; Robinson et al., 1980; Arnett et al., 1994). In both the Wisconsin Card Sorting Task in humans and set-shifting tasks in mice, the dorsolateral and orbital prefrontal cortex is critical for successful performance (Weinberger et al., 1986; Berman et al., 1995; Brigman et al., 2005; Bissonette et al., 2008). In the most common version of the murine set-shifting task, mice are required to select a bowl in which to dig for a food reward. Bowls can be discriminated from each other according to different stimulus dimensions such as texture and odor. Successful completion of the task requires the animal to shift between stimuli dimensions to successfully retrieve the food reward. The ability to extract knowledge from different stimuli dimensions suggests that the mouse is capable of using at least some aspects of higher-order cognitive functions seen in human executive functioning (Chudasama, 2011). Numerous different transgenic mouse models of AD have shown deficits in set-shifting tasks (Zhuo et al., 2007, 2008; Marchese et al., 2013).

Reversal learning is another way that aspects of executive function are modeled in the mouse. While less complex than attentional set shifting, reversal learning does require both cognitive flexibility and impulse control, thus tapping into components of human executive function (Chudasama, 2011; Stopford et al.,

2012). There are many different variations of reversal learning tasks in mouse behavior, but they all work on the same principle. The animal first learns that a particular response to a stimulus will be rewarded, while a response to a different stimulus will be unrewarded. Then the stimulus-reward is switched so that the previously unrewarded stimulus becomes the rewarded stimulus. The animal must learn to reverse responses in order to receive the reward. Wild type control mice are able to quickly adjust their response in order to obtain the reward. However, animals with prefrontal cortex lesions display profound deficits in reversal learning (Chudasama, 2011; Izquierdo and Jentsch, 2012). Similarly, many different AD mouse models have shown impairment in reversal learning (Angelo et al., 2003; Dong et al., 2005; Filali et al., 2012; Cheng et al., 2013; Musilli et al., 2013; Papadopoulos et al., 2013).

Another aspect of executive function that is modeled in mice is response inhibition. Response inhibition is required for the appropriate control of an individual's behavioral actions in response to a stimulus (Robbins, 1996; Humby et al., 1999; Perry and Hodges, 1999; Romberg et al., 2013a). The five choice serial reaction time task (5-CSRTT) is a behavioral task that measures the response inhibition component of executive function (5-CSRTT is also used to model aspects of attention, see below section) in mice (Robbins, 2002; Bari et al., 2008; Chudasama, 2011; Romberg et al., 2013a). The 5-CSRTT can test two different aspects of response inhibition: (1) a failure to withhold the impulsive urge to respond while anticipating correct response (premature responses) and (2) a failure to disengage from repeating past correct responses (perseveration responses) (Chudasama, 2011). Several mouse models of AD have shown deficits in response inhibition using the 5-CSRTT (Romberg et al., 2011, 2013b).

MODELING ATTENTION

Several behavioral tasks have been developed for modeling attention in mice that provide reliable measures comparable to the neuropsychological assessments used in AD. The most widely used of these tasks is the 5-CSRTT (Muir, 1996; Humby et al., 1999; Robbins, 2002; De Bruin et al., 2006; Gibson et al., 2006; Lambourne et al., 2007; Pattij et al., 2007). This task employs an operant box with nose poke holes on the front wall of the chamber. Animals are trained to respond to brief flashes of light corresponding to five different spatial locations on this front side of the chamber and correct responses are rewarded with a food pellet released to a feeder box at the rear of the chamber. Touchscreen versions of this task are also available in which the nose poke holes and stimulus lights are replaced with an LCD screen (Romberg et al., 2011; Bussey et al., 2012). For both standard and touchscreen versions of the task, multiple trials are run each day and both the duration of the stimulus itself or the interval between the stimulus and response can be manipulated to increase the attention demands placed on the animal. Sustained attention is measured by examining when the animal responds to a different (incorrect) hole than where the stimulus light appeared (called errors of commission), fails to respond within the allotted time to the stimulus (errors of omission), and the speed with which the animal responds (reaction time). Aspects of selective attention can also be modeled with the 5-CSRTT by introducing brief bursts of white noise that the animal must ignore while still detecting the visual stimulus as it is presented (Robbins, 2002; Bari et al., 2008). The rodent 5-CSRTT is analogous to Leonard's 5-CSRTT used in humans (Wilkinson, 1963). Both tasks require subjects (mouse and human respectively) to utilize sustained attention divided among multiple spatial locations across which a large number of trials and errors of commission, omission, and reaction time are scored. Another task that can be considered somewhat analogous to the 5-CSRTT is the human Continuous Performance Tests (CPT) of sustained attention (Beck et al., 1956). In this task, the subjects are asked to respond to signal and non-signal events across numerous trials, and scores of hits, misses, rejections, and false alarms are recorded. Errors of commission in the 5-CSRTT are thought to be analogous to CPT false alarms rates. Similarly, errors of omission in the 5-CSRTT are thought to be analogous to CPT misses. AD mice such as the 3×Tg-AD mice have been shown to have deficits in sustained attention using the 5-CSRTT (Romberg et al., 2011). However, the homology between mouse and human versions of these tasks is far from perfect, and caution should be used when drawing conclusions from the rodent 5-CSRTT and applying them to human attention (Young et al., 2009).

MODELING EPISODIC MEMORY

Episodic memory refers to the ability to encode and recall personal past events and experiences. Episodic memory has also been referred to as the "what, when, and where" aspect of a particular experience. Modeling AD-related deficits in episodic memory in mice is a less well-explored area than that of other aspects of working memory, executive function, or attention. Historically, episodic memory was thought to be unique to humans (Tulving and Markowitsch, 1998). However, work over the past few decades on episodic-like memory across a number of animal species has suggested otherwise, and several mouse behavioral tasks designed at assessing episodic-like memory have been developed (Clayton and Dickinson, 1998; Clayton et al., 2001; Griffiths and Clayton, 2001; Morris, 2001; Davis et al., 2013a,b). One such task is the What-Where-Which Task (WWWhich). This task is an adaptation of the NOR task. While the NOR task itself is too simplistic a task to be considered a true episodic memory task (rather it is considered a task of recognition memory), the WWWhich task is able to model episodic-like memory. In the WWWhich task, the animal must integrate the location of a particular object with specific contextual cues to form an episodic-like memory (Davis et al., 2013a,b). Several studies employing the WWWhich task have observed performance deficits related to the aging process and to AD disease state in several transgenic mouse lines (Davis et al., 2013a,b). While the WWWhich task models episodic memory in the mouse, it is not very comparable to any of the episodic memory tasks commonly used in neuropsychological testing for AD. This is largely because the human tasks rely on language as a foundational construct for assessment. Obviously, there exists no such component in the WWWhich task for mice. Therefore, caution should be used when attempting to correlate any preclinical finding concerning episodic memory in mice to that of human cognition.

SUMMARY OF MOUSE BEHAVIORAL TESTS

All of the rodent behavioral tasks discussed in this section have been specifically developed to assess deficits in cognitive domains related to what is seen in human AD. Just as multiple neuropsychological tests assessing different cognitive domains are often used clinically to provide a global cognitive profile, so multiple behavioral tasks assessing different cognitive domains should ideally be used when characterizing the profile of AD-related cognitive impairment in a particular mouse model of AD.

TEMPORAL PROGRESSION OF THE COGNITIVE DEFICITS SEEN IN THE COMMONLY USED AD MOUSE MODELS

Cognitive decline is a defining feature of AD, and many mouse models have been developed that recapitulate aspects of the cognitive impairments seen in AD (Elder et al., 2010; Hochgrafe et al., 2013; Platt et al., 2013). Although no one animal model fully replicates the progression of cognitive impairments seen in the human disease, AD mouse models have been invaluable in advancing our knowledge of the disease. It should be noted that most of the AD mouse models are representative of the familial form of AD (FAD) which accounts for only a small percentage of the total AD cases each year (Campion et al., 1999). In addition, the contributions of both background strain and likely overexpression of mutant human APP genes on brain development and function must always be considered with regard to observed cognitive deficits in the various AD mouse models. Each transgenic mouse model of AD provides different insights into aspects of AD pathogenesis and the cognitive deficits associated with the disease. A generalized time course of the development of cognitive deficits across the various mouse models is depicted in **Figure 1B**. For each specific mouse model the temporal time course and progression of cognitive deficits in each cognitive domain can be different. In addition, in some models, cognitive deficits can be detected prior to the appearance of significant neuropathology. Careful forethought is therefore required in the selection of an optimal model displaying the AD related cognitive deficits desired based on the specific research interests of the investigator. An overview of the progressive cognitive deficits and the time of appearance of amyloid pathology is presented in **Table 3** for five mouse models that contain amyloid precursor protein (APP) mutations and in **Table 4** for five other common mouse models that contain APP and presenilin (PS1) mutations, or APP/PS1/Tau mutations. These tables are by no means an all-encompassing list of mouse models; rather they are simply meant to be examples of some of the commonly used mouse models of AD that are characterized by APP mutations. For recent reviews of additional AD mouse strains not included here, see (Ashe and Zahs, 2010; Elder et al., 2010; Epis et al., 2010; Hall and Roberson, 2012; Platt et al., 2013).

PDAPP

(Promoter: Platelet-Derived (PDGF) Promoter, Symbol: Tg (APPV717F) 109Ili, MGI ID: 2151935)

The PDAPP mouse was first described by Games in 1995 and is considered one of the earliest mouse models of AD (Chartier-Harlin et al., 1991; Games et al., 1995). In this model, the cognitive deficits first present themselves in spatial working memory at 4 months of age when assessed by MWM testing (Hartman et al., 2005). These deficits in working memory in the MWM are present throughout the rest of the life span for this model (Chen et al., 2000; Daumas et al., 2008). Deficits in recognition memory appear to develop after the working memory deficits in this model, as the first reported deficits in the NOR task are at 6 months old (Dodart et al., 1999). The cognitive deficits in recognition memory do not appear as robust as those in working memory in this model as there are inconsistent reports in the literature (Dodart et al., 1999, 2002; Chen et al., 2000). The cognitive defects in this model appear to present themselves before the appearance of plaque deposition (first appear at approximately 6 months) or other gross amyloid pathologies (Games et al., 1995; Hsiao et al., 1996; Schenk et al., 1999; Chen et al., 2000; Morgan, 2003).

TG2576

(Promoter: Hamster PrP Promoter, Symbol: Tg (APPSWE) 2576Kha, MGI ID: 2385631)

In the Tg2576 mouse (Hsiao et al., 1996), the first presentation of cognitive deficits is seen at 5 months of age in spatial working memory (Arendash et al., 2004). These spatial working memory deficits are generally accepted to be present across the rest of the life span for this model (Hsiao et al., 1996; Westerman et al., 2002; Arendash et al., 2004). However, methodology used to assess spatial memory appears to be very important, as several different reports have failed to observe these same deficits at various ages (Hsiao et al., 1996; Arendash et al., 2001a; King and Arendash, 2002). Non-spatial working memory tasks show a similar time course progression, first appearing at 3–5 months of age and persisting across the lifespan (Hsiao et al., 1996; Chapman et al., 1999; King and Arendash, 2002; Lalonde et al., 2003; Ohno et al., 2004). Deficits in recognition memory do not appear until much later, first appearing at 12 months of age in the NOR task (Oules et al., 2012; Yassine et al., 2013).

APP23

(Promoter: Thy-1, Symbol: Tg (Thy1-APP) 3Somm, MGI ID: 2447146)

The APP23 mouse model was reported in 1997 (Sturchler-Pierrat et al., 1997). In this model, the cognitive deficits begin to first appear in both recognition memory and spatial working memory at 3 months of age. The deficits appear to be progressive with age, and at 12 months old the animals also show cognitive deficits in a reference memory version of the Barnes maze (Prut et al., 2007). This model develops non-spatial working memory deficits very late in the progression of the disease (only after 19 months of age) (Lalonde et al., 2002; Dumont et al., 2004). Interestingly, cognitive performance in passive avoidance memory tasks follows the same progression as non-spatial working memory deficits in this model, unimpaired at 15 months of age and then developing deficits between 19 and 20 months of age (Kelly et al., 2003).

TgCRND8

(Promoter: PrP, Symbol: Tg (PRNP-APPSweInd) 8Dwst, MGI ID: 3589475)

The TgCRND8 model, described by Chishti et al. (2001), exhibits early cognitive impairment that spans across multiple cognitive domains (Chishti et al., 2001). TgCRND8 are impaired on spatial

Table 3 | Progression of cognitive deficits in APP mouse models of AD.

Strain	Passive avoidance	Fear Conditioning	Y/T-Maze Alternation	NOR	RAWM	MWM	Barnes Maze	Age in Months
PDAPP: [1]					[2]	[2]		1-2m
				[3]		[4]		3-5m
				[3] [5]	[6]			6-8m*
				[3]		[4]		9-11m
				[6]		[6]		12-14m
					[2]	[4]		15-18m
				[6]		[6]		≥19m
Tg2576: [7]		[8]	[9]					1-2m
	[10]	[8]	[10] [7]		[11]	[12]		3-5m
			[13] [11]	[14]	[12] [11]	[15] [11]	[14]	6-8m
	[10]	[8]	[7, 9]			[7] [10]		9-11m*
	[10]	[16]		[17]		[15] [10]		12-14m
		[18]	[16] [11]	[17] [14]	[19]	[15]	[14]	15-18m
	[10]		[10]			[15] [10]		≥19m
APP23: [20]	[21]					[21]		1-2m
	[21]			[22]		[21]		3-5m
	[21]			[23]		[21]		6-8m*
								9-11m
							[24]	12-14m
	[25]		[26]			[26]		15-18m
	[25]		[27]			[25]		≥19m
TgCRND8: [28]			[29]					1-2m
		[30]		[31-33]		[28]	[32]	3-5m*
		[30]	[29]		[34]	[35]		6-8m
			[29]			[36]		9-11m
		[30]				[36]		12-14m
								15-18m
								≥19m
J20: [37]				[38]				1-2m
		[39]		[38] [40]	[41]	[42]		3-5m
		[39]		[43]	[44]	[45]		6-8m*
			[46]	[47]	[48]	[46]		9-11m
		[49]	[49]	[49]	[44]	[50]		12-14m
								15-18m
					[44]			≥19m

Data not found | Same as control | Increased from control | Decreased from control

The strain is presented in the left column and age is presented in the right column. Black cells represent impairment, light gray cells represent no impairment compared to controls, dark gray cells represent increases from control (very few such cases), and white cells represent time points where no data are available for the respective behavior. The asterisk appearing in the age column represents when diffuse amyloid plaques are first observable in the brain for that particular mouse model of AD. The numbers within each cell correspond to the following references: 1: (Games et al., 1995), 2: (Nilsson et al., 2004), 3: (Dodart et al., 1999), 4: (Hartman et al., 2005), 5: (Dodart et al., 2002), 6: (Chen et al., 2000), 7: (Hsiao et al., 1996), 8: (Dineley et al., 2002), 9: (Chapman et al., 1999), 10: (King et al., 1999), 11: (Arendash et al., 2001a), 12: (Arendash et al., 2004), 13: (Ohno et al., 2004), 14: (Yassine et al., 2013), 15: (Westerman et al., 2002), 16: (Corcoran et al., 2002), 17: (Oules et al., 2012), 18: (Lassalle et al., 2008), 19: (Morgan et al., 2000), 20: (Sturchler-Pierrat et al., 1997), 21: (Van Dam et al., 2003), 22: (Huang et al., 2006), 23: (Heneka et al., 2006), 24: (Prut et al., 2007), 25: (Kelly et al., 2003), 26: (Lalonde et al., 2002), 27: (Dumont et al., 2004), 28: (Chishti et al., 2001), 29: (Hyde et al., 2005), 30: (Hanna et al., 2012), 31:(Ambree et al., 2009), 32: (Gortz et al., 2008), 33:(Richter et al., 2008), 34: (Lovasic et al., 2005), 35: (Janus, 2004), 36: (Hanna et al., 2009), 37: (Mucke et al., 2000), 38: (Harris et al., 2010), 39: (Saura et al., 2005), 40: (Simon et al., 2009), 41: (Lustbader et al., 2004), 42: (Cheng et al., 2007), 43: (Cisse et al., 2011), 44: (Du et al., 2011), 45: (Palop et al., 2003), 46: (Murakami et al., 2011), 47: (Escribano et al., 2009), 48: (Fang et al., 2012), 49: (Karl et al., 2012), 50: (Galvan et al., 2006).

Table 4 | Progression of memory deficits in other mouse models of AD (APP + PS1/Tau).

Strain	Passive avoidance	Fear Conditioning	Y/T -Maze Alternation	Novel Object Recognition	RAWM	MWM	Barn es Maze	Age in Months
APP/PS1: [1]								1-2m
					[2]			3-5m*
		[3] [4]	[5, 6]		[7]	[6]	[4]	6-8m
		[3]			[8]	[3]		9-11m
	[9]	[10]			[2]	[11]		12-14m
						[11]	[12]	15-18m
								≥19m
APP + PS1: [13]		[14]				[15]		1-2m
		[14]	[13, 16]		[17]	[16]		3-5m
					[15] [16]	[15] [13]		6-8m*
		[14]				[13]		9-11m
				[18]				12-14m
			[16, 17]		[19]	[17]		15-18m
					[20]	[21]		≥19m
APP/PS1KI: [22]								1-2m
								3-5m
				[23]	[23]			6-8m*
				[23]	[23]			9-11m
								12-14m
				[23]	[23]			15-18m
				[23]	[23]			≥19m
5xFAD: [24]			[24]					1-2m*
		[25]	[24] [26]	[26]		[25]		3-5m
		[27]	[26, 28]	[18]		[25]		6-8m
		[27]	[26]			[29]		9-11m
		[27]	[26, 28]					12-14m
								15-18m
								≥19m
3xT-gAD: [30]				[31]		[31]		1-2m
	[31]		[32]			[31] [33]		3-5m*
		[34]	[35]	[31]		[34]		6-8m
	[35]		[32]	[31]		[36]		9-11m
	[37]		[32]	[37, 38]		[33]	[39]	12-14m
				[40]		[36]		15-18m
								≥19m

Data not found | Same as control | Increased from control | Decreased from control

The strain is presented in the left column and age is presented in the right column. Black cells represent impairment, light gray cells represent no impairment compared to controls, dark gray cells represent increases from control (very few such cases), and white cells represent time points where no data are available for the respective behavior. The asterisk appearing in the age column represents when diffuse amyloid plaques are first observable in the brain for that particular mouse model of AD. The numbers within each cell correspond to the following references: 1: (Jankowsky et al., 2001), 2: (Park et al., 2006), 3: (Cramer et al., 2012), 4: (Reiserer et al., 2007), 5:(Cao et al., 2007) 6:(Lalonde et al., 2004), 7: (Volianskis et al., 2010), 8: (Sood et al., 2007), 9: (Zhang et al., 2011), 10: (Knafo et al., 2009), 11: (Lalonde et al., 2005), 12: (O'leary and Brown, 2009), 13: (Holcomb et al., 1999), 14: (Dineley et al., 2002), 15: (Trinchese et al., 2004), 16: (Arendash et al., 2001a), 17: (Arendash et al., 2001b), 18: (Tohda et al., 2012), 19: (Morgan et al., 2000), 20: (Wilcock et al., 2004), 21: (Sadowski et al., 2004), 22: (Flood et al., 2002), 23: (Webster et al., 2013), 24: (Oakley et al., 2006), 25: (Ohno et al., 2006), 26: (Shukla et al., 2013), 27: (Devi and Ohno, 2010), 28:(Devi and Ohno, 2012), 29: (Urano and Tohda, 2010), 30: (Oddo et al., 2003), 31: (Clinton et al., 2007), 32: (Carroll et al., 2007), 33: (Gimenez-Llort et al., 2007), 34: (Billings et al., 2005), 35: (Nelson et al., 2007b), 36: (Pietropaolo et al., 2008), 37: (Filali et al., 2012), 38: (Arsenault et al., 2011), 39: (Stewart et al., 2011), 40: (Halagappa et al., 2007).

working memory tasks starting at 3 months of age. These deficits are seen in the MWM and progress with age of the animal (Janus et al., 2000; Chishti et al., 2001; Gortz et al., 2008; Richter et al., 2008; Ambree et al., 2009). Reference memory deficits via Barnes maze testing are also present at 3 months of age (Gortz et al., 2008; Richter et al., 2008; Ambree et al., 2009). Similar to this observed temporal time course of spatial working memory and reference memory deficits are the development of deficits in both recognition memory and fear conditioning (Gortz et al., 2008; Richter et al., 2008; Ambree et al., 2009; Hanna et al., 2012). Deficits in alternation tasks develop by 6 and 9 months in Y-maze and T-maze alternation tasks, respectively (Hyde et al., 2005).

J20

(Promoter: Platelet-Derived (PDGF), Symbol: Tg (PDGFB-APPSwInd) 20Lms, MGI ID: 3057148)

The J20 mouse model was developed by (Mucke et al., 2000).This model is unique in that the first presented cognitive deficits are observed very early (at 1–2 months of age) in recognition memory (Harris et al., 2010). These deficits in recognition memory are present when assessed at several other time points (Escribano et al., 2009; Simon et al., 2009; Cisse et al., 2011). However, they do not appear to progress with the age of the animal and there has even been a report of no recognition memory deficits in old animals in advanced stages of the disease (Karl et al., 2012). Early memory deficits can also be observed in spatial working memory at 3 months of age when assessed by the MWM and the RAWM tasks (Lustbader et al., 2004; Cheng et al., 2007; Meilandt et al., 2008). These spatial working memory deficits are present across the rest of this model's lifespan (Palop et al., 2003; Galvan et al., 2006; Cisse et al., 2011; Du et al., 2011; Murakami et al., 2011; Fang et al., 2012). Fear conditioning deficits appear consistent with the presentation of spatial working memory and recognition memory deficits in this model (Saura et al., 2005). Interestingly, this model does not appear to display working memory deficits on tasks of alternation such as the Y-maze (Murakami et al., 2011; Karl et al., 2012).

APP/PS1

(Promoter: PrP, Symbol: Tg (APPswe, PSEN1de9) 85Dbo, MGI ID: 3524957)

The cognitive deficits in the APP/PS1 mouse model, first described by Jankowsky et al. (2001), have been well characterized. Cognitive deficits are first seen at 3 months of age in the RAWM spatial working memory task and are also reported by 6 months of age in the MWM (Cao et al., 2007; Ding et al., 2008). Further, these deficits have been well characterized across the lifespan of this mouse model in water based spatial working memory tasks (Lalonde et al., 2005; Park et al., 2006; Cao et al., 2007; Sood et al., 2007; Ding et al., 2008; Volianskis et al., 2010; Zhang et al., 2011; Cramer et al., 2012; Ma et al., 2012). Impairments in reference memory develop by 6 months and persist through the rest of the life of this model (Reiserer et al., 2007; Bernardo et al., 2009; O'leary and Brown, 2009). Deficits in associative learning have also been described in fear conditioning tasks starting at 6–8 months of age (Knafo et al., 2009; Cramer et al., 2012). Similarly, passive avoidance deficits have also been described at 12 months of age (Zhang et al., 2011). No deficits were seen in alternation tasks of working memory for this model (Lalonde et al., 2004; Cao et al., 2007).

APP + PS1

(Promoter: Hamster PrP Promoter, Symbol: Tg (APPSWE) 2576kha, MGI ID: 2385631) × (Platelet-Derived (PDGF), Symbol: Tg (PDGFB-PSEN1M146L) 2Jhd, MGI ID: 2447326)

Holcomb described a mouse model in 1998 that has been widely used to study cognitive deficits related to AD (Holcomb et al., 1998). The first observable deficits in this model are shown in associative learning and present themselves between 4 and 5 months of age (Dineley et al., 2002). The progression of the spatial working memory impairment in this model is relatively slow compared to most other models. The first reported impairment in spatial working memory was observed using 6-month-old animals (Trinchese et al., 2004). However, these cognitive deficits are not robust at this age, as others have observed no such deficit (Holcomb et al., 1999; Arendash et al., 2001a). By 15 months of age the spatial working memory is consistently impaired throughout the rest of the life span (Morgan et al., 2000; Arendash et al., 2001b; Gordon et al., 2001; Sadowski et al., 2004; Wilcock et al., 2004). Similarly, deficits in recognition memory occur later in this model, first observed at 12 months of age (Mori et al., 2013). No deficits were observed in alternation tasks of working memory (Holcomb et al., 1998; Arendash et al., 2001a).

APP/PS1 KI

(Promoter: Endogenous, Symbol: Apptm1.1Cep, MGI ID: 2652346) × (Promoter: Endogenous, Symbol: Psen1tm1Dgf, MGI ID: 3608968)

The APP/PS1 knock-in mouse model (first described in Flood et al., 2002) uses endogenous promoters to drive the expression of humanized amyloid beta sequence, and AD-like pathology and cognitive deficits develop in the absence of APP or PS1 overexpression (Flood et al., 2002). The earliest reports of cognitive deficits are reported at 7 months in this model (Bruce-Keller et al., 2011). However, the majority of cognitive deficits appear later. We have shown previously that the cognitive deficits in spatial working memory (assessed by RAWM testing) first appear at 9 months of age (Webster et al., 2013). These deficits are followed by impairments in associative memory (appearing by 14 months of age Thibault et al., 2012) and in recognition memory (not developing until 15 months of age Webster et al., 2013).

5×FAD

(Promoter: Thy-1, Symbol: Tg (APPSwFlLon, PSEN1*M146L* L286V) 6799Vas, MGI ID: 3693208)

The 5×FAD model, first described by Oakley et al. (2006), develops progressive cognitive deficits with age. This model develops cognitive deficits by 3 months of age in spatial working memory (Ohno et al., 2006; Urano and Tohda, 2010). These working memory deficits are followed temporally with the development of associative learning impairment in fear conditioning (Ohno et al., 2006; Devi and Ohno, 2010) as well as the development of deficits in a working memory version of the Y-maze (Oakley et al., 2006; Devi and Ohno, 2012; Shukla et al., 2013). As with several of the other models (PDAPP, Tg2576, APP/PS1, and APP/PS1 KI) this model develops deficits in recognition memory

later than the observed deficits in spatial working memory (Tohda et al., 2012).

3×Tg-AD

(Promoter: Thy-1, Symbol: Tg (APPSwe,tauP301L) 1Lfa, MGI ID: 2672831) × (Promoter: Endogenous, Symbol: Psen1tm1Mpm, MGI ID: 1930937)

The 3×Tg-AD mouse model, developed by Oddo et al. (2003), shows progressive cognitive impairments starting at a young age. The first deficits observed in this model are those of associative learning deficits, which begin between 3 and 5 months of age. These are then followed by deficits in spatial working memory at 6 months of age in the MWM task. Both Y-maze alternation and contextual fear conditioning impairment follow a similar temporal time course. Then deficits in recognition memory present themselves between 9 and 11 months of age. Finally, reference memory impairment in the Barnes maze task is observed at 12 months of age.

TEMPORAL PROGRESSION OF AD-LIKE NON-COGNITIVE BEHAVIORAL ABNORMALITIES SEEN IN THE COMMONLY USED MOUSE MODELS

While most AD research has focused on the neurobiological mechanisms underlying the cognitive deficits seen in AD pathogenesis, there is a wide range of non-cognitive neuropsychiatric symptoms also associated with the disease. Indeed, these non-cognitive symptoms are seen as a very important concern among the family members of the patients and caregivers alike (Tan et al., 2005). These non-cognitive symptoms are often more difficult to deal with, as they compose important sources of distress and psychological burden on the family members/caregivers and can drastically affect the quality of life of patients by leading to institutionalization (Hope et al., 1998; Shin et al., 2005). Non-cognitive neuropsychological symptoms associated with AD include activity disturbances, affective disturbances, aggression, stereotypic behavior, circadian rhythm disturbances, and anxiety (Ancoli-Israel et al., 1989; Okawa et al., 1991; Vitiello et al., 1992; Bliwise, 1994; Satlin et al., 1995; Van Someren et al., 1996; Hope et al., 1998; Harper et al., 2004; Shin et al., 2005; Tan et al., 2005). While there has been less emphasis placed on the modeling of these non-cognitive neuropsychological disturbances in murine models of AD, several of the commonly used mouse models of AD do show a number of these disruptions. Non-cognitive symptoms associated with AD shown in the mouse include increased locomotor activity (Dodart et al., 1999; King et al., 1999; Arendash et al., 2001b; Dumont et al., 2004; Hyde et al., 2005; Cheng et al., 2007; Gil-Bea et al., 2007; Pietropaolo et al., 2008; Sanchez-Mejia et al., 2008; Ambree et al., 2009; Cisse et al., 2011; Mori et al., 2013), anxiety (Moechars et al., 1996, 1999; Lalonde et al., 2003, 2004; Gil-Bea et al., 2007; Reiserer et al., 2007; Lassalle et al., 2008; Espana et al., 2010; Bedrosian et al., 2011; Cisse et al., 2011; Murakami et al., 2011; Filali et al., 2012), circadian disturbances (Huitron-Resendiz et al., 2002; Vloeberghs et al., 2004; Wisor et al., 2005; Ambree et al., 2006; Sterniczuk et al., 2010; Bedrosian et al., 2011), and increased aggression (Moechars et al., 1996, 1998; Van Dorpe et al., 2000; Ambree et al., 2006; Vloeberghs et al., 2006; Pugh et al., 2007; Alexander et al., 2011).

LOCOMOTOR ACTIVITY

Most of the commonly used AD mouse models exhibit increased locomotor activity (Dodart et al., 1999; King et al., 1999; Arendash et al., 2001b; Dumont et al., 2004; Hyde et al., 2005; Cheng et al., 2007; Gil-Bea et al., 2007; Pietropaolo et al., 2008; Sanchez-Mejia et al., 2008; Ambree et al., 2009; Cisse et al., 2011; Mori et al., 2013). These disturbances include hyperactivity, stereotypic behaviors, and home cage activity disturbances (Dodart et al., 1999; Janus and Westaway, 2001; Auld et al., 2002; Dumont et al., 2004; Hyde et al., 2005; Cheng et al., 2007; Gil-Bea et al., 2007; Gimenez-Llort et al., 2007; Pietropaolo et al., 2008; Cisse et al., 2011) and have been linked to altered APP metabolism, amyloid levels, and disease progression (Van Someren et al., 1996; Harper et al., 2004). These activity disturbances do not seem to be constant, but rather present themselves with more severity at different times of the day. For example, in the TgCRND8 mice, the deficits seem most severe near the end of their wake cycle and less severe at other times of the day (Ambree et al., 2006). Further, these disturbances increase with the age of the animals and with the severity of the disease. Likewise, in the APP23 mice there have been reports of increased activity in the second half of the nocturnal phase (end of the active phase of the wake cycle) (Vloeberghs et al., 2004). These increased activity disturbances at the end of the activity cycle have been suggested to be similar to the exacerbation of activity behavioral symptoms observed in human AD patients late in the afternoon and evening time termed sundowning syndrome (Vitiello et al., 1992; Bliwise, 1994). Other AD mouse models that show activity disturbances are the PDAPP, TG2576, J20, APP + PS1 [Tg2576 + PS1 (M146L)], and 3×Tg-AD mouse models of AD. The onset of the disturbances is different for each model, with the J20 and the TgCRND8 mice developing disruptions earliest (approximately 1 month of age), followed by PDAPP and TG2576 (approximately 3 months of age), and the PS1 [Tg2576 + PS1 (M146L)], 3×TgAD, and APP23 mice developing last (approximately 6–9 months of age) (Dodart et al., 1999; King et al., 1999; Arendash et al., 2001b; Dumont et al., 2004; Hyde et al., 2005; Cheng et al., 2007; Gil-Bea et al., 2007; Gimenez-Llort et al., 2007; Pietropaolo et al., 2008; Sanchez-Mejia et al., 2008; Ambree et al., 2009; Harris et al., 2010; Cisse et al., 2011; Mori et al., 2013).

CIRCADIAN RHYTHM AND SLEEP DISRUPTIONS

Similar to the reported activity disturbances, circadian rhythm disruptions are also observed in many of the AD mouse models (Huitron-Resendiz et al., 2002; Vloeberghs et al., 2004; Wisor et al., 2005; Ambree et al., 2006; Sterniczuk et al., 2010; Bedrosian et al., 2011). Circadian rhythm disturbances have also been well described in human AD patients (Ancoli-Israel et al., 1989; Okawa et al., 1991; Vitiello et al., 1992; Van Someren et al., 1996; Auld et al., 2002; Harper et al., 2004). These disturbances are characterized by the AD patient's propensity to frequently awaken during the nighttime and to increase the amount of time slept during the day (Ancoli-Israel et al., 1989; Okawa et al., 1991). While this behavior is in itself a non-cognitive behavior, it has been reported to possibly have important effects on patient cognition (Smith, 1985; Graves et al., 2001). Several mouse models of AD also display sleep disruption. Other similarities between the

sleep disturbances seen clinically and those seen in the AD mouse models are: (1) as the severity of the disease progresses, the worse the sleep disturbances become (Smith, 1985; Graves et al., 2001; Huitron-Resendiz et al., 2002, 2005) and (2) the greater the sleep disruptions are, the more severe the cognitive decline seems to be (Huitron-Resendiz et al., 2002, 2005).

ANXIETY DISTURBANCES

Anxiety disturbances have been reported in many of the AD mouse models (Moechars et al., 1996, 1999; Lalonde et al., 2003, 2004; Gil-Bea et al., 2007; Reiserer et al., 2007; Lassalle et al., 2008; Espana et al., 2010; Bedrosian et al., 2011; Cisse et al., 2011; Murakami et al., 2011; Filali et al., 2012). The prevailing thought is that these anxiolytic-like behaviors stem from disinhibitory tendencies resulting from the underlying AD pathology (Lalonde et al., 2003; Ognibene et al., 2005). Both APP function (as these anxiolytic behaviors are more common in APP transgenic mice) (Moechars et al., 1996, 1999; Lalonde et al., 2003, 2004; Lassalle et al., 2008; Murakami et al., 2011; Filali et al., 2012) and disruption of the cholinergic system (Apelt et al., 2002; Klingner et al., 2003; Luth et al., 2003) because of its well-known role in behavioral inhibition (and disruption in several of the AD mouse lines) have been proposed as underlying causes of this behavioral abnormality. The temporal time course of these anxiety-like disturbances can vary depending on the mouse model. For example, in some models the behavioral disturbances start early at 1–2 months of age or 3–6 months of age for the J20 and the APP/PS1 models, respectively (Lalonde et al., 2004; Reiserer et al., 2007; Harris et al., 2010). Other models such as the TG2576 develop the anxiety-like disturbances later at the age of 9–11 months of age (Gil-Bea et al., 2007). It is also important to note that not all murine AD models exhibit anxiety disturbances (Arendash et al., 2001b; Lalonde et al., 2002; Webster et al., 2013). Still, the majority of models do display these disturbances and the prevailing thought is that this behavioral phenotype of disinhibition may be akin to the disinhibition seen in AD patients (exemplified by unacceptable behavior and inappropriate euphoria) (Daffner et al., 1992; Chung and Cummings, 2000).

AGGRESSIVE BEHAVIORS

Increased aggressive behaviors are another common behavioral symptom of AD and present themselves in as much as 65% of AD patients (Burns et al., 1990b). As with other non-cognitive behavioral symptoms of AD, increased aggression can be emotionally stressful to both the patient and caregivers (Murman et al., 2002a,b). The exact mechanism that underlies these increased aggressive behaviors is not known but proposed mechanisms deal with dysregulation of different neurotransmitter systems such as serotonin, norepinephrine, dopamine, and GABA (Arsland, 1995; Meltzer et al., 1998). Many AD mouse models also display increased aggressive behavior (Moechars et al., 1996, 1998; Van Dorpe et al., 2000; Ambree et al., 2006; Vloeberghs et al., 2006; Pugh et al., 2007; Alexander et al., 2011). For example, in the TG2576 mouse increased aggressive behaviors display themselves in both the frequency of attacks on other home cage mice as well as on the latency to first attack when interacting with a novel mouse (Alexander et al., 2011). The APP23 mouse model of AD also shows increased aggressive behaviors (Vloeberghs et al., 2006). These aggressive disruptions appear to develop later than the onset of cognitive deficits in this model (Kelly et al., 2003; Van Dam et al., 2003; Vloeberghs et al., 2006). The aggression alterations in the APP23 model appear by 6 months of age (after amyloid pathology and behavioral deficits) and seem to remain relatively constant throughout the rest of the course of disease (Vloeberghs et al., 2006). This suggests that perhaps aggressive deficits correlate best with moderate to severe stages of the disease, which is also what is observed clinically in human AD (Senanarong et al., 2004).

DEPRESSIVE SYMPTOMS

Depressive symptoms/behaviors are a very common comorbidity with AD. The exact prevalence of this comorbidity is not known but is believed to range from as low as 2% to as high as 85% (variability likely due to methods of assessment, diagnostic criteria, stage of AD participants, and other factors) (Mendez et al., 1990; Burns et al., 1990a; Migliorelli et al., 1995a,b; Devanand et al., 1996; Cummings, 2000; Apostolova and Cummings, 2008). Numerous meta-analysis studies have linked depression and AD (Chen et al., 1999; Charlson and Peterson, 2002; Ownby et al., 2006; Lenoir et al., 2011; Diniz et al., 2013), and several even consider late-life depression a significant risk factor for future development of AD (Butters et al., 2000, 2008; Diniz et al., 2013). Generally, depressive symptoms precede the onset of AD (Devanand et al., 1996) and usually worsen with the progression of the disease (Mega et al., 1996). Similarly, patients with a history of depression prior to a diagnosis of AD are much more likely to experience depressive episodes in the course of AD (Pearlson et al., 1990; Strauss and Ogrocki, 1996). Despite this well documented connection between depression and AD and having a wide range of potentially applicable tools to study depression in animal models (Seligman et al., 1975; Jolly et al., 1999; Song and Leonard, 2005; Flint and Shifman, 2008; Nestler and Hyman, 2010), very little work has been devoted to determining the range of depressive behavioral symptoms in the commonly used mouse models of AD. Depressive-like behaviors have been reported in at least one mouse model of AD (Filali et al., 2009), and likely exist in many of the other commonly used models.

CONCLUSIONS

No one animal model fully replicates the pathogenesis of AD, but rather only model different aspects of the disease. Consequently, no one model recapitulates all of the cognitive deficits observed in human AD. Further, the anatomical makeup and cognitive ability of mice make it difficult to model all of the intricacies of higher-order cognitive function exclusive to humans. Instead, each mouse model allows us insight into different aspects of cognition related to AD. Several important points should be taken away from the preceding discussion of the temporal development of cognitive deficits in the various mouse models of AD. First, the temporal time course and progression of cognitive deficits in a specific cognitive domain/behavioral task can be quite different among the different mouse models. Investigators should use careful forethought in selection of an optimal model and planning experiments based on the progression of that model's

specific deficits. Secondly, most models display deficits in spatial working memory earlier than the deficits in other cognitive domains. Similarly, most of the studies using mouse models of AD have focused on understanding/correcting the cognitive deficits associated with the disease. However, AD is not just a memory disorder, rather it is a complex disease with many different non-cognitive neuropsychiatric symptoms which are an important source of distress and a psychological burden on family members and caregivers alike. These non-cognitive symptoms are present across many of the different mouse models of AD and more emphasis should be placed on understanding/correcting these deficits, as well as the cognitive aspects of the disease. It is our hope that this comprehensive review of the spectrum of behavioral deficits present in commonly used AD mouse models and how well they model human cognitive and non-cognitive symptoms will assist investigators in selecting an appropriate mouse model to investigate different aspects of AD pathology and disease progression.

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Aging and chronic administration of serotonin-selective reuptake inhibitor citalopram upregulate Sirt4 gene expression in the preoptic area of male mice

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Sexual dysfunction and cognitive deficits are markers of the aging process. Mammalian sirtuins (SIRT), encoded by *sirt 1-7* genes, are known as aging molecules which are sensitive to serotonin (5-hydroxytryptamine, 5-HT). Whether the 5-HT system regulates SIRT in the preoptic area (POA), which could affect reproduction and cognition has not been examined. Therefore, this study was designed to examine the effects of citalopram (CIT, 10 mg/kg for 4 weeks), a potent selective-serotonin reuptake inhibitor and aging on SIRT expression in the POA of male mice using real-time PCR and immunocytochemistry. Age-related increases of *sirt1, sirt4, sirt5, and sirt7* mRNA levels were observed in the POA of 52 weeks old mice. Furthermore, 4 weeks of chronic CIT treatment started at 8 weeks of age also increased *sirt*2 and *sirt*4 mRNA expression in the POA. Moreover, the number of SIRT4 immuno-reactive neurons increased with aging in the medial septum area (12 weeks = 1.00 ± 0.15 vs. 36 weeks = 1.68 ± 0.14 vs. 52 weeks = 1.54 ± 0.11, $p < 0.05$). In contrast, the number of sirt4-immunopositive cells did not show a statistically significant change with CIT treatment, suggesting that the increase in sirt4 mRNA levels may occur in cells in which *sirt4* is already being expressed. Taken together, these studies suggest that CIT treatment and the process of aging utilize the serotonergic system to up-regulate SIRT4 in the POA as a common pathway to deregulate social cognitive and reproductive functions.

Keywords: serotonin, aging, reproduction, sirtuins, cognition

Introduction

Aging of the central nervous system deregulates homeostatic mechanisms responsible for sexual behavior (Davidson et al., 1983), feeding (Weindruch et al., 2001), sleep (Nakamura et al., 2011) and cognition (Barrientos et al., 2012). Sexual dysfunction and cognitive loss are prominent markers of the aging process. The preoptic area (POA) is involved in the hypothalamic-pituitary-gonadal (HPG) axis for the control of reproduction (Larsson and Heimer, 1964). Gonadotropin-releasing hormone (GnRH) is a pivotal molecule synthesize by neurons in the POA that regulates the release of gonadotropins (LH, luteinizing hormone and FSH, follicular stimulating hormone) that are important for reproduction and reproductive behaviors (Tsutsumi and Webster, 2009). The POA including the GnRH neurons receive serotonergic (5-hydroxytrypramine, 5-HT) innervations

(Van De Kar and Lorens, 1979; Jennes et al., 1982). Pharmacological manipulations and lesions of the serotonergic system has a negative tone on reproduction (Verma et al., 1989; Kondo and Yamanouchi, 1997; Olivier et al., 2011) and cognitive function (Sibille et al., 2007). During aging, sex steroid deprivation shifts the homeostasis of the HPG axis which results in increase circulating LH and GnRH levels (Chakravarti et al., 1976). Furthermore, increases in LH are associated with decline in cognitive performance (Casadesus et al., 2007). Moreover, an age-related decline in the serotonergic system also leads to cognitive dysfunction (Meltzer et al., 1998). The POA is involved in social cognition (Driessen et al., 2014). Hence, to understand the mechanism of reproductive aging and cognitive loss, it is important to examine the serotonergic system in the POA during aging.

The family of seven sirtuin (SIRT) proteins is involved in the aging mechanism, which may include reproductive aging (Duan, 2013). Sirt activity is governed by its co-activator, nicotinamide adenine dinucleotide (*nad*), inhibitor nicotinamide (*nam*) and the intermediary conversion enzyme nicotinamide mononucleotide adenylyltransferase (*nmnat-1*) (Denu, 2005). All seven SIRT proteins are expressed in the brain (Dali-Youcef et al., 2007). SIRT proteins are involved in energy balance, reproduction and in brain aging (Duan, 2013). SIRT4 controls glutamate metabolism through glutamate dehydrogenase (Haigis et al., 2006), overexpression of which alters synaptic activity similar to serotonin-depleted models (Michaelis et al., 2011). In addition, serotonin1b (5-HT1b) receptor knockout mice, up-regulate *sirt5* in adult male mice, causing early onset of brain aging (Sibille et al., 2007).

Whether the 5-HT system regulates SIRT in the POA, which could affect the HPG axis, reproduction and cognition has not been examined. Treatment with citalopram (CIT), a potent selective-serotonin reuptake inhibitor, shows deficits in sexual behavior in adult mice (Soga et al., 2010) and sexual dysfunction in humans(Montejo et al., 2001), a condition that mimics aging, which has decreased 5-HT synthesis (Hussain and Mitra, 2000). CIT is extremely selective for its transporter, biosynthetic enzymes and receptors and is used pharmacologically to increase endogenous 5-HT levels at the synapse, although, chronic treatment decreases 5-HT synthesis (Moret and Briley, 1996; Bezchlibnyk-Butler et al., 2000; Stenfors et al., 2001). Therefore, this study was design to examine the effect of CIT and aging on *sirt* mRNA and SIRT expression in the POA of male mice using real-time PCR and immunocytochemistry respectively.

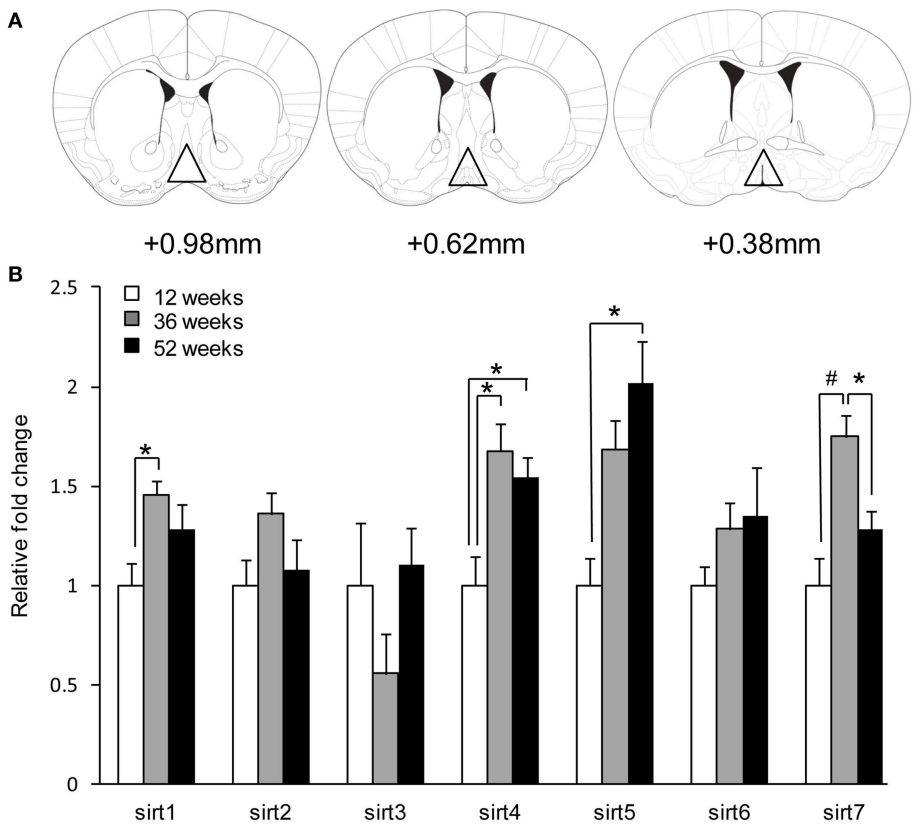

FIGURE 1 | Aging induces *sirt* gene expression in the preoptic area. (A) Representative brain sections depicting areas dissected for gene expression studies in the POA. **(B)** Quantitative real-time PCR revealed differential *sirt* expression changes. Data are expressed as mean ± SEM. Statistical analysis was carried out using one-way analysis of variance (12 weeks control, $n = 9$; 36 weeks, $n = 8$; 52 weeks, $n = 10$). $^{*}P < 0.05$ and $^{\#}P < 0.01$ vs. control.

Materials and Methods

Animals

Male C57BL/6N mice (CLEA Japan, Inc Tokyo, Japan) aged 12 weeks (weeks) ($n = 33$), 36 weeks ($n = 8$), and 52 weeks ($n = 16$) were maintained under standard conditions at the animal facility of the Brain Research Institute, Monash University Malaysia. These conditions include constant temperature (22°C) and lighting (12 h light/12 h dark cycle with lights on from 12:00 a.m.) with food and water available *ad libitum*. All procedures were approved by Animal Ethics Committee of Monash University (SOBSB/MY/2010/45) and were accordance with the Guidelines for the Care and Use of Animals by Monash University.

Chronic Citalopram Treatment

Mice were administered with CIT [10 mg/kg body weight (BW) in 50 μl, C7861, Sigma-Aldrich, Singapore; $n = 15$] or 50 μl vehicle (distilled water; $n = 15$) daily at 9 a.m. by intraperitoneal (i.p) injections for 4 weeks beginning at 8 weeks of age until 12 weeks of age. Mice were used for two studies; gene expression study and immunocytochemical study. For the gene expression study [12 weeks (vehicle), $n = 9$ and 12 weeks CIT, $n = 9$] using the POA and compared with gene expressions of intact aged mice (36 weeks, $n = 8$ and 52 weeks, $n = 10$). For the immunocytochemical study, the POA of adult mice (12 weeks, $n = 6$, 12 weeks CIT, $n = 6$) and mid-age group (52 weeks, $n = 6$) was used. In this experiment, 52 weeks C57BL/6 male mice represented the reproductive aging model. This strain of mice at 52 weeks begin to exhibit declining male fertility (Fox et al., 2006) characterized by an increase in abnormal spermatozoa leading to ejaculatory disorders (Fabricant and Parkening, 1982), decrease in pheromone production (Wilson and Harrison, 1983), and a decrease in sexual arousal (Bronson and Desjardins, 1982). Additionally, at 52 weeks old these mice begin to experience age-related cognitive decline (Pettan-Brewer et al., 2013).

Real-time PCR Quantification of *sirt1-7*, *nam* and *nmnat-1* in the POA

At various ages (12weeks, 12weeks CIT, 36weeks, and 52weeks), animals were deeply anaethesized with an i.p. injection of ketamine xylazine (4.5 mg/kg/BW) followed by rapid removal of the brain and snap frozen. The POA (bregma +0.98 to +0.26, 8–11 sections/brain) was cut on a cryostat (60 μm/section) and each section further dissected with a sterile blade under naked eye (**Figure 1A**). Total RNA from these tissues was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and transcribed using High Capacity Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocols. Quantitative real-time PCR was performed on a ABI 7300 (Applied Biosystems Foster City, CA, USA) using 2X Power SYBR Green PCR mix (Applied Biosystems), and 0.2M primers for *sirt1-7*, *nam,* and *nmnat-1* (**Supplementary Table 1**) in a final volume of 10 μl. The resulting PCR products were validated using an ABI PRISM 310 Genetic Analyzer and Sequence Analysis Software (Applied Biosystems) and ran on a 2.5% agarose gel with ethidium bromide used for visualization.

SIRT4 Immunocytochemistry

Male mice at 12 weeks (control, $n = 6$; 12 weeks CIT, $n = 6$) and 52 weeks of age (control, $n = 6$) were anaesthesized with an i.p. injection of ketamine xylazine (4.5 mg/kg) and perfused transcardially with 4% paraformaldehyde (PFA) in

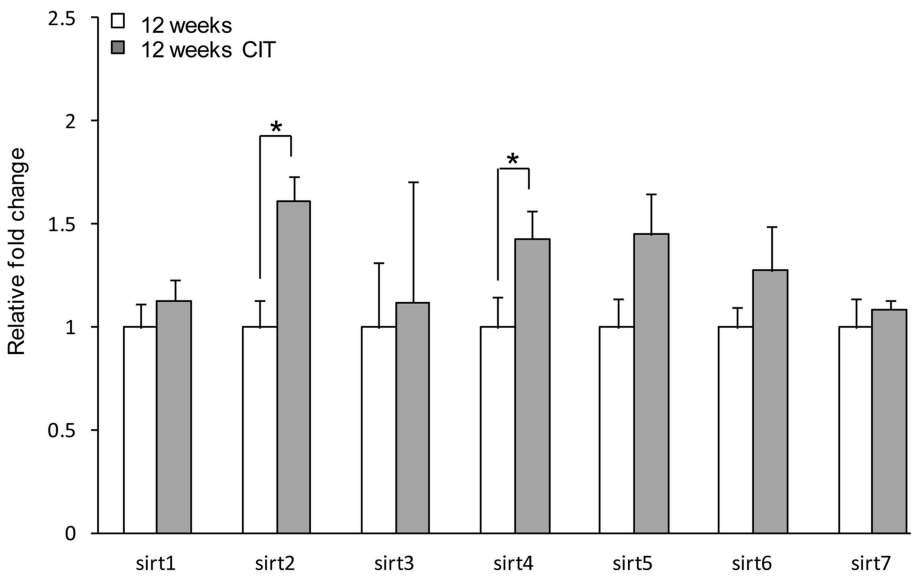

FIGURE 2 | Chronic citalopram (CIT) treatment up-regulates *sirt2* and *sirt4* in the preoptic area. CIT was administered at 10 mg/kg/bodyweight daily in male mice for a duration of 4 weeks leading to relative mRNA changes in *sirt2* and *sirt4* expression in the POA. There was no difference in sirt3, sirt5, sirt6, or sirt7 mRNA levels in the POA after CIT treatment. Data are expressed as mean ± SEM. Statistical analysis was carried out using one-way analysis of variance (12 weeks control, $n = 9$; 12 weeks CIT, $n = 9$). *$P < 0.05$ vs. control.

0.1M phosphate buffer (pH 7.5). The brains were removed, postfixed for 4 h in 4% PFA, and cryoprotected in 30% sucrose overnight. The POA (bregma +0.98 to +0.26 mm) was then sectioned at 30 μm in the coronal plane into three equal series. A set of sections was incubated for 20 min at 60°C in sodium citrate (pH 6) to unmask antigen epitopes. Prior to incubation in blocking solution (0.25% bovine serum albumin and 1.0% Triton X in 0.1M PBS) for 1 h with tissues were washed twice in 0.1M phosphate buffer saline (PBS) for 10 min each. Subsequently, the same 0.1M PBS wash was conducted on tissues prior to every incubation step. Sections were then incubated for 48 h at 4°C in a polyclonal goat anti-SIRT4 antibody (ab10140, Abcam, USA) at 1:500 dilution in blocking solution. Next, tissues were incubated with biotinylated rabbit anti-goat (Vector Laboratories, Burlingame, CA, USA) at 1:300 dilution in blocking solution for 1 h. Following this, sections were incubated in avidin-peroxidase (1:100 Vector Laboratories) and immunoreactive signal was observed using nickel-enhanced 3,3′-diaminobenzidine hydrochloride (Sigma). Sections were thoroughly washed in PBS and mounted on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA), air-dried, dehydrated in ethanol followed by xylene. Finally, the slides were coverslipped with DPX mounting medium.

Double-label Immunofluorescence

Coronal POA sections (30 μm) from male mice at 12 weeks ($n = 3$) were used for double immunohistochemistry with NeuN or GFAP and antisera to SIRT4 as described above. Tissue sections were incubated in Alexa Fluor 488 Anti-Goat (1:200, A11055, Molecular Probes) for 1 h and then incubated with either polyclonal rabbit antibody against GFAP (1:500, G9269, Sigma) or mouse monoclonal antibody against NeuN (1:500, MAB377, Millipore-Chemicon, Billerica, MA, USA) for 24 h. Sections incubated with antisera to GFAP were incubated with Alexa Fluor 594 Anti-Rabbit (1:200, A11012, Molecular Probes) while those with antisera to NeuN were incubated with Alexa Fluor 594 Anti-Mouse (1:200, A11005, Molecular Probes). After a final 0.1M PBS wash, sections were mounted on SuperFrost plus slides (Fisher Scientific) and coverslipped with VECTASHIELD mounting medium (H-1000, Vector Laboratories).

Absorption Test and SIRT4 Antibody Specificity

The SIRT4 antibody employed in this study recognizes amino acids 302–314 at the C-terminal. For testing antibody specificity, two procedures were carried out. Firstly, an absorption test was carried out using intact 12 weeks mice POA sections ($n = 3$) at 1:500 SIRT4 antisera pre-absorbed overnight with SIRT4 protein (AB23185; amino acids 302–314, 1 μg/ml, Abcam) in immunohistochemical procedures. Secondly, the primary SIRT4 antibody was omitted from the primary incubation solution. Both pre-absorbed and omission of SIRT4 antisera did not produce any immunoreactive staining.

SIRT4 Immunoreactive Analysis

POA sections were viewed using bright-field microscopy (Nikon Eclipse 50i) and images were captured in TIF format (Nikon, Tokyo, Japan).The distribution of SIRT4 immunoreactivity was mapped throughout the mouse forebrain and brainstem. Cell counts for SIRT4 immunoreactive cells in the POA of 12 weeks, 12 weeks CIT treated, and 52 weeks (90 μm apart) were carried out using Image Pro Plus (Media Cybernetics Incorporation, Bethesda, USA). The POA consisting of the medial septum (MS), organum vasculosum of the lamina terminalis (OVLT) and the anterior hypothalamic area (AHA) were defined as per unit area of 500,000 pixels, 400,000 pixels, and 50,000 pixels respectively using Image Pro Plus. For each animal, two anatomically matched tissues per area were captured and used for cell counts. Cell counts were carried out by a researcher blind to the treatment and age. A single SIRT4 immunostained cell in a single focal plane was quantified by Image Pro Plus as 120 pixels. Therefore, any clusters of immunoreactive cells quantified by Image Pro Plus were divided by 120 pixels to obtain cell number counts, only immunostained cells with a full size (120 pixels) nuclei were counted in each image of the POA to make adjustments for double counts, in order to obtain true SIRT4 positive cell numbers. Data is expressed as mean number of identifiable SIRT4-immunoreactive cells.

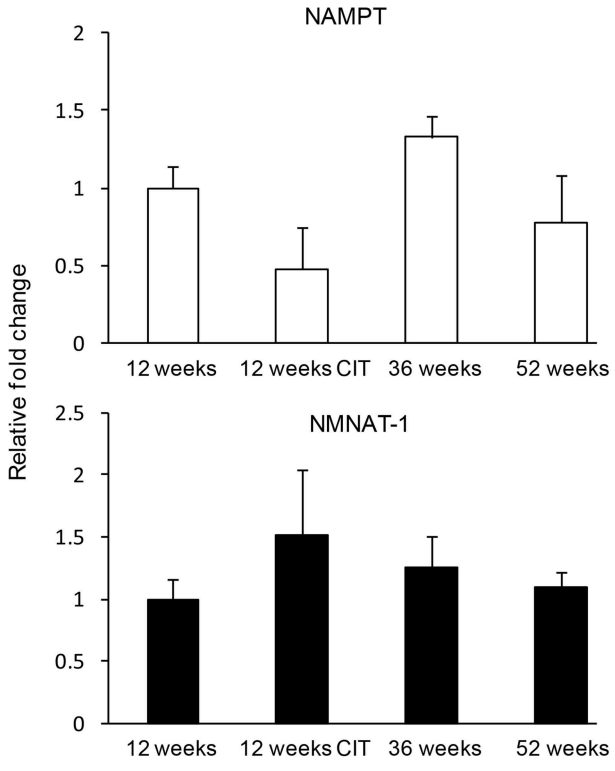

FIGURE 3 | Nicotinamide adenine dinucleotide (NAD) biosynthethic enzymes were not affected by chronic citalopram (CIT) treatment or aging. CIT was administered at 10 mg/kg/bodyweight daily in male mice for 4 weeks duration. Data are expressed as mean ± SEM. Statistical analysis was carried out using one way analysis of variance (12 weeks control, $n = 9$; 12 weeks CIT, $n = 9$) aging animals used were 36 weeks ($n = 8$) and 52 weeks ($n = 10$).

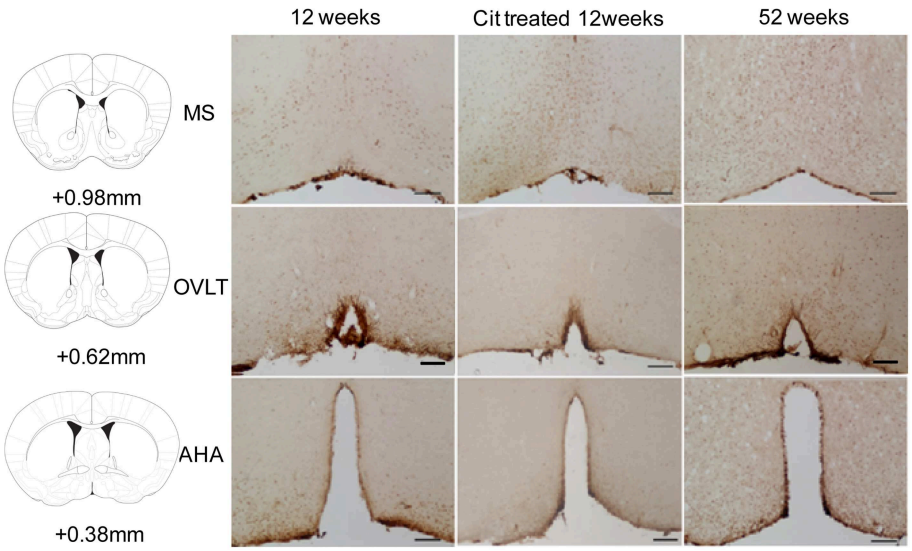

FIGURE 4 | Representative images of SIRT4 immunoreactivity in the medial septum (MS), organum vasculosum of the lamina terminalis (OVLT) and anterior hypothalamic area (AHA) from control, citalopram (CIT) treated and mid-aged samples (52 weeks). Scale bar = 100 μm.

Double-label Immunofluorescence Analysis

The co-localization of NeuN or GFAP with SIRT4 immunoreactive cells was viewed with a 20X objective lens under a fluorescent microscope (Nikon Eclipse 90i, Tokyo, Japan). Sections were viewed using a Texas red filter to observe NeuN and GFAP labeled cells while a fluorescein-isothiocyanate filter was used to observe SIRT4 immunolabeled cells. Images of co-localized cells, SIRT4/NeuN and SIRT4/GFAP, were further captured using a laser scanning confocal microscope (C1si, Nikon) and software (NIS Elements AR v4.0, Nikon). The images were captured at 1024 pixel density using a 20X objective and 3X digital zoom function at every 1.25 μm interval to cover the entire neuronal volume.

Statistical Analysis

Statistical analysis for the effect of age and treatment (CIT) on sirtuin and NAD was carried out using one-way Analysis of variance (ANOVA) PASW statistic software (Version 17.0 Chicago, IL USA), followed with *post-hoc* analysis, Tukey's test, for comparison of multiple age groups. The effect of CIT was further analyzed using unpaired Student's *t*-test. Significant main interactions from One-Way ANOVA were further analyzed using Student's *t*-test. Statistical analysis for the effect of age and CIT on SIRT4 protein expression was carried out using Two-Way ANOVA using age and region (MS, OVLT and AHA) as factors followed by *T*-test to determine significance. Data are presented as means ± SEM. Significant difference was considered when $p < 0.05$.

Results

Effects of Aging on *sirt* Expression in the POA

Aging did not alter *sirt2*, *sirt3,* and *sirt6* gene expression in the POA of male mice. There was an increase in *sirt4* and *sirt5* mRNA expression in the POA during aging [*sirt4*; 12 weeks 1.00 ± 0.15 vs. 36 weeks 1.68 ± 0.14 vs. 52 weeks 1.54 ± 0.11, $F_{(2,\ 27)}$= 7.20, $p < 0.01$] and [*sirt5*; 1.00 ± 0.14 vs. 36 weeks 1.69 ± 0.15 vs. 52 weeks 2.02 ± 0.21, $F_{(2,\ 27)}$= 10.40, $p < 0.01$] (**Figure 1B**). *Post-hoc* analysis revealed an increase in *sirt4* (12 weeks vs. 52 weeks; $p < 0.05$) and *sirt5* (12 weeks vs. 52 weeks; $p < 0.05$). *sirt1* and *sirt7* showed an increase only during 36 week [sirt1; 12 weeks 1.00 ± 0.11 vs. 36 weeks 1.46 ± 0.07 vs. 52 weeks 1.28 ± 0.12, $F_{(2,\ 27)} = 4.93$, $p < 0.05$ and *sirt7;* 12 weeks 1.00 ± 0.14 vs. 36 weeks 1.75 ± 0.11 vs. 52 weeks 1.28 ± 0.09, $F_{(2,\ 27)} = 11.26$, $p < 0.01$] (**Figure 1B**). *Post-hoc* analysis for *sirt7* revealed a decrease at 52 weeks compared to 36 weeks of age (36 weeks vs. 52 weeks; $p < 0.05$).

Effects of CIT on *sirt* Expression in the POA

Chronic CIT treatment induced an increase in *sirt2* (12 weeks 1.00 ± 0.13 vs. 12 weeks CIT 1.61 ± 0.12, $p < 0.05$) and *sirt4* (12 weeks 1.00 ± 0.15 vs. 12 weeks CIT 1.43 ± 0.13, $p < 0.05$) expression in the POA (**Figure 2**). There was no difference in *sirt3, sirt5, sirt6, and sirt7* levels in the POA after CIT treatment (**Figure 2**).

Effects of Aging and CIT on *nampt* and *nmnat-1* Expression in the POA

CIT and aging had no effect on *nam* and *nmnat-1* expression in the POA (**Figure 3**).

Effects of Aging and CIT on SIRT4 Protein in the POA

SIRT4 immunoreactive cells were observed in the MS, OVLT and AHA regions (**Figure 4**). We observed SIRT4 protein localized mainly in neurons compared to glial cells in the POA (**Figure 5A**). SIRT4 co-localization with glial cells were observed in areas close to the third ventricle of the preoptic area whereas

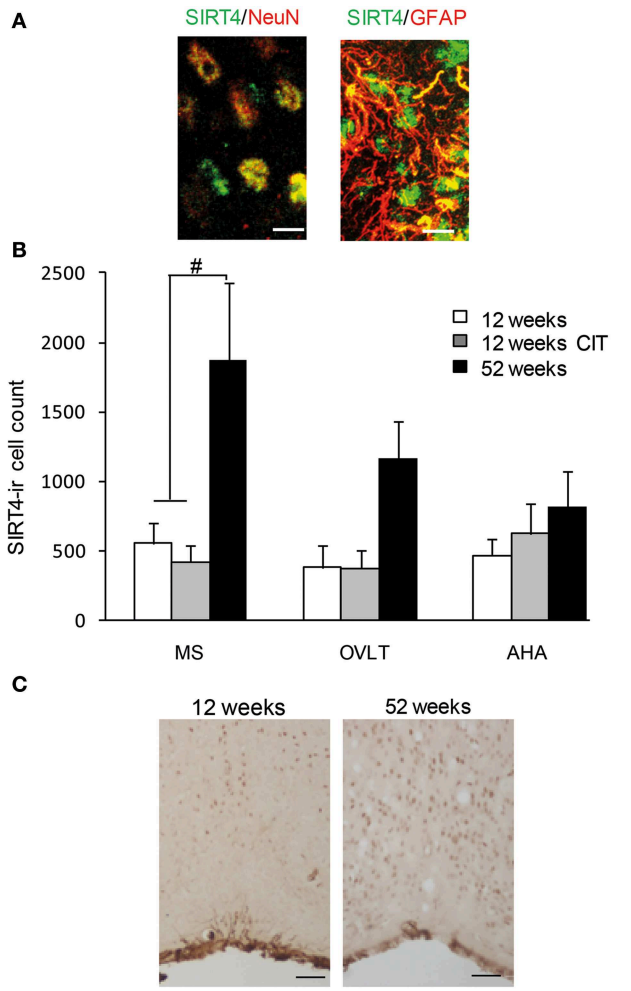

FIGURE 5 | Aging but not citalopram (CIT) induced SIRT4 immunoreactive cells in the medial septum (MS). (A) SIRT4 in the POA was observed to be highly co-localized with neuronal nuclei (NeuN) compared to glial fibrillary acidic protein (GFAP) Scale bar = 10 μm. **(B)** SIRT4 immunoreactive cell counts revealed up-regulation of SIRT4 during aging in the MS but not in the organum vasculosum of the lamina terminalis (OVLT) and anterior hypothalamic area (AHA). However, CIT treatment did not alter any SIRT4 immunoreactive cell counts. **(C)** High magnification image of SIRT4 staining in MS of 12 weeks and 52 weeks. Scale bar = 50 μm. Data are expressed as mean ± SEM. Statistical analysis was carried out using analysis of variance, $^{\#}P < 0.01$ vs. control.

the vast majority of SIRT4 immunoreactivity was observed in neurons. There was no immunostaining when the antibody was preadsorbed with its corresponding antigen or when the antibody was excluded.

There was an increase in SIRT4 immunoreactive cell counts in the MS of 52weeks aging male mice (12 weeks 421 ± 116, 12 weeks CIT 559 ± 146, 52 weeks 1521 ± 306) [Age effect in the MS, $F_{(1, 16)} = 16.98$, $p < 0.01$] and *T*-test revealed that SIRT4 immunoreactivity at 52 weeks was higher than 12 weeks ($p < 0.05$) and 12 weeks CIT treated mice ($p < 0.01$) (**Figures 5B,C**). CIT treatment did not alter SIRT4 immunoreactivity in the MS. Age and CIT treatment did not alter SIRT4 expression in the OVLT and AHA (**Figure 5B**).

Discussion

In this study, we observed an age-related up-regulation of *sirt1*, *sirt4*, *sirt5,* and *sirt7* gene expression in the POA. The expression patterns of *sirt1*, *sirt4*, *sirt5,* and *sirt7* gene were different at 36 and 52 weeks, which suggest that different regulatory factors might be involved in their control. Higher levels of *sirt4* and *sirt5* mRNA at 52 weeks may be linked with a decrease in 5-HT (Hussain and Mitra, 2000) and a decrease in testosterone levels (Eleftheriou and Lucas, 1974; De Marte and Enesco, 1986) which are observed during the aging process. In contrast to *sirt4* and *sirt5* expression pattern in the POA, the expression level of *sirt7* was higher in 36 weeks than in 52 weeks old animals. This could be due to an overall decrease in transcription activity in the brain at 52 weeks or due to hypothalamic neurodegeneration that begins at 36 weeks (Bourre and Piciotti, 1992). Since *sirt1* is an age induced gene (Duan, 2013), the increase expression of *sirt1* in the POA of 36 weeks old animals was not unexpected. *Sirt 1* gene expression may be regulated by site-specific modulation in the brain. As *sirt* 1 gene is a regulator of metabolic functions, centrally through the hypothalamus (Duan, 2013), up-regulation of sirt1 gene expression may be due to metabolic changes at 36 weeks in the POA.

CIT treatment up-regulated only *sirt2* and *sir4* gene in the POA. Sirt1 remained unchanged following CIT treatment as it does not respond to SSRI-class of antidepressant (Kishi et al., 2011). This suggests that the effect of CIT on *sirt1* might involve factors aside from 5-HT that change in the male HPG axis during aging (Veldhuis, 2008). CIT treatment may affect *sirt2* gene through 5-HT signaling in the POA, since *sirt2* gene up-regulation is also seen in patients during remission state of depression (Abe et al., 2011). On the other hand, *sirt4* gene expression is regulated by age and CIT in the POA. A decrease in 5-HT during aging (Hussain and Mitra, 2000) has been linked with neurodegenerative diseases (Glorioso et al., 2011), decreased gonadotropin release and cognitive loss (Alzheimer's disease) (Simpkins et al., 1977; Meltzer et al., 1998). Antidepressants like CIT and fluvoxamine cause sexual dysfunction in rodents (Montejo et al., 2001; Soga et al., 2010), and human (Waldinger et al., 2001). Recent studies have shown that chronic SSRI treatment could decrease 5-HT content and signaling in the brain (Delgado et al., 1990; Hervás and Artigas, 1998), rather than simply facilitating synaptic 5-HT availability (de Jong et al., 2006; Geddes et al., 2015). Therefore, age-related and CIT induced sexual dysfunction and cognitive loss due to the decline in 5-HT in the POA may be mediated by an up-regulation of *sirt4* gene expression.

We speculate that the up-regulation of sirt4 gene expression in the POA might occur through one or several potential mechanisms, such as: (1) the blockade of 5-HT uptake by chronic CIT treatment could decrease presynaptic 5-HT content or desensitize postsynaptic 5-HT receptors; (2) the aging process might be associated with a decrease in 5-HT synthesis or a decline in postsynaptic 5-HT receptors (**Figure 6**). According to

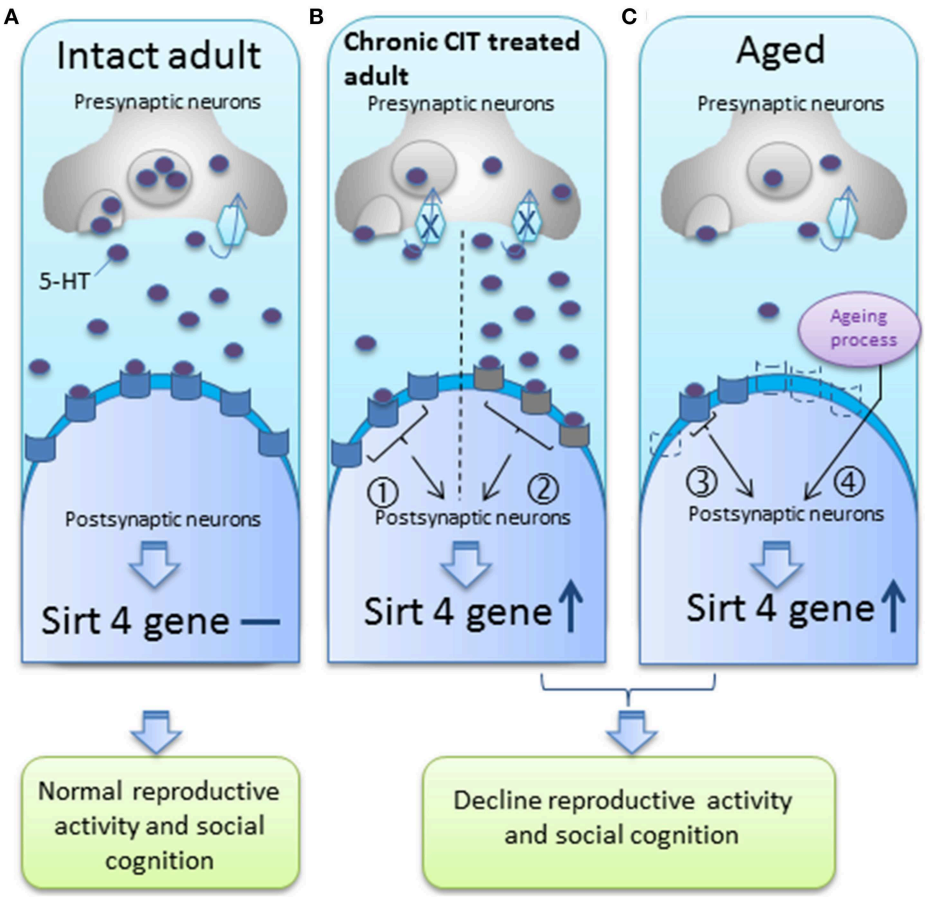

FIGURE 6 | A hypothetical diagram of sirt4 gene regulation by serotonin (5-HT) in the preoptic area (POA) of intact adult, chronic citalopram (CIT) treated adult and aged animals. (A) In the intact adult, sirt4 gene expression levels do not change; **(B)** in the chronic CIT treated adult, the inhibition of 5-HT re-uptake results in decreased presynaptic 5-HT content (pathway ①) or desensitization of postsynaptic receptors that could subsequently be less activated in the presence of normal, or even increased, synaptic 5-HT concentrations (pathway ②), either of which can result in decreased 5-HT neurotransmission and increased sirt4 gene expression; **(C)** in aged animals, the serotonergic system may be impaired by decreased 5-HT synthesis (pathway ③) or a decrease in postsynaptic 5-HT receptors (dotted receptor) (pathway ④) to explain sirt4 upregulation, or an unknown age related effect could increase sirt4 gene expression. These 5-HT signaling pathways could facilitate sirt4 gene expression in the POA leading to reproductive and cognitive failure. 5-HT receptors Desensitized 5-HT receptors Decrease 5-HT receptors 5-HT transporter re-uptake X Inhibition of re-uptake ↑ increase — No change in gene expression, ① decrease 5-HT neurotransmission, ② desensitized receptor, ③ decrease 5-HT neurotransmission, ④unknown age-related factors could activate sirt4 gene expression.

our model, any of these events could explain a change in sirt4 expression associated with a decrease in serotonergic tone.

The localization of SIRT4 primarily in adult neurons is similar to other members of the SIRT family; SIRT1 and SIRT2 in neurons (Houtkooper et al., 2012). The roles of SIRT1, 2 and 4 in neuronal and glial development have been reported (Prozorovski et al., 2008; Park et al., 2012; Komlos et al., 2013). SIRT4 might function in tandem with other SIRT during early development of glial cells and aging of neurons in the POA. Unlike the increase in *sirt4* mRNA, our failure to observe an increase in the number of SIRT4 immunostained cells in the POA following CIT treatment could be due to the methodology used to detect protein levels. Although, immunohistochemistry is an accepted semi-quantitative measure of protein levels; a subtle change in protein levels within cells could go undetected using immunocytochemistry and thereby result in unaltered SIRT4 cell numbers.

The POA is involved in social cognitive functions such as paternal behavior, social recognition and reproductive behavior (Ferguson et al., 2002). An age-related decline in cognitive and reproductive functions (Meltzer et al., 1998) might be associated with decline in the serotonergic system. The POA is known to project to brain regions important for cognitive functions such as the dorsal raphe that harbors 5-HT neurons and the

hippocampus (Sava and Markus, 2008). This suggests that the anatomical and functional connection between the POA and the hippocampus and the dorsal raphe might be involved in age-related cognitive impairment. Inversely, 5-HT neurons are known to project to GnRH neurons in the POA (Jennes et al., 1982). A decrease in 5-HT during aging could decrease GnRH levels through the activation of sirt, which could cause a decrease in LH receptors and GnRH receptors in the hippocampus, resulting in deregulation of the social cognitive functions.

Hence, we suggest that the age-related and CIT-induced activation of *sirt4* gene expression, might be initiated by the decline in 5-HT in the POA which leads to reproductive dysfunction and cognitive deficits.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fgene.2015.00281

Supplementary Table 1 | Sequences of primers used for real-time PCR.

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Using the zebrafish model for Alzheimer's disease research

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Rodent models have been extensively used to investigate the cause and mechanisms behind Alzheimer's disease. Despite many years of intensive research using these models we still lack a detailed understanding of the molecular events that lead to neurodegeneration. Although zebrafish lack the complexity of advanced cognitive behaviors evident in rodent models they have proven to be a very informative model for the study of human diseases. In this review we give an overview of how the zebrafish has been used to study Alzheimer's disease. Zebrafish possess genes orthologous to those mutated in familial Alzheimer's disease and research using zebrafish has revealed unique characteristics of these genes that have been difficult to observe in rodent models. The zebrafish is becoming an increasingly popular model for the investigation of Alzheimer's disease and will complement studies using other models to help complete our understanding of this disease.

Keywords: zebrafish, Alzheimer disease, presenilins, gamma-secretase, amyloid beta-protein precursor, MAPT

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most prevalent form of dementia. It was estimated in 2010 that 36 million people were living with AD and this figure is predicted to increase to 66 million by 2030 (World Alzheimer report's, 2010). The main clinical feature of AD is progressive memory loss. However, AD can also be characterized by the impairment of speech and motor ability, depression, delusions, hallucinations, and aggressive behavior (Voisin and Vellas, 2009). Despite these recognizable behavioral differences it is actually difficult during the early stages of the disease to correctly diagnose someone as having AD (Blennow et al., 2006). There is significant neuronal loss in several brain regions in AD patients (Regeur et al., 1994; West et al., 1994). This is usually accompanied by extracellular aggregates of the amyloid-beta peptide and intracellular aggregates of the tau protein called neurofibrillary tangles (NFTs).

Alzheimer's disease can be classed as familial (FAD, usually with onset <65 years) or sporadic (SAD, onset >65 years). We have acquired most of our knowledge on the pathogenesis of AD through studies of FAD mutations. FAD is attributed to mutations in three genes, *PRESENILIN 1* (*PSEN1*), *PRESENILIN 2* (*PSEN2*), and the *AMYLOID BETA A4 PRECURSOR PROTEIN* (*APP*). The PSEN proteins are essential for γ-secretase activity that cleaves transmembrane domain proteins within lipid bilayers (reviewed in McCarthy et al., 2009). The PSEN proteins and four other proteins comprise γ-secretase complexes. PSEN (1 or 2), nicastrin (NCT), anterior pharynx defective 1 (APH1a or APH1b), and presenilin enhancer 2 (PSENEN) form active γ-secretase complexes in cellular membranes. These complexes are responsible for the cleavage of a number of single-pass transmembrane proteins such as APP and NOTCH. APP is initially cleaved by α- or β-secretases to release APPα or APPβ fragments, respectively. The subsequent cleavage of APP by γ-secretase after β-secretase cleavage liberates amyloid-β (Aβ) peptides of various lengths. The longer Aβ-42 peptide is prone to aggregation and is suggested to form toxic oligomers and fibrils that eventually deposit as amyloid plaques in the brain. Deposits of plaques and NFTs are common occurrences in the brains of those with FAD.

The common, late onset form of AD (accounting for >90% of cases) occurs sporadically (Blennow et al., 2006). Sporadic AD (SAD) has a complex etiology and is associated with many risk factors including old-age and possession of the ε4 allele of the *APOLIPOPROTEIN E* (*APOE*) gene. Human genome wide association studies have revealed a number of possible loci associated with risk for SAD [reviewed by Rademakers and Rovelet-Lecrux, 2009). There are numerous other risk factors associated with an individual's lifestyle that can also increase the likelihood of developing AD. These overlap considerably with the risk factors for cardiovascular disease (Kotze and van Rensburg, 2012) including hypertension, hypercholesterolemia and obesity (Martins et al., 2006). The molecular and cellular triggers for the onset of SAD are yet to be completely resolved, however, there have been many hypotheses suggested to explain how AD is initiated. Of the many hypotheses that have been postulated over the years, some that are gaining momentum attribute AD to vasculature dysfunction (Stone, 2008; Marchesi, 2011), oxidative stress (Nunomura et al., 2001), mitochondrial dysfunction (Selfridge et al., 2012) or hypoxia (Oresic et al., 2011).

The common observation of amyloid plaques in AD brains led to the formulation of the amyloid hypothesis in 1992 (Hardy and Higgins, 1992) that continues to dominate research thinking as a unifying hypothesis for FAD and SAD. The hypothesis suggests that toxic oligomerization of Aβ peptides is the initiating factor that triggers a cascade of subsequent cellular abnormalities such as inflammation and oxidative stress (Verdile et al., 2004). These secondary phenomena ultimately lead to neuronal dysfunction, degeneration and death. Although still widely accepted, doubts have arisen regarding its validity following several failed

clinical trials of drugs intended to reduce Aβ levels (refer to www.alzforum.org regarding information on AD drugs in clinical trials).

Rodent models of AD have been exploited extensively and have given considerable insight into this disease. However, we still do not understand the events that trigger the neurodegeneration evident in AD. Research using the zebrafish model has revealed particular characteristics of the various genes implicated in AD that have been difficult to observe in other animal models (Liao et al., 2012; Newman et al., 2012). This review will focus on how the zebrafish has been used to study the genes and various cellular pathways implicated in FAD and SAD pathogenesis and will conclude by examining the limitations of the zebrafish and what the future may hold for use of this model in AD research.

THE ZEBRAFISH MODEL

The zebrafish are a small, hardy freshwater fish native to India and often kept in home aquaria. They were originally used as a model organism for the study of vertebrate development. However, over the last decade the zebrafish model has become increasingly employed for investigating a wide variety of human diseases (Lieschke and Currie, 2007). The zebrafish has a number of characteristics that make it a versatile animal model. Although they lack the advanced cognitive behaviors evident in rodent models, their transparent embryos, rapid development *ex utero* and large reproductive capacity (100+ embryos per spawning) provide obvious advantages over mammalian models. Furthermore, multiple genes can be manipulated simply and effectively in the zebrafish at physiologically relevant levels (Newman et al., 2012), which cannot be currently achieved in rodent models. While, in general, rodents more closely model human physiology than fish, zebrafish nevertheless are vertebrates and so are more relevant to understanding human biology than invertebrate models as *Drosophila melanogaster* and *Caenorhabditis elegans*. Zebrafish embryos are particularly manipulable due to their large size, ready availability and the ability to exploit changes in their development for assay of particular gene activities (Nornes et al., 2009). Thus, zebrafish embryos can often present a felicitous vertebrate system in which to examine the molecular and cellular functions of genes implicated in AD.

The zebrafish genome is extensively annotated. The zebrafish (teleost-bony fish) evolutionary lineage separated from the human (tetrapod) lineage approximately 450 million years ago (Kumar and Hedges, 1998). Teleosts appear to have undergone an additional round of genome duplication since their separation from the tetrapod lineage followed by loss of many of the duplicated genes (Catchen et al., 2011). However, in most cases, zebrafish genes can be identified that are clear orthologs of human genes. Zebrafish possess genes orthologous to the human genes that are thought to play essential roles in AD. The *psen1* (Leimer et al., 1999) and *psen2* (Groth et al., 2002) genes are orthologs of human *PSEN1* and *PSEN2*, respectively, while the *appa* and *appb* genes are "co-orthologs" of human *APP* (Musa et al., 2001). Zebrafish hold orthologous genes for the components of the gamma-secretase complex, *PSENEN* (*psenen*; Francis et al., 2002; Campbell et al., 2006), *NCTN* (*ncstn*; Strausberg et al., 2002) and *APH1b* (*aph1b*; Francis et al., 2002). While orthologs of β-secretase (*BACE1* and *BACE2*) have also recently been identified in zebrafish: *bace1* (Moussavi Nik et al., 2012) and *bace2* (van Bebber et al., 2013). The *microtubule-associated protein tau* (*MAPT*) gene in humans encodes the tau protein and our laboratory identified co-orthologs of this gene in zebrafish, *mapta*, and *maptb* (Chen et al., 2009). Zebrafish also possess co-orthologs of *APOE*: *apoea* and *apoeb* (Babin et al., 1997; Woods et al., 2005). Genes arising by duplication can have overlapping functions. This can be disadvantageous when analyzing gene function as particular loss-of-function phenotypes may be obscured unless the function of both duplicate genes is blocked. Alternatively, duplicate genes can have partially non-overlapping functions and this can facilitate functional analysis as loss-of-function phenotypes may be restricted to particular cells or tissues.

Zebrafish are an advantageous model for genetic and molecular studies. Zebrafish embryos are genetically malleable by injection of morpholino antisense oligonucleotides, mRNA, transgenes and more recently by genome engineering systems (reviewed in Hwang et al., 2013; Schmid and Haass, 2013; Hisano et al., 2014). These technologies can make both subtle and drastic changes in gene expression with the effects observed in the developing transparent embryos. Morpholinos are designed to bind to particular sites in transcripts from a gene of interest. Binding of a morpholino can either block mRNA translation (knockdown) or interfere with correct splicing of exons (e.g., Draper et al., 2001; Nornes et al., 2008; Berger et al., 2011). Injection of sense mRNA can allow overexpression of a particular gene of interest. The effects of morpholino and mRNA injection generally only persist during embryogenesis (2–3 days post-fertilization). Transgenic zebrafish can be generated using efficient vectors such as the Tol2 transposase system (Kawakami et al., 2000) to insert genes under the control of tissue specific promoters. Conditionally expressed transgenics can also be generated using the Cre/loxP (Hans et al., 2009) and GAL4-UAS (Halpern et al., 2008) systems for gene function analysis at particular time points. The absence of technology available to generate targeted mutations in the zebrafish genome was previously a disadvantage of the zebrafish model relative to rodents. In recent times however, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENS) and the type II prokaryotic CRISPR (clustered regularly interspaced short palindromic repeats)/Cas systems have been developed for targeted modification of gene sequences in the zebrafish genome (Hwang et al., 2013; Schmid and Haass, 2013).

A search of the available scientific literature in the PubMed database using the term "zebrafish + alzheimer's disease" revealed 119 publications with 98 being original research papers (23rd May, 2014). **Figure 1** displays the research areas in which these papers have been published. There is an obvious concentration of research on Aβ and Presenilin function in zebrafish.

USING ZEBRAFISH TO INVESTIGATE GENES MUTATED IN FAD

THE PRESENILINS

The genes implicated in FAD have been extensively studied over the last 25 years, although our understanding of their normal functions is still far from complete. The *PSEN* genes

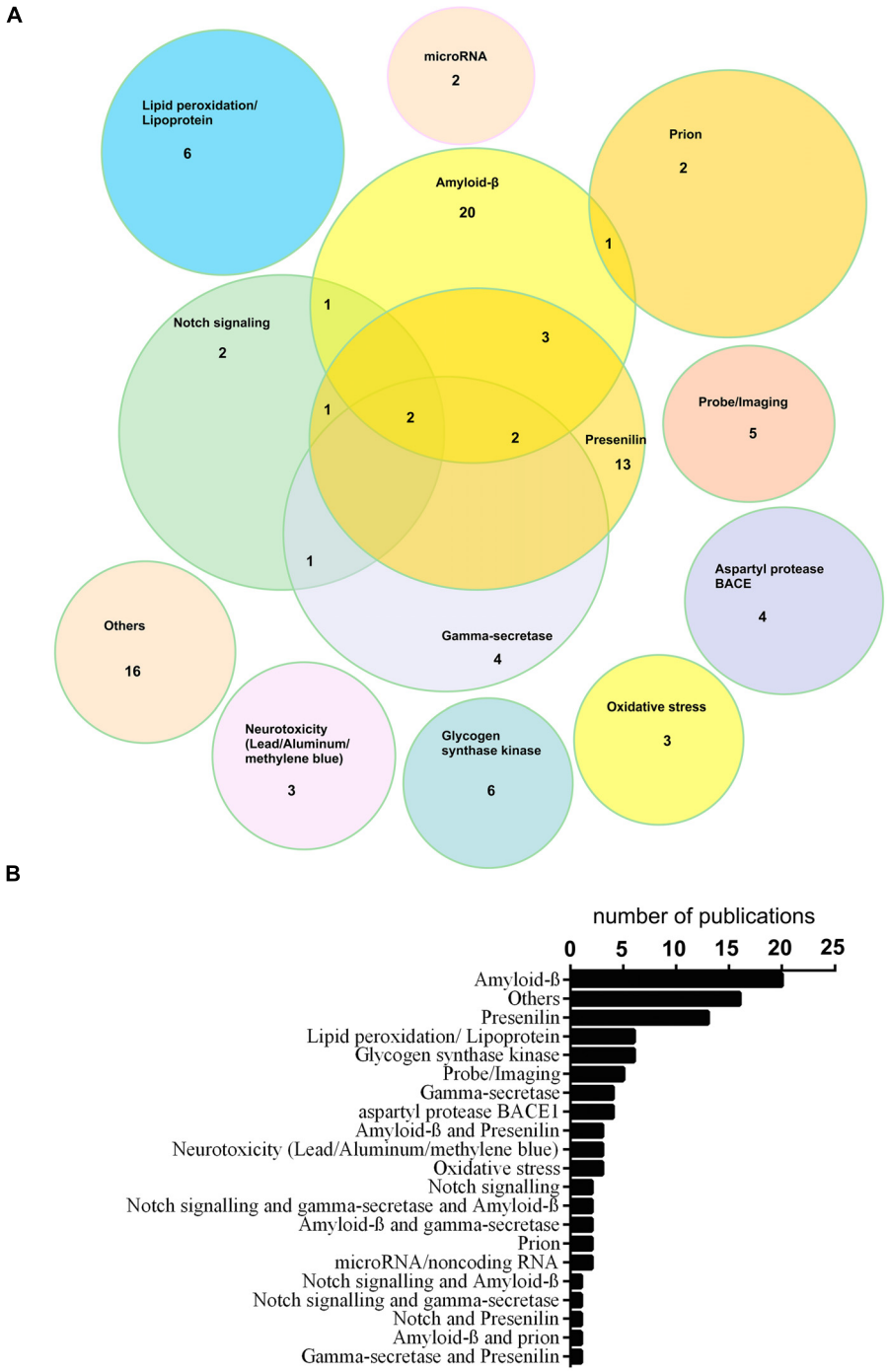

FIGURE 1 | Text mining analysis of the scientific literature to detect papers on the application of Zebrafish in Alzheimer's disease research. The PubMed database was mined for zebrafish and Alzheimer's disease using the search term, "zebrafish+alzheimer's disease," by the MedScan Reader 5 tool implemented in the Pathway Studio 9 Package (Nikitin et al., 2003b; Novichkova et al., 2003a). MedScan Reader 5 collects information and processes undertaken data using natural language processing (NLP). The language interprets these to logical concepts and extracts functional relationships between proteins, small molecules, and cellular processes (Nikitin et al., 2003b; Novichkova et al., 2003a). 119 publications were found. Abstracts of these publications were studied and review papers were removed such that 98 papers remained. **(A)** Venn diagram and **(B)** bar graph showing numbers of research papers in different areas. A concentration of research on Amyloidβ and Presenilin function is apparent.

play important roles in development. The zebrafish (as well-characterized model for studies of developmental biology) has been employed to investigate the normal functions of these genes. The *psen1* and *psen2* genes are ubiquitously expressed in zebrafish embryos (Leimer et al., 1999; Groth et al., 2002), implying a functional importance of these genes during development. Injection of morpholinos to knockdown *presenilin* gene expression revealed similar and dissimilar phenotypes compared to Presenilin knockout mice (*Psen1*$^{-/-}$). *Psen1*$^{-/-}$ mice die during development (Shen et al., 1997). To overcome this, conditional *Psen1* knockout mouse models have been developed (reviewed in van Tijn et al., 2011). Zebrafish embryos injected with a *psen1* translation-blocking morpholino are viable and have similar phenotypes (Nornes et al., 2003; Campbell et al., 2006; Nornes et al., 2008) to *Psen1*$^{-/-}$ mice such as aberrant somite formation and Notch signaling defects (Shen et al., 1997; Wong et al., 1997) Interestingly, a zebrafish mutant (discovered in a TILLING screen) lacking Psen1 activity is viable (Sundvik et al., 2013). These mutant fish have been used to study the brains histaminergic system (Sundvik et al., 2013). This system is essential in mediating cognitive functions affected in AD. Analysis of histamine neuron numbers in the *psen1*$^{-/-}$ zebrafish brains revealed that *psen1* in zebrafish is a regulator of histaminergic neuronal development (Sundvik et al., 2013). These mutant fish will be a valuable tool for further analysis of *psen1* function in zebrafish.

Blockage of Psen2 protein translation in zebrafish embryos (Nornes et al., 2008, 2009) appears to have major effects on Notch signaling in comparison to *Psen2*$^{-/-}$ mice which are viable and show only a minor phenotype (Herreman et al., 1999). Furthermore, loss of Psen2 expression affects the production of dorsal longitudinal ascending (DoLA) interneurons in the developing spinal cord of zebrafish larvae (Nornes et al., 2009). As loss of Psen1 does not affect DoLAs this observation provides an *in vivo* assay for Psen2 function. This assay was used to demonstrate a functional interaction between Psen1 and Psen2 in zebrafish (Nornes et al., 2009).

In the absence of technology available to analyze *PSEN* FAD missense mutations in zebrafish, our laboratory previously attempted to model *PSEN1* splicing mutations by injecting morpholinos that target splice acceptor sites of the orthologous *psen1* gene. We attempted to cause exon skipping to exclude exon 8 or 9 from zebrafish *psen1* transcripts to mimic the effects of the human *PSEN1 L271V* and Δ9 mutations, respectively,. However, instead of the (at that time) expected exon exclusion, injection of morpholinos blocking splice acceptor sites lead to the failure of introns 7 and 8 to be correctly spliced out of transcripts (Nornes et al., 2008). The inclusion of intron sequence lead to premature truncation of the open reading frame (ORF) after exons 6 and 7, respectively. It appeared that the aberrant transcripts induced by morpholino injection were able to evade nonsense-mediated decay and be translated into truncated protein molecules. Assays developed in our laboratory (discussed in the next section) demonstrated that truncation of the zebrafish *psen1* transcript ORF after exons 6 and 7 had potent dominant negative effects on Psen1 activity and could also suppress Psen2 activity (Nornes et al., 2008). These putative truncated proteins were subsequently expressed in embryos by injection of synthetic mRNAs. Injection of mRNAs coding for proteins truncated after exon 6- or 7-derived sequence had the same dominant effect on Psen1 activity as the morpholino-induced aberrant *psen1* transcripts. Recently we published results where we expanded the original study to analyze a series of *psen1* ORF truncations (i.e., after exon 4, 5, 6, 7, 8; Newman et al., 2014). We found differential, dominant activation of Notch signaling and APP cleavage amongst the different truncations of Psen1. Interestingly, similar effects were also observed after injection of synthetic mRNAs encoding equivalent truncations of human PSEN1. This supports the evolutionary conservation of function of these truncated proteins. A particularly important discovery from this study was that the activity of a form of Psen1 truncated after exon 4 sequence of the ORF) cannot be observed in the absence of normal endogenous Psen1. This implies that mutant forms of PSEN1 require endogenous normal PSEN1 to exert their effects. A similar observation was made recently by Heilig et al. for various FAD mutant forms of the PSEN1 protein in mouse embryo fibroblasts (Heilig et al., 2013).

OTHER COMPONENTS OF THE γ-SECRETASE COMPLEX

There has been limited functional analysis of the zebrafish genes ortholgous to the non-PRESENILIN components of the γ-secretase complex. Blockage of translation of Psenen or Aph1b proteins in zebrafish causes phenotypes including defective somitogenesis and reduced neuron formation as expected for loss of Notch signaling (that requires γ-secretase activity; Campbell et al., 2006). Furthermore, blockage of Psenen translation leads to destabilization of Psen1 protein and causes a much greater induction of apoptosis in developing embryos than blocking translation of Psen1, Psen2, or Aph1b (Campbell et al., 2006). Simultaneous inhibition of p53 translation was able to block the induction of apoptosis (Campbell et al., 2006).

THE AMYLOID-BETA PRECURSOR PROTEIN

The zebrafish *APP* "co-orthologs," *appa* and *appb* have widespread and overlapping expression from mid-gastrulation in the developing embryo (Musa et al., 2001). At 24hpf both genes are expressed in the developing forebrain and other tissues with only *appb* expressed in the spinal cord (Musa et al., 2001). In a zebrafish study by Lee and Cole a section of *appb* regulatory sequence was fused to GFP (Lee and Cole, 2007). Dissimilar to other expression studies, they observed expression of *appb* in the developing vasculature. More recently, Liao et al. (2012) isolated transposon gene trap integrations that contained RFP in the *appa* gene and in the closely related a*myloid beta precursor-like protein 2 gene (aplp2)*. The gene traps caused fusions to RFP of the extracellular domains of both of the encoded proteins. The fusion proteins of these genes were accumulated in the vasculature. However, they could not detect the transcripts of these genes in the endothelial cells of the vasculature. Instead, transcripts were detected in neuronal cells. This suggests that these proteins are synthesized in neuronal cells and then accumulate in the vasculature.

Translation blocking morpholinos have also been employed to investigate the function of the Appa and Appb proteins (Joshi et al., 2009). Inhibition of Appa had little effect on the developing

embryo while Appb translation inhibition resulted in defective convergent extension cellular movements and a reduced body length. These defects could be rescued by injecting Appb deficient embryos with mRNA coding for human APP. The rescue by human APP was more effective than injection of an mRNA encoding a human *APP* FAD mutant (the APP Swedish double mutation *K595N* and *M596L*). Loss of Appb activity has also been shown to cause defective neural development (Song and Pimplikar, 2012) including defective axonal out-growth patterning and synapse formation (Abramsson et al., 2013). In the study by Song and Pimplikar, only full-length human APP but not truncated forms could rescue the neuronal defects, revealing that both intracellular and extracellular domains of human APP are required for normal function (Song and Pimplikar, 2012). These studies demonstrate that zebrafish embryos can be exploited for the analysis of different mutant forms of human *APP*.

AMYLOID-BETA FUNCTION AND TOXICITY STUDIES IN ZEBRAFISH

Although Aβ was discovered over 25 years ago (Goate and Hardy, 2012) and many studies have examined its claimed toxicity, the non-pathological functions of the Aβ peptide are still poorly understood. A study by Cameron et al. (2012) demonstrated that high levels of Aβ can increase cerebrovascular branching in the developing zebrafish hindbrain. They then completed a follow up study to determine whether lowering Aβ levels would have the opposite effect (Luna et al., 2013). From 2 days post fertilization (dpf) APP-deficient (by morpholino injection) and Aβ deficient (endogenous Aβ production blocked using a β-secretase inhibitor) larvae presented with cerebrovascular defects. Interestingly, these defects could be rescued by treating the embryos with the human Aβ-42 peptide but not the shorter cleavage product of APP named p3 (that arises from cleavage of APP by α-secretase and γ-secretase). These results suggest that Aβ may play a role in maintaining normal cerebrovascular function. This interesting finding using the zebrafish model provided further evidence that caution should be taken when treating AD with drugs designed to inhibit Aβ production such as BSIs and γ-secretase inhibitors (GSIs).

As mentioned above the amyloid hypothesis of AD proposes that aggregations of Aβ peptides are toxic. Therefore, there has been a considerable effort put towards developing and discovering Aβ-lowering compounds to counter the toxicity. Inhibition of β-secretase, also known as beta-site APP cleaving enzyme (BACE1), is of prime interest for the development of amyloid-lowering drugs. BACE1 has become an attractive target for drug development as, in comparison to the lethality observed in *Psen1* null mice, *Bace1* knock-out mice are viable (Roberds et al., 2001; Luo et al., 2003) and have subtle phenotypes (Harrison et al., 2003; Willem et al., 2006). Furthermore no phenotypes have been reported for knockout of the *Bace1* homolog, *Bace2* and deletion of *Bace2* in mice did alter Aβ generation (Dominguez et al., 2005). Recently, *bace1* and *bace2* loss-of-function mutations have been introduced into the zebrafish genome by genome editing using ZFNs (van Bebber et al., 2013). *bace1* mutants had an increased number of mechanosensory neuromasts and, similar to observations in the mouse, *Bace1* knockout caused a decrease in myelination. *bace2* mutants had a distinct melanocyte migration phenotype that was not observed in the *bace1* mutants. There was no effect on myelination or neuromasts in the *bace2* mutants. The phenotypes observed in the single mutants were not enhanced further in the Bace1/2 double knockout. Together the data from this study suggests that, in zebrafish, Bace1 and Bace2, have distinct non-redundant physiological functions. These specific phenotypes observed in zebrafish *bace1* and *bace2* mutants have provided important information on BACE1 and BACE2 function and support that zebrafish can be a useful *in vivo* system for determining, e.g., whether BACE1 inhibitors also inhibit BACE2 function and vice versa. *In vivo* evaluation of BACE inhibitors is integral for establishing these drugs as a therapeutic option for AD.

The zebrafish is an excellent system for the screening of chemical libraries. Embryos and larvae placed into microtiter plates can be treated with various chemicals in their aqueous support medium. This strategy can be employed to reveal therapeutic compounds for various disease states modeled in the fish. The only published study that has generated a transgenic Aβ toxicity model in zebrafish involved fusing the human Aβ-42 sequence to the promoter of the *mitfa* (*nacre*) gene (Newman et al., 2010). This would drive expression of human Aβ-42 specifically in the melanin-containing pigment cells (melanocytes) of the zebrafish in the hope that it would produce an easily identifiable disrupted pigmentation pattern phenotype without being lethal to the zebrafish larvae. A disrupted pattern did become evident in the adult fish. However, unfortunately it only became apparent after 16 months which is too late for drug screening and was too late for breeding the old, infertile fish. Aβ toxicity can also be analyzed in zebrafish simply by exposing embryos to amyloid-beta in their supportive aqueous environment. Treatment of embryos with 2.5 μM Aβ-40 caused defective development including that of the vasculature and also accelerated cell senescence (Donnini et al., 2010). This system could be expanded to test Aβ-42 (more aggregative form) and also to find compounds that ameliorate these observed defects.

γ-secretase inhibitors (GSIs) have been investigated as a therapeutic option to inhibit the production of Aβ peptides. However, the use of GSIs comes with the potential side affects of also affecting Notch signaling. Testing the toxicity of GSIs on zebrafish has revealed important information on how Notch related pathways are adversely affected by GSIs (Yang et al., 2010).

ASSAYS FOR AD-RELEVANT CELLULAR PATHWAYS AND PROCESSES IN ZEBRAFISH

γ-SECRETASE ACTIVITY ASSAYS

There are a number of cellular pathways and processes that are aberrant in AD. The zebrafish are a useful system for investigating molecular events such as γ-secreatse activity and autophagy that have been implicated in AD pathogenesis.

There are over 70 proteins that are known to be substrates of γ-secreatse activity (Lleo and Saura, 2011). In zebrafish, γ-secretase activity was initially analyzed by observing changes in the expression of genes known to be downstream targets of Notch signaling such as *hairy-related 6* (Bernardos et al., 2005; Campbell et al., 2006; Arslanova et al., 2010) and *neurogenin* (Campbell et al.,

2006). However, gene expression can be under different transcriptional control in different regions of the embryo (Campbell et al., 2006; Arslanova et al., 2010). Therefore, using whole embryos in quantitative PCR analysis is not informative to assess changes in gene expression in response to various factors. To overcome this, changes in gene expression in a particular region of the embryos have been assessed by whole-mount *in situ* transcript hybridization (Campbell et al., 2006; Nornes et al., 2008; Arslanova et al., 2010). However, this is not normally used as a quantitative technique since many variables can influence the strength of the staining signal such as fixation conditions and incubation times. Consequently, use of this technique to assay relative differences in Notch signals under various treatments requires stringent controls.

Recently, the first assay to assess directly γ-secretase cleavage activity was developed by Wilson and Lardelli (2013). α- and β-secretase cleavage of Appa provide substrates for subsequent γ-secretase cleavage. Unfortunately these cleaved forms of Appa cannot be detected in zebrafish embryos prior to 48 hpf which currently makes monitoring endogenous Appa cleavage in manipulated zebrafish embryos difficult. Therefore, a fragment of Appa equivalent to the membrane-embedded remnant of APP following β-secretase cleavage was fused to GFP and expressed transiently in zebrafish embryos by the use of Tol2 vector transgenesis system. This construct is co-expressed with a set ratio of free GFP (for signal normalization). Western immunoblotting is then used to assess the ratio of the Appa:GFP substrate to free GFP (the γ-secretase cleavage product itself is too unstable to be observed). Once an Appa:GFP/ free GFP ratio is determined for a protein sample from a pool of manipulated embryos (e.g., drug treatment, morpholino or mRNA injection) it can then be compared to control embryos to determine how that particular manipulation is affecting γ-secretase cleavage activity.

ASSAYING PROTEIN DEGRADATION PATHWAYS IN ZEBRAFISH

Excess or aberrant cellular proteins can be degraded by the ubiquitin-proteasome system (UPS). Since protein aggregation is implicated in many neurodegenerative diseases (Ross and Poirier, 2004) it is unsurprising that problems with UPS function have been implicated in neurodegenerative disease such as AD (West et al., 1994; Nikitin et al., 2003a; Novichkova et al., 2003b) and Parkinson's disease (Olanow and McNaught, 2006). Zebrafish have been used to investigate UPS function in Parkinson's disease (Regeur et al., 1994); however, no analyses in zebrafish have yet examined the UPS with respect to AD.

Autophagy is an important mechanism required for the degradation of dysfunctional and unwanted cellular components (including incorrectly folded and aggregated proteins) through the actions of lysosomes. Indeed, autophagy has been identified as a pathway for the degradation of accumulated Aβ peptides (Nilsson et al., 2013). Recently, the Presenilin proteins were suggested to have a major role in autophagy with FAD mutations in human PSEN1 inhibiting this function (Lee et al., 2010). These authors presented evidence showing that PSEN1 acts as a chaperone in the ER for a transmembrane protein required for acidification of the lysosomes, the v-ATPase V0a1 subunit [Lee et al., 2010; however, other reports have disputed this finding (Coen et al., 2012; Zhang et al., 2012)]. They also demonstrated that this function of PSEN1 is dependent on the full-length PSEN1 holoprotein rather than the endoproteolysed form that is active in the γ-secretase complex. Furthermore, a γ-secretase inhibitor and loss of another γ-secretase complex component (NCT) had no effect on autophagy (Lee et al., 2010) suggesting that this function of PSEN1 is independent from its function in γ-secretase complexes.

Autophagy can be analyzed in zebrafish by observing induction of the LC3-II protein by western immunoblotting using a human antibody against LC3 that cross-reacts with zebrafish Lc3 (He et al., 2009; He and Klionsky, 2010). Transgenic zebrafish have also been developed that express GFP fused to Lc3 (He et al., 2009). As Presenilin protein expression can easily be manipulated in the zebrafish these autophagy assay are a useful tool for further investigation of the involvement of the Presenilins in autophagy.

USING THE ZEBRAFISH TO INVESTIGATE OTHER ASPECTS OF ALZHEIMER'S DISEASE ETIOLOGY

ANALYSIS OF HYPOXIA IN THE ZEBRAFISH

There is accumulating evidence suggesting that hypoxia is an important initiating factor in the pathogenesis of AD. Under hypoxic conditions the electron transport chain in the mitochondria increases free radical production that leads to increased oxidative stress (Bell et al., 2007). Biomarkers of hypoxia can differentiate between people with mild cognitive impairment that progress to AD and those who do not (Oresic et al., 2011). The risk factors for cardiovascular disease and AD are similar (Kotze and van Rensburg, 2012) and it is anticipated that vasculature problems would affect oxygenation of the brain. Interestingly, Aβ levels in serum have been shown to be elevated after cardiac arrest (Zetterberg et al., 2011).

Zebrafish are an advantageous system for analysis of the effects of hypoxia on various biological functions. Zebrafish embryos and adults can be exposed to real hypoxia by depleting their water environment of oxygen or to chemical mimicry of hypoxia through, e.g., sodium azide treatment (Moussavi Nik et al., 2011). Similarly to what is observed in humans (Lukiw et al., 2001; Pluta, 2005; De Gasperi et al., 2010; Zhang and Le, 2010), hypoxia upregulates *psen1*, *psen2*, *appa*, *appb*, and *bace1* in zebrafish adult brain and larvae (Moussavi Nik et al., 2012). This suggests that Aβ is produced as a protective response to hypoxia in both human and zebrafish cells – a response conserved over 450 Mya of evolutionary time. Note, however, that while all the enzymes required to cleave Aβ from Appa and/or Appb are present in zebrafish, the existence of Aβ itself has not yet been directly demonstrated (e.g., through immunoblotting or mass spectrometry). The study by Moussavi Nik et al. (2012) also demonstrated that, unlike in mammals, F2-isoprostanes are not a good marker of oxidative stress in zebrafish and that the upregulation of *catalase* gene expression can be a better alternative marker for demonstration of oxidative stress in zebrafish (Jin et al., 2011; Tseng et al., 2013).

APOE

The APOE ε4 allele has been identified has the main genetic risk factor for SAD. APOE is important for clearance of amyloid-beta

from the brain (Huang and Mucke, 2012), while the AD risk-associated ε4 allele has been shown to impair the clearance of Aβ (Deane et al., 2008) and, more recently, to affect the integrity of the blood–brain barrier (Bell et al., 2012). There has been little research investigating APOE function in zebrafish. Expression studies of *apoea* (Raymond et al., 2006) and *apoeb* (Pujic et al., 2006) revealed expression in the developing retina and yolk syncytial layer. Furthermore, *apoeb* has also been observed in microglial cells (Veth et al., 2011), developing fins and epidermis (Monnot et al., 1999; Tingaud-Sequeira et al., 2006), regenerating fin tissue (Monnot et al., 1999), macrophages (Lien et al., 2006), liver, intestine, and ovary (Levi et al., 2012).

MAPT

The MAPT is the main component of the NFTs found in AD brains. Various dysfunctions of the tau protein are found in other neurodegenerative disorders such as frontotemporal dementia (FTD), corticobasal degeneration and progressive supranuclear palsy (Pittman et al., 2006). Diseases with tau-like pathology are collectively termed "tauopathies." A review by Bai and Burton (2011) discussed how the zebrafish has been used to investigate these diseases. A number of MAPT protein isoforms exist as a result of alternative splicing of *MAPT* transcripts. These isoforms can be classified into two groups, 3R or 4R, depending on the number of tubulin-binding motifs. It appears that an overall one-to-one ratio of 3R to 4R transcripts is required for normal functioning of the MAPT protein in the brain (Goedert and Spillantini, 2006). In most tauopathies this ratio is found to be changed (Goedert and Spillantini, 2006) and altered splicing of *MAPT* is also suggested to occur in AD brains (Conrad et al., 2007).

Transgenic zebrafish expressing human *MAPT* were generated and investigated prior to identification of the zebrafish ortholog(s) of *MAPT*. In these studies human *MAPT* was specifically expressed in zebrafish CNS neurons (Bai et al., 2007; Paquet et al., 2009). Bai et al. used the promoter of the *enolase 2* gene to drive the expression of MAPT 4R in zebrafish neurons at approximately eightfold higher levels than what is observed in human brain. This resulted in accumulations of tau protein (resembling NFTs) in the zebrafish brain. In the study by Paquet et al., the *HuC* promoter was employed to drive expression of a Gal4:VP16 fusion protein in neurons. This protein was then bound to UAS sites in a bidirectional promoter transcribing the DsRed fluorescent marker protein gene and a mutant form of human MAPT associated with FTD, *TAU-P301L*. The transgenic zebrafish larvae showed biochemical changes consistent with those observed in human tauopathies. However, it should be noted that no comparisons of phenotype were made between the non-mutant and mutant forms of human *MAPT* in the zebrafish. Furthermore, the expression levels of the transgenes relative to the endogenous zebrafish *mapt* genes were not assessed in these studies. Despite these limitations these transgenic zebrafish models provide a useful system to investigate whether chemical inhibitors can modulate the observed tauopathy-associated changes.

The zebrafish "co-orthologs" of the human *MAPT* gene, *mapta* and *maptb* have similar but not completely overlapping patterns of expression in developing embryos (Chen et al., 2009). They are both predominantly expressed in the developing CNS while only *maptb* has strong expression in the trigeminal ganglion and dorsal sensory neurons of the spinal cord. *Mapta* is spliced into 4R–6R isoforms while *maptb* is spliced mainly into 3R isoforms. This expression is in contrast to mouse *Mapt* which is mainly expressed as 3R in the brain (and hence may not be a good model of MAPT function for human pathologies). Manipulation of the expression of the zebrafish *mapt* isoforms may therefore be advantageous for understanding the function of 3R and 4R MAPT and the role(s) the 3R:4R ratio plays in pathogenesis.

THE FUTURE FOR MODELING ALZHEIMER'S DISEASE IN ZEBRAFISH

The zebrafish is rapidly emerging as an attractive model for AD research. They are an ideal model for drug testing prior to clinical testing in rodents. However, there are still aspects of this model that require better understanding. For the zebrafish system to be used to model aspects of AD pathobiology we need to better understand zebrafish brain structure and function and also gain a deeper understanding of adult zebrafish brain physiology. Work so far has revealed that the zebrafish brain does have a reasonable level of conservation of basic structure when compared to mammals as well as similar neuroanatomical and neurochemical pathways to those that play roles in human disease (reviewed in Santana et al., 2012). We have revealed various aspects of presenilin gene biology using the zebrafish that would otherwise be difficult to observe/analyze in other models. However, to analyze effectively future transgenic and mutant zebrafish models of AD we need to strengthen our understanding of the functions in zebrafish of some of the orthologs of the key genes implicated in human AD pathogenesis such as MAPT and APOE.

Whether the zebrafish can be employed to model a late-onset disease like AD is debatable since zebrafish have a profound capacity for regeneration and this must impact on the development of neurodegenerative phenotypes. Neurogenesis in the adult zebrafish brain is much more abundant than is observed in mammals (Kizil et al., 2012) consequently making analysis of neuronal loss difficult. Despite these limitations the recent availability and feasibility of using genome editing technologies presents an exciting opportunity to develop zebrafish genetic models of neurodegenerative diseases such as AD. ZFNs, TALENs and CRISP/Rs have been validated for use in the zebrafish [reviewed by Hwang et al., 2013; Schmid and Haass, 2013) and it is inevitable that FAD mutations will be introduced into zebrafish FAD gene orthologs.

Animal models are a useful tool in investigating the causes and pathologies of human diseases. Obviously such models can never reflect the complete pathology that is observed in human cases. The complexity of the human brain makes AD a particularly difficult disease to model in animals. However, by using a number of different models including the zebrafish, we can exploit the unique characteristics of each to unravel the molecular basis of this disease.

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Phospholipase A_2 – nexus of aging, oxidative stress, neuronal excitability, and functional decline of the aging nervous system? Insights from a snail model system of neuronal aging and age-associated memory impairment

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The aging brain undergoes a range of changes varying from subtle structural and physiological changes causing only minor functional decline under healthy normal aging conditions, to severe cognitive or neurological impairment associated with extensive loss of neurons and circuits due to age-associated neurodegenerative disease conditions. Understanding how biological aging processes affect the brain and how they contribute to the onset and progress of age-associated neurodegenerative diseases is a core research goal in contemporary neuroscience. This review focuses on the idea that changes in intrinsic neuronal electrical excitability associated with (per)oxidation of membrane lipids and activation of phospholipase A_2 (PLA_2) enzymes are an important mechanism of learning and memory failure under normal aging conditions. Specifically, in the context of this special issue on the biology of cognitive aging we portray the opportunities offered by the identifiable neurons and behaviorally characterized neural circuits of the freshwater snail *Lymnaea stagnalis* in neuronal aging research and recapitulate recent insights indicating a key role of lipid peroxidation-induced PLA_2 as instruments of aging, oxidative stress and inflammation in age-associated neuronal and memory impairment in this model system. The findings are discussed in view of accumulating evidence suggesting involvement of analogous mechanisms in the etiology of age-associated dysfunction and disease of the human and mammalian brain.

Keywords: aging, age-associated memory impairment (AMI), lipid peroxidation, phospholipases A2, neuronal excitability, neuronal plasticity, *Lymnaea stagnalis*

INTRODUCTION

Most organisms age, humans and the vast majority of other complex animals included. That is, the life functions required for their survival and reproduction weaken as they grow older due to natural processes that are still only partially understood and involve a complex combination of chemical, genetic, metabolic, molecular, physiological, ecological, and evolutionary features (see for instance Kirkwood and Austad, 2000; Ljubuncic and Reznick, 2009; Masoro and Austad, 2011).

Aging generally affects all aspects of an organism's biology including, in the case of humans and complex multicellular animals, the nervous system's ability to execute its control processes and support learning and memory functions. For example, declining learning and memory performance, weakening sensory, and motor functions, and slowing of reaction times are common symptoms of old age in humans and many vertebrate and invertebrate model systems (Burke and Barnes, 2006; Chawla and Barnes, 2007; Murakami, 2007; Partridge, 2008; Yeoman et al., 2012; Haddadi et al., 2014). Some of these changes, particularly in our own species, may involve pathological processes such as Alzheimer's and Parkinson's disease. However, even in the healthy aging brain where loss of neurons and circuits to cell death is generally not a significant factor, functional performance tends to decline with age (Peters et al., 1994; West et al., 1994; Rapp and Gallagher, 1996; Rasmussen et al., 1996; Gazzaley et al., 1997; Merrill et al., 2000, 2001; Keuker et al., 2003; Burke and Barnes, 2006). Why this happens, particularly what makes healthy aging neurons change the way in which they work and communicate with each other is the focus of this article. Specifically, we explore the idea that processes triggered by oxidative stress ensuing at the level of phospholipid bilayer membranes of cells and organelles play a central role in age-associated deterioration of the healthy aging nervous system (Note: while this review centers on non-pathological mechanisms of brain aging it should be kept in mind that many consider normal and pathological aging as two causally related extremes of a continuum in which it is not always clear where normal aging ends and pathological aging starts. See for instance Hung et al., 2010; Dai et al., 2014).

The idea that oxidative stress is a major factor in biological aging has a long history and extensive experimental support (Harman, 1956; Pérez et al., 2009; Hekimi et al., 2011; Speakman and Selman, 2011; Zimniak, 2011; Alcedo et al., 2013). Yet, despite

its prominence in the conceptual framework of biological aging, understanding of how oxidative stress translates to functional decline of the aging nervous system remains incomplete. Here we examine how (per)oxidation of methylene-interrupted poly-unsaturated fatty acids[1] (PUFAs) and subsequent activation of members of the phospholipase A_2 (PLA_2) family of fatty acylases may play a central role in declining excitability of aging neurons and age-associated memory impairment (AMI; see **Figure 1** for conceptual framework; see **Figure 2** for mechanisms and potential implications of PUFA peroxidation).

NEUROBIOLOGICAL CORRELATES OF NORMAL AGING

Considerable effort has been put into identifying neurobiological correlates of normal brain aging for several decades. The picture emerging from these studies is that structural and functional changes occurring in the normal aging brain are generally much more low-key and variable than those found in pathological aging brains. The list of physiological and biochemical symptoms of neuronal aging is extensive and includes evidence for oxidative stress, impaired energy and redox metabolism, Ca^{2+} signaling perturbation, accumulating damage to proteins, lipids, nucleic acids and organelles, declining dendritic complexity, alterations in synaptic density, distribution and function, changes in gene regulation, and molecular processes underlying remodeling of synapses (Barnes, 1994; Burke and Barnes, 2006; Mattson and Magnus, 2006; Esiri, 2007; Shankar, 2010; Toescu and Vreugdenhil, 2010; Yeoman et al., 2012).

While cell autonomous processes likely constitute an important facet of neuronal aging, evidence for significant cell non-autonomous (i.e., organismal) dimensions of neuronal aging is growing. For instance, it is evident that brain aging has vascular, immunological and endocrinological dimensions (Joseph et al., 2005; Aïd and Bosetti, 2011; Yirmiya and Goshen, 2011; Alcedo et al., 2013; Rincon and Wright, 2013; Sadagurski and White, 2013; Oakley and Tharakan, 2014; Tarumi and Zhang, 2014).

[1]Key concept 1 | Methylene-interrupted PUFAs. Poly-unsaturated fatty acids with one or more pairs of hydrogen unsaturated carbons in *cis*-arrangement separated by a hydrogen-saturated (bis-allylic) methylene bridge. Because the methylene moiety's bis-allylic C–H bonds are weaker than ordinary C–H bonds they are prone to free radical attack. The main PUFAs with more than one bis-allylic methylene site in the plasmamembranes of neurons are arachidonic acid (AA), docosahexanoic acid (DHA), and eicosapentaenoic acid (EPA).

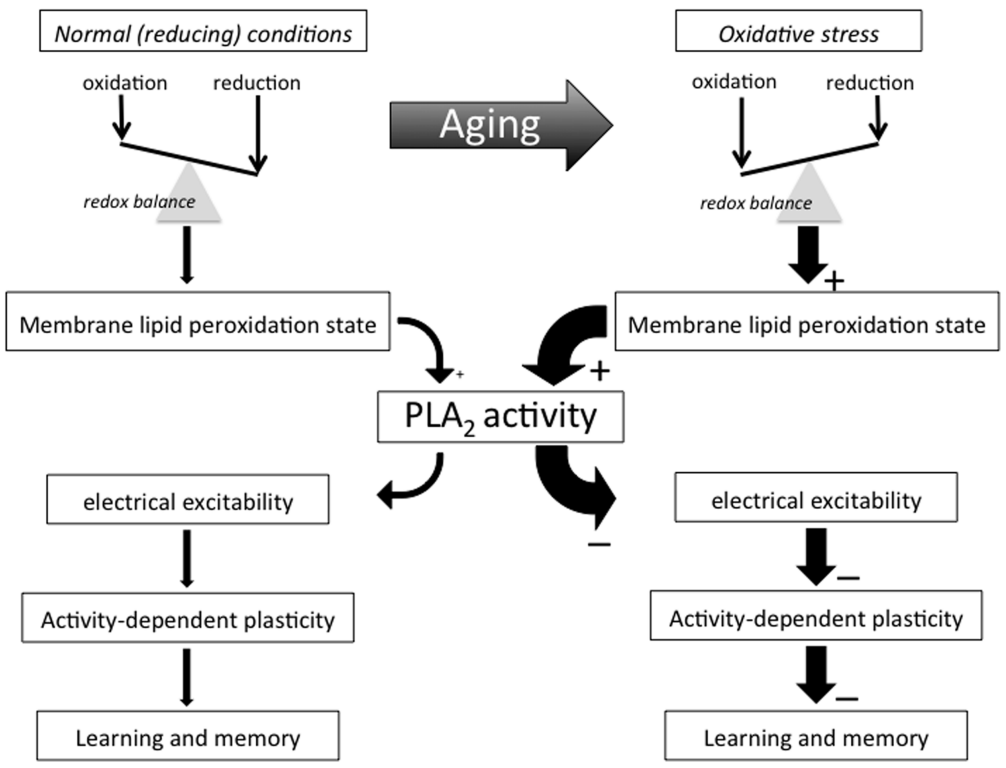

FIGURE 1 | Conceptual framework. This flow diagram outlines key concepts and hypotheses addressed in this essay and illustrate the way we propose they contribute to AMI. Many important biological processes involve redox reactions and many of life's macromolecules are sensitive to electrophilic attacks that may impair their ability to perform their normal functional or structural roles. Few of life's building blocks are as sensitive to oxidation as the poly-unsaturated fatty acids (PUFAs) making up much of cell and organelle membranes. Organisms generate electrophiles like reactive oxygen and nitrogen species as part of many essential live processes, including metabolism, host defense, and intercellular signaling processes. Active maintenance of an appropriate redox balance is therefore of the utmost importance for organismal function and survival. Biological aging tends to be associated with shifts in redox balance away from reducing towards oxidative conditions in many of an organism's molecular, cellular, and organismal systems and domains, including an advanced state of membrane PUFA peroxidation that recruits PLA_2 and induces a (PLA_2-dependent) decline in neuronal excitability and activity-dependent plasticity that manifests itself as AMI.

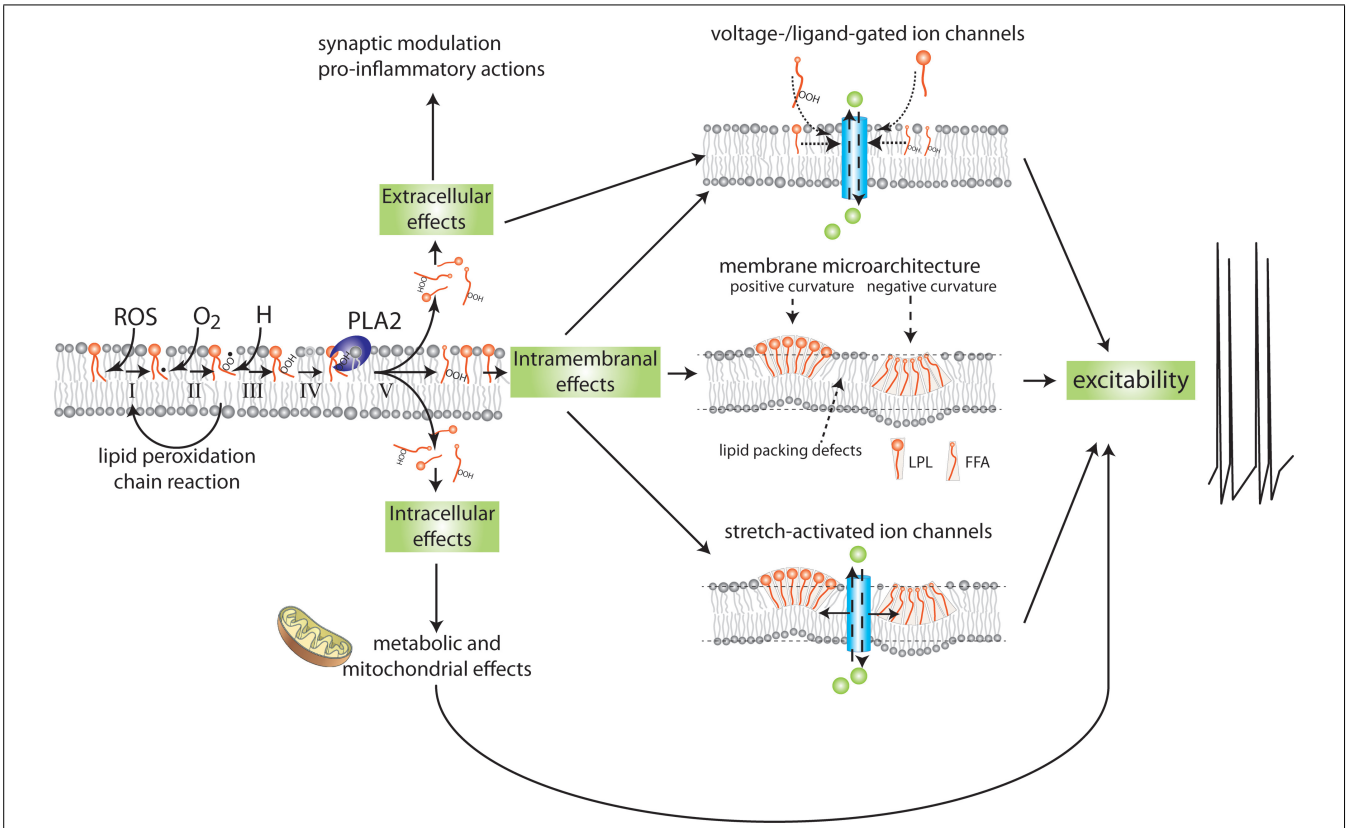

FIGURE 2 | Mechanism and possible implications of membrane lipid peroxidation and phospholipase A_2 activation. PUFAs are highly susceptible to (per)oxidation. Peroxidized PUFAs are prone to excision by members of the PLA_2 family of enzymes. The products of PLA_2's enzymatic activity, free fatty acids (FFAs) and lysophospholipids (LPLs), have a wide range of extracellular, intracellular, and intramembranal biological activities. This highly simplified diagram indicates a few of the mechanisms and targets with special relevance to the nervous system. FFAs and LPLs can themselves act as signaling molecules and modify gating behavior of various types of ion channels. In addition they can act as substrate for other lipid-based intra- and extracellular signaling cascades, including signaling systems regulating inflammation. Moreover, through their effect on membrane microarchitecture they may change functions of integral membrane proteins such as stretch-activated ion channels, alter lipid micro domain organization, disrupt organization of transmembrane signaling complexes, or alter other cellular processes such as membrane fission and fusion that are important in the regulation of intrinsic neuronal excitability, synaptic transmission, and molecular mechanisms underlying neuronal plasticity. See **Figure 6** for details on mechanisms involved in PUFA peroxidation.

One particularly intriguing organismal dimension to normal brain aging emerging in recent decades is the concept that the brain at least in part regulates its own destiny through insulin-like peptide signaling systems coupling nutrient availability and dietary conditions to the control of cell and organismal metabolism (e.g., Alcedo et al., 2013; Sadagurski and White, 2013). Furthermore, while there seems little doubt that many of the phenomena associated with neuronal aging are of a deteriorative nature, evidence for functional preservation and compensation has also been reported (see Barnes, 1994; Burke and Barnes, 2006 for review).

THE NEED FOR SIMPLIFICATION: *Lymnaea stagnalis* AS A MODEL FOR INTEGRATIVE MOLECULE-TO-BEHAVIOR INVESTIGATIONS OF NEURONAL AND BEHAVIORAL AGING

How all dietary, (bio)chemical, metabolic, molecular, cellular, physiological, system-, and organismal-level processes factor into biological aging of neurons and the nervous system and contribute to AMI remains one of the most important unsolved puzzles of neuroscience. Answering fundamental questions of this kind in a system as complex, dynamic, and inherently adaptive as the nervous system is not a trivial problem. With the goal of reducing the complexity of such undertaking, we adopted the pond snail *L. stagnalis* as our research platform (**Figure 3**). Since *Lymnaea* is not as widely known as other model systems in neuronal aging and AMI we will briefly summarize its most relevant features before continuing with the main focus of this review.

Lymnaea stagnalis is a freshwater pulmonate snail with a long and varied track record as a model system in a wide range of basic and applied biological research, including the study of behavioral, neural, and molecular aspects of associative learning and memory. It has a relatively simple central nervous system (CNS, **Figure 4**) of about 20,000–25,000 neurons organized in 11 ganglia. The CNS contains many individually identifiable neurons and well-mapped, behaviorally characterized neural circuits supporting direct behavior-to-molecule inferences about the foundations of neuronal function and dysfunction (see for instance Benjamin et al., 2000; Taylor and Lukowiak, 2000; Brembs, 2003; Benjamin

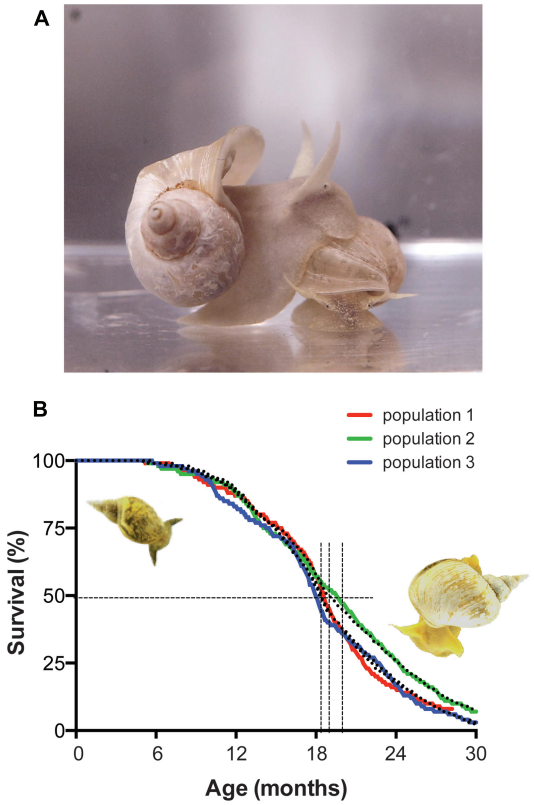

FIGURE 3 | *Lymnaea stagnalis*, the animal and its survival characteristics. (A) *L. stagnalis* is an obligate freshwater aquatic gastropod of the clade *Euthyneura*. The animals are bimodal breathers capable of absorbing oxygen through their skin and through ventilating a simple lung cavity to air. **(B)** Survival characteristics of populations of *Lymnaea* held in our aquatic facility. Shown are the census data for three different populations, representative for normal population survival in the facility (colored lines) fitted by a Weibull power law failure model (dotted lines). Under the conditions in our facility the median population age hovers around 1.5 years (vertical dotted lines median age ± 95% confidence interval) and individual maximum age routinely exceeds 2 years. Under standard culture conditions we maintain in our in house facility (i.e., *ad libitum* feeding of standard diet at 17–19°C water temperature under a 12:12 light:dark regimen) survival characteristics of healthy captive populations show very little variation and reach an average median age of 593 ± 13 days and average maximal age of 880 ± 10 days (arithmetic means ± SE of the mean, $n = 15$; for more details on aquaculture conditions see Watson et al., 2012a).

and Kemenes, 2010) including the biochemical, molecular, cell biological, and physiological foundations of neuronal aging and AMI.

Under laboratory conditions, *Lymnaea* can be effectively reared to ages and quantities required for aging research. Depending on variables like feeding regimen, animal density, day length and water temperature, the maximal age of the animals routinely exceeds 2.5 years with median ages of around 1.5 years under these conditions and age-dependent mortality follows Gompertz and Weibull survival characteristics indicative of aging (**Figure 3B**; see also Janse et al., 1988; Slob and Janse, 1988).

Lymnaea's application in aging studies dates back to the 1980s with the publication of several neurophysiological studies (Frolkis et al., 1984; Nagy et al., 1985; Janse et al., 1986), the first report of age effects on associative memory performance (Audesirk et al., 1982) and work on the general biology and population-level aspects of the species' aging process (Janse et al., 1988; Slob and Janse, 1988). Since then publications on various cell biological, endocrinological, neurophysiological, biophysical, behavioral, and reproductive aspects of aging in *Lymnaea* have appeared (Frolkis et al., 1989, 1991; Janse et al., 1989, 1999; Wildering et al., 1991; Klaassen et al., 1998; Janse and van der Roest, 2001; Arundell et al., 2006; Patel et al., 2006, 2010). Its use in research of the molecular and cellular foundations of AMI started in earnest with Hermann et al. (2007) followed by a series of other publications from our laboratory linking AMI and neuronal excitability changes to oxidative stress, inflammation, lipid peroxidation and declining glutathione (GSH) availability, and identifying PLA_2 as a central player in these phenomena (Watson et al., 2012a,b, 2013, 2014; Hermann et al., 2013; Beaulieu et al., 2014).

Lymnaea has quite a broad behavioral and sensory repertoire and can be taught to change many of its behaviors in response to a variety of chemical, mechanical, and even visual cues (Audesirk et al., 1982; Lukowiak et al., 1996; Sakakibara et al., 1998; Kawai et al., 2004; reviewed in Benjamin et al., 2000; Brembs, 2003; Lukowiak et al., 2006; Benjamin, 2012). Neurobiological substrates of several behavioral paradigms of associative learning and memory have been well characterized. In recent decades most research has focused on two training paradigms: classical conditioning of the animals' feeding behavior, a paradigm with critical involvement of a pair of interneurons called cerebral giant cells (CGCs), and operant conditioning of their aerial respiration, a paradigm with a multi-modal neuron called right pedal dorsal 1 (RPeD1) at its center (**Figures 4** and **5**; Spencer et al., 1999; Staras et al., 1999; for detailed review on the mechanisms and circuitry underlying these behaviors and learning paradigms see Benjamin et al., 2000; Brembs, 2003; Lukowiak et al., 2006; Benjamin, 2012). Using these two learning models, detailed insights in the molecular, cellular, neuronal network, and behavioral aspects of learning and memory have been obtained (Ribeiro et al., 2003, 2005; Kemenes et al., 2006a; Nikitin et al., 2006; Sadamoto et al., 2010; Wan et al., 2010; Dalesman and Lukowiak, 2012; Rosenegger and Lukowiak, 2013; reviewed in Benjamin et al., 2000 and Benjamin, 2012). Importantly, these studies show that, like other invertebrates, *Lymnaea* learning shares many key behavioral, molecular, and functional facets with learning in vertebrate model systems.

LIPID MEMBRANES AS A KEY THEATER OF AGING AND AGE-ASSOCIATED DECLINE OF THE NERVOUS SYSTEM

A large body of evidence indicates that oxidative stress is a prominent feature of both pathological and non-pathological forms of nervous system aging (Choi, 1995; Mattson and Magnus, 2006; Torres et al., 2011; Lopez et al., 2013; Orr et al., 2013; Haddadi et al., 2014). How oxidative stress contributes to age-associated disease processes of the brain and translates to functional decline of normal healthy aging neurons is not well understood. Here we explore the theory that processes associated with the high level of unsaturation of the lipid portion of neuronal membrane

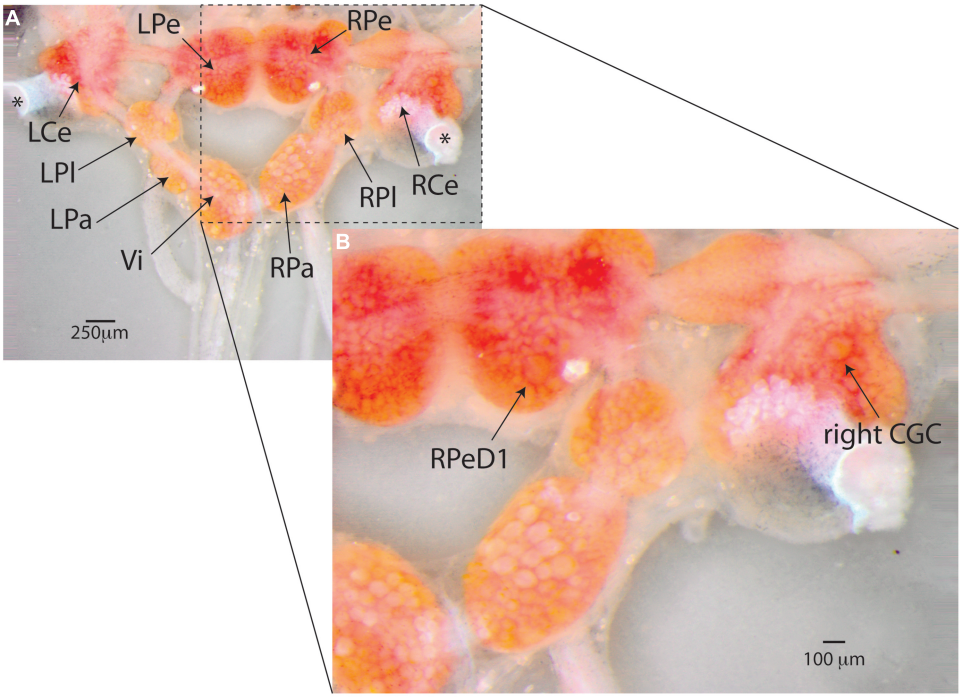

FIGURE 4 | *Lymnaea's* central nervous system and its identified neurons. (A) *Lymnaea's* central nervous system (CNS) is comprised of a circumesophagal ganglionic ring made up of nine ganglia plus two buccal ganglia that are not part of the inner ganglionic ring (LCe, left cerebral ganglion; RCe, right cerebral ganglion; LPe, left pedal ganglion; RPe, right pedal ganglion; LPl, left pleural ganglion; RPl, right pleural ganglion; LPa, left parietal ganglion; RPa, right parietal ganglion; Vi, visceral ganglion; the paired left and right buccal ganglia are not shown in this image. To expose location of neurons discussed in this paper, the cerebral commissure connecting LCe and RCe has been cut and LCe and RCe have been folded out laterally). Inside the ganglia somata of many neurons can be recognized. Many of these neurons are individually identifiable. **(B)** Enlargement of the boxed area in **(A)** showing the location of the two identified neurons that are the main focus of the paper. Right pedal dorsal 1 (RPeD1), located on dorsal surface of the RPe ganglion is a multi-modal neuron involved in the control of *Lymnaea's* aerial respiration and an important locus of operant-conditioning induced plasticity of the animals' respiratory behavior. The cerebral giant cell (GCG) indicated here is one of a bilaterally symmetrical pair of interneurons instrumental in the control of *Lymnaea's* feeding behavior. The CGCs are important loci of reward-conditioning of the animals feeding behavior. These composite images were generated from 15–20 images taken at different focal depths using DMap focus stacking algorithm in Zerene Stacker build T201404082055 (Zerene Systems, Richland, WA, USA).

constitute a key link between aging, oxidative stress, and the age-associated physiological and behavioral decline frequently observed in normal healthy aging animals and the etiology of age-associated disorders of the brain. Central to this idea are the inordinate sensitivity of methylene-interrupted PUFAs to electrophilic attacks and subsequent activation of PLA_2's, a family of enzymes involved in maintenance of membrane phospholipid integrity and membrane-associated lipid signaling processes (**Figure 2**).

Eukaryote cell and organelle membranes are functionally complex, dynamically regulated assemblies of mostly glycerophospholipids, fatty acids (FAs), sterols, proteins, glycoproteins, and glycolipids (Alberts et al., 2002). While most of life's structures are sensitive to oxidation, few are as susceptible to oxidative damage as phospholipid bilayer membranes (Zimniak, 2011). This elevated susceptibility to oxidation is due to a number of factors that conspire, as a manner of speaking, to create "perfect storm" conditions. First, under normal conditions solubility of molecular oxygen and many reactive oxygen and nitrogen species (RONS) in the lipid microenvironment of membranes is quite high (Subczynski and Wisniewska, 2000). Second, as mentioned, PUFAs are inordinately sensitive to attack by RONS and other electrophiles (Hulbert et al., 2007). Third, PUFAs that have undergone free radical attack are themselves reactive free radicals and are, because of the tightly packed molecular arrangement of phospholipids in bilayer membranes, capable of propagating potentially catastrophic lipid peroxidation cascades (Forlenza et al., 2007; Ballatori et al., 2009; Currais and Maher, 2013; Sultana et al., 2013). Neuronal membranes contain high levels of PUFAs such as arachidonic acids (AA), docosahexaenoic acids (DHA), and eicosapentaenoic acids (EPA), and are therefore particularly sensitive to (per)oxidation making lipid peroxidation potentially one of the major instruments of free radical-mediated injury and cell death in the nervous system.

Parenthetically, the idea that phospholipid membranes are one of the primary molecular theaters of aging and longevity has gained substantial traction in recent decades (for reviews see Pamplona, 2008; Zimniak, 2011). In fact, the observation that the level of lipid membrane unsaturation correlates inversely with longevity in mammals, birds, and even invertebrates led to formulation of the "Membrane hypothesis of aging" which essentially sees lipid

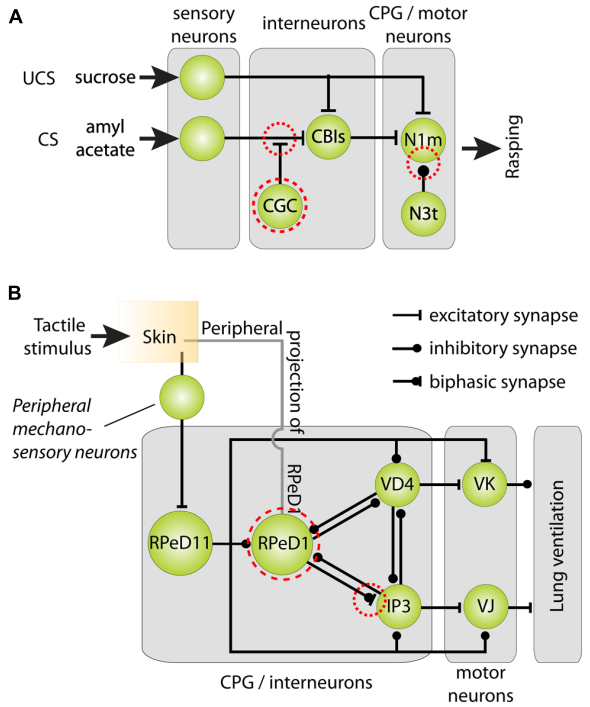

FIGURE 5 | Cellular models of classical reward and operant aversive conditioning in *Lymnaea*. **(A)** Parsimonious model of the neural mechanisms underlying classical chemical reward conditioning of *Lymnaea's* feeding behavior. Behavioral conditioning by peripheral application of sucrose as unconditioned stimulus (UCS) and amylacetate as conditioned stimulus (CS) induces a delayed persistent depolarization of the cerebral giant cells (CGC). This depolarization facilitates throughput of peripheral CS connections with cerebral-buccal interneurons (CBIs) thereby gating throughput of the CS to Neuron 1 medial (N1m) interneurons of the feeding circuit's central pattern generator (CPG) that play a pivotal role in the initiation of the three phase rasping cycle. At the same time, the spike frequency of the CPG interneuron N3t is reduced after behaviorally conditioning thereby releasing N1m from its inhibition. **(B)** Parsimonious model of the neural mechanisms underlying aversive operant conditioning of *Lymnaea's* aerial respiratory behavior. Aerial respiration involves opening and closing of an orifice giving access to the lung cavity (the pneumostome). The CPG driving the animals' aerial respiration behavior consists of three identified interneurons: right pedal dorsal 1 (RPeD1), visceral dorsal 4 (VD4) and (wide-acting) input 3 neuron (IP3). Spiking activity in RPeD1 initiates rhythmic pattern of spiking activity in VD4 and IP3 that in turn trigger electrical activity in, respectively, Visceral K (VK) pneumostome closer motor neurons and visceral J (VJ) pneumostome opener motor neurons. Aversive operant conditioning through tactile stimulation of the pneumostome area induces a hyperpolarization of RPeD1 and reduces the latter's ability to trigger rhythmic activity in IP3. Mechanosensory conditioning of the respiratory CPG's electrical activity through tactile stimulation of the pneumostome area involves both afferent projections of RPeD1 ("peripheral projections of RPeD1") and excitation of the 'whole-body withdrawal interneuron' RPeD11 by identified mechanosensory neurons. Dashed red circles in both circuit diagrams indicate neural loci of plasticity. Circuit diagrams shown were adapted from Benjamin et al. (2000), Taylor and Lukowiak (2000) and Benjamin and Kemenes (2010).

membranes as one of the weakest and therefore highest maintenance links in the biochemical armature of living organisms (Hulbert et al., 2007; Ungvari et al., 2011, 2013; Munro and Blier, 2012; Munro et al., 2013).

LIPID PEROXIDATION INDUCED PHOSPHOLIPASE A_2 ACTIVITY AND ITS CONSEQUENCES

One characteristic cellular response triggered by lipid peroxidation is activation of one or more PLA_2 family members that catalyze cleavage of FAs from the sn-2 position of glycero-phospholipids in plasma- and organelle membranes (Brown et al., 2003; Sun et al., 2004; Farooqui and Horrocks, 2006; Chen et al., 2008; Dennis et al., 2011). Although the precise underlying molecular mechanisms of lipid-peroxidation induced PLA_2 activation are still a matter of debate (see for instance Greenberg et al., 2008), it is generally thought to involve peroxidation induced changes in fatty acyl chain geometry (Niki, 1990; Salgo et al., 1992; Rashba-Step et al., 1997; Greenberg et al., 2008).

Activation of PLA_2 leads to the production of (peroxidized) free FAs (FFAs) and lysophospholipids (LPLs), both classes of molecules with potentially substantial biological activity (**Figure 6**; see below; Brown et al., 2003; Farooqui and Horrocks, 2006). For example, the molecular geometry of LPLs and FFAs is quite different from their mutual parent molecule (i.e., a phospholipid). They may therefore also cause changes in the micro-architecture and biophysical properties of phospholipid membranes and potentially affect membrane-associated signaling functions (**Figure 2**; Yu et al., 1992; Choi and Yu, 1995; Choe et al., 1995; Patel et al., 2001; reviewed in Piomelli et al., 2007). In addition, PLA_2 mediated FFA liberation is the starting point for the generation of an extensive array of lipid-based neuromodulators. That includes FFAs themselves (e.g., AA and DHA) and LPLs (e.g., lysolecithin) but also encompasses an extensive array of biologically active molecules such as eicosanoids, isoprostanes, and neuroprostanes generated by a variety of lipid metabolizing pathways. Many of these pathways act as direct effectors of signal transduction in the nervous system through either direct interaction with ion channels or binding to G-protein-coupled or nuclear receptors (reviewed in Piomelli et al., 2007; Sultana et al., 2013).

Poly-unsaturated fatty acid peroxidation generally also leads to the formation of electrophilic aldehydes such as acrolein, malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE; Pamplona, 2008, 2011). These highly reactive aldehydes can seriously compromise molecular integrity of cells and tissues through the formation of molecular adducts with phospholipids, proteins, and DNA. However, recent evidence suggests that these lipid-derived aldehydes may also induce adaptive responses driven to decrease oxidative damage and improve antioxidant defenses (see for instance Pizzimenti et al., 2013).

ANTIOXIDANT DEFENSES

Organisms are not defenseless against the actions of pro-oxidants. Rather, they posses a wide and varied array of enzymatic and non-enzymatic instruments to defend their structures and functions against oxidation that includes catalases, peroxidases, and dismutases, the GSH system and various other non-enzymatic anti-oxidants like vitamin C and vitamin E.

ENZYMATIC MECHANISMS

Because of its relatively low redox potential and high abundance, the tripeptide GSH is a versatile anti-oxidant involved as primary

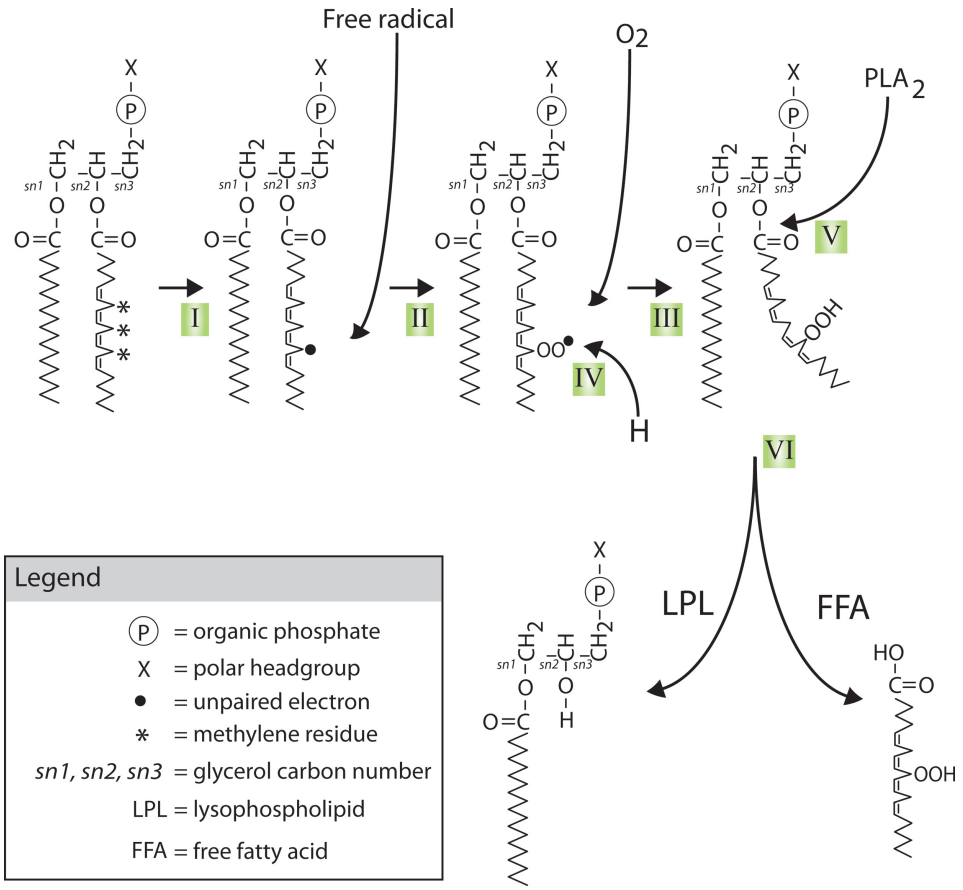

FIGURE 6 | Mechanisms of PUFAs peroxidation and hydrolysis. Neuronal glycerophospholipids contain high levels of methylene-separated PUFAs such as the 20:4 omega-6 fatty acid arachidonic acid (AA) shown attached at the second carbon of the phospholipid glycerol backbone (the sn-2 position) in the diagram. These PUFAs are very sensitive to free radical attack at their bis-allylic C-H bonds (indicated by *) and subsequent (per)oxidation. This process starts with (I) the formation of a C-centered radical (indicated by •) followed by (II) recombination with molecular oxygen that is normally present in abundance in lipid bilayers. The lipid peroxide radicals formed in step II can start a peroxidation chain reaction unless (IV) defused by reduction through for instance donation of a proton by vitamin E or neutralization by other chemical mechanisms. Peroxidation of these PUFAs (V) recruits members of the PLA_2 family of phospholipases. PLA_2 mediated hydrolysis of peroxidized PUFAs (VI) generates peroxidized FFAs and LPLs.

redox buffering of many intra- and extra-cellular compartments, including lipid membranes, and mitochondria (Mari et al., 2009; Ribas et al., 2014). In addition to its antioxidant actions, GSH also plays an important role in detoxification processes, including the detoxification of potentially harmful products of lipid peroxidation such as aforementioned aldehydes. This process is mediated by members of the glutathione-*S*-transferase (GST) family of enzymes (Zimniak, 2008; Ribas et al., 2014). GSH is one of the most abundant molecules in healthy cells and tissues. Its availability is a function of usage, *de novo* synthesis and redox regeneration. The rate-determining step in GSH synthesis is catalyzed by the enzyme γ-glutamylcysteine synthetase (GCL; Chen et al., 2005; Genovese et al., 2007). Perturbation of GCL activity has been shown to significantly reduce GSH availability. In the context of the current focus of lipid bilayer redox homeostasis as a instrument of neuronal aging it is thought provoking that several studies report age-related declines in in GSH levels and GCL activity in various cell types, including neurons (Toroser and Sohal, 2005, 2007; Zhu et al., 2006; Jacob et al., 2013; Watson et al., 2014). This suggests that anti-oxidant failure either with or without escalation of pro-oxidant activity may be a key facet of the progressive lipid peroxidation stress characteristic of aging.

NON-ENZYMATIC MECHANISMS

Non-enzymatic antioxidants such as Vitamins A, C, and E also play key roles in the defense against oxidative stress, including the maintenance of membrane redox homeostasis. Of these three vitamin families, Vitamin E is considered the major antioxidant present in membranes. Vitamin E is comprised of two families of closely related lipid-soluble compounds, the tocopherols, and tocotrienols. Alpha-tocopherol is the most abundant Vitamin E analog and according to recent insights has a preference for non-lipid raft PUFA-containing domains (Atkinson et al., 2010; Lemaire-Ewing et al., 2010). Alpha-tocopherol associates with membranes such that its active hydroxyl moiety is located at the lipid/water interface and not within the hydrocarbon matrix (Marquardt et al., 2013). As a consequence,

α-tocopherol appears to exert its anti-oxidant activity at the surface of a membrane, possibly through the interception of diffusing ROS, or by means of terminating lipid radicals (Marquardt et al., 2013).

NON-TRADITIONAL, NON-ANTIOXIDANT ACTIONS OF α-TOCOPHEROLS

In addition, several studies provide an alternative, non-antioxidant, scenario for α-tocopherol's beneficial effects. That is, it is suggested that incorporation of α-tocopherols in membranes can structurally stabilize the membrane against lipid-peroxidation induced curvature stresses or normalize fatty acid signaling by acting as FFA trap through its ability to form hydrogen-bridges and engage in Vanderwaals interactions with FFAs (Atkinson et al., 2008; Lemaire-Ewing et al., 2010; Marquardt et al., 2013). Especially α-tocopherol's acyl tail has been shown to play a critical role in its membrane stabilizing effect (Kagan, 1989). Methylation of this hydroxyl group disrupts α-tocopherol's ability to engage in hydrogen-bridge formations. In this respect it is interesting to note that work from our lab shows that only non-methylated α-tocopherol is capable of reversing a decline in neuronal excitability due to age- or experimentally induced oxidative stress (Watson et al., 2012b).

LIPID PEROXIDATION AND (BRAIN) AGING

Considering that neuronal membranes tend to have a high PUFA content it is not surprising that lipid peroxidation and its byproducts are increasingly implicated in brain aging. For instance, accumulation of MDA, 4-HNE, acrolein is commonly reported in aged brains of vertebrate and invertebrate model systems (Terman and Brunk, 2006; Zhu et al., 2006; Watson et al., 2012b). Moreover, PLA_2s are also increasingly implicated in normal brain aging and the etiology of age-associated neurodegenerative disorders (Farooqui and Horrocks, 2006; Ong et al., 2010). How the products of PLA_2 activity exert their detrimental effects in the aging brain is, however, not always clear but may involve one or more of the scenarios outlined in the preceding sections.

INTRINSIC ELECTRICAL PROPERTIES AS A SUBSTRATE OF PLASTICITY

This review focuses on the role of intrinsic excitability of neurons in age-associated decline in behavioral plasticity. Decades of research on the cellular and molecular foundations of learning and memory have led to a "synapse-centric" view of learning and memory mechanisms that holds that memories are formed and stored through activity-dependent alterations of synaptic strength and neuronal circuit architecture (Bliss and Collingridge, 1993; Kandel, 2001). In this context non-synaptic mechanisms of plasticity, i.e., mechanisms involving changes in intrinsic electrical properties of neurons, have received much less attention. Yet, experimental evidence that changes in intrinsic neuronal electrical properties could be serving as part or whole of an engram beyond merely facilitating activity-dependent synaptic modulation goes back as far as the 1970s and the concept has gained substantial traction in recent years (reviewed in Benjamin et al., 2008; Mozzachiodi and Byrne, 2010; Sehgal et al., 2013; D'Angelo, 2014; for review of early work in this area see Byrne, 1987). For example, numerous studies have shown that eye blink trace conditioning in rodents induces an increase in intrinsic excitability of hippocampal CA1 and CA3 neurons through reduction of spike frequency adaptation caused by action potential afterhyperpolarizations (AHP) mediated by Ca^{2+}-dependent outward K^+ currents[2] (Coulter et al., 1989; Moyer et al., 1992, 2000; Disterhoft et al., 1996; Thompson et al., 1996; Oh et al., 2010; Sehgal et al., 2014; for review, see Christian and Thompson, 2003). There is also substantial evidence of non-synaptic forms of plasticity in invertebrates, particularly in gastropod mollusks. For instance, Alkon (1974, 1979) demonstrated that associative learning in the nudibranch snail *Hermissenda crasicornis* involved an increase in excitability of type B photo-receptor cell associated with a decrease in the types of voltage- and Ca^{2+}-sensitive K^+ currents mediating the AHP and spike frequency adaptation. In *Lymnaea,* long-term memory (LTM) for classical conditioning of feeding has been associated with a persistent Na^+-current dependent depolarization of the CGCs, two interneurons that act as gate-keepers to the feeding circuit and that play an essential role in chemosensory behavioral conditioning of the animals feeding behavior (Jones et al., 2003; Kemenes et al., 2006b; Nikitin et al., 2008, 2013). In the marine gastropod *Aplysia californica*, changes in intrinsic excitability of identified neurons in the central pattern-generating (CPG) network for feeding have been associated with the network's behavioral conditioning induced motor output (Sieling et al., 2014).

More examples of non-synaptic plasticity can be found in the literature (see reviews by Byrne, 1987; Benjamin et al., 2008; Mozzachiodi and Byrne, 2010; Sehgal et al., 2013; D'Angelo, 2014). For the purpose of the current review it is suffice to say that the idea of intrinsic excitability regulation as a non-synaptic instrument of plasticity is now on firmer ground than ever before. The balance of this paper focuses on the idea that the mechanisms controlling intrinsic neuronal excitability, particularly those underlying the control of repetitive action potential firing, are a key target of oxidative stress-associated aging processes and a root cause of functional decline of normal aging neurons and brains.

EVIDENCE FOR AGE-ASSOCIATED ALTERATIONS IN INTRINSIC ELECTRICAL PROPERTIES

Considering that neuronal electrical excitability is fundamental to brain function, substantial research on the neurobiological basis of behavioral aging has focused on the possibility that aging neurons undergo changes in intrinsic excitability. Neuronal excitability is

[2]Spike frequency adaptation, Action potential afterhyperpolarization (AHP), Ca^{2+}-activated K^+ currents – Spike frequency adaptation is a process by which the action potential firing rate of a neuron declines with time despite the maintenance of a constant excitatory stimulus. The action potential of most neurons is followed by an HP, a period of varying length of time during which the cells' membrane potential is lower than the resting potential preceding the action potential and during which the cell is less excitable than before. Various ionic mechanisms may contribute to the AHP including a class of outward K^+ currents activated either exclusively or in part by increase of intracellular free Ca^{2+} levels (i.e., Ca^{2+}-activated K^+ currents). The AHP as part of a negative feedback loop linking spiking frequency and opening of voltage-gated Ca^{2+} channels to activation of inhibitory Ca^{2+}-activated K^+ currents is a key component of spike frequency adaptation in most neurons (see Lewis et al., 1986; Sah, 1996; Sah and Davies, 2000 for more detail).

a complex function of a neuron's shape, the repertoire, distribution, and density of voltage- and ligand-gated ion channels in their plasma-membrane, the activity of ion pumps and various intra- and extracellular signaling systems and state variables, including intracellular Ca^{2+}-, metabolic-, and redox homeostasis (Bean, 2007; Beck and Yaari, 2008; Vacher et al., 2008; Nusser, 2012). There is evidence for age-associated changes in many of these parameters. It is beyond the scope of this paper to review this vast and diverse literature. For a more in-depth treatment of this subject the reader is referred to many excellent reviews available in this area (see Barnes, 1994; Burke and Barnes, 2006; Mattson and Magnus, 2006; Chawla and Barnes, 2007; Thibault et al., 2007; Mattson et al., 2008; Oh et al., 2010; Toescu and Vreugdenhil, 2010; Yeoman et al., 2012). Instead in the next paragraphs we briefly review key insights gained from vertebrate and invertebrate model systems suggesting that intensification of spike frequency adaptation mechanisms is an important facet of the normal neuronal aging process.

EVIDENCE FROM VERTEBRATE MODEL SYSTEMS

Most of the work on age-related intrinsic neuronal excitability changes in vertebrates has been done on the rodent hippocampus. On balance, the evidence from a large body of *in vitro* electrophysiological studies on different hippocampal regions of young and old mice, rats and rabbits suggest that most intrinsic neuronal electrical properties such as resting membrane potential, membrane time constant, input resistance, rheobase current, action potential threshold, and various aspects of action potential dynamics change very little across the lifespan (reviewed in Barnes, 1994; Burke and Barnes, 2006; Chawla and Barnes, 2007). However, "*a number of consistent changes do occur*" (Barnes, 1994). One of those changes involves alterations in voltage-gated Ca^{2+} currents, intracellular Ca^{2+}-homeostasis, Ca^{2+}-activated outward K^+ currents and interactions between them enhancing the AHP and associated spike frequency adaptation of older neurons (Moyer et al., 1992; Tombaugh et al., 2005; reviewed in Oh et al., 2010). It should be noted, however, that even though the *in vitro* evidence for a role of these mechanisms in cognitive decline associated with normal brain aging is compelling, the idea is not undisputed (for review of this issue, see Burke and Barnes, 2006). Other studies report changes in voltage-activated Ca^{2+}- or Na^+-channel properties to age-associated decline in intrinsic excitability of various types of rodent neurons (Scott et al., 1988; Randall et al., 2012; Branch et al., 2014; for review see Annunziato et al., 2002; Foster, 2012).

EVIDENCE FROM INVERTEBRATE MODEL SYSTEMS

Several studies, particularly in gastropod mollusks, have looked into the question of intrinsic neuronal excitability changes in invertebrate neurons. For example, Frolkis et al. (1991, 1995) reported increased voltage-gated Ca^{2+} current densities and changes in other aspects of intrinsic electrical excitability in aged *Lymnaea* neurons. The same group reported changes in voltage-gated K^+ currents amplitudes and inactivation kinetics in old *Lymnaea* neurons (Frolkis et al., 1989). Patel et al. (2006) reported a decline in intrinsic excitability associated with a decline in input resistance and an increase in AHP magnitude and duration in CGGs of older *Lymnaea* and Klaassen et al. (1998) observed a decline in excitability associated with a reduction in input resistance in aging RPeD1. We observed a decline in intrinsic excitability of CGCs in aged learning-impaired older animals and concluded that "*this decline in excitability is not due to changes in passive neurophysiological properties but rather involves alterations in active properties of the cells*" (Hermann et al., 2007). An age-associated decline in input resistance associated with lengthening membrane time constant was reported for at least three identified neurons in *Aplysia* (Rattan and Peretz, 1981).

Age-associated neurophysiological changes linked to behavioral impairment have been demonstrated in *Caenorhabditis elegans*, *Aplysia*, and *Lymnaea*. For instance, using a classical reward conditioning learning paradigm, we demonstrated in *Lymnaea* that a selective age-related impairment of appetitive LTM is associated with a decline in intrinsic excitability of the CGCs that is reflected in more pronounced spike frequency adaptation (**Figure 7**; Hermann et al., 2007). Along a similar vein, we also showed that age-related LTM impairment in aerial respiration operant-conditioning model is associated with enhanced spike frequency adaptation properties of RPeD1, an interneuron instrumental in the execution of this behavior and one of the cellular substrates of LTM in this learning paradigm (Watson et al., 2013). In addition, Janse et al. (1999) showed a correlation between reproductive senescence and reduced electrical excitability of the caudodorsal cells (CDCs), neuroendocrine cells producing *Lymnaea's* ovulation hormone and other neuropeptides involved in the regulation of reproductive behavior (Janse et al., 1999). In *Aplysia*, studies from the Peretz lab and a recent study by Kempsell and Fieber (2014) have linked age-associated behavioral and homeostatic deficiencies to declining neuronal excitability (Rattan and Peretz, 1981; Peretz, 1988; Skinner and Peretz, 1989). Interestingly, in the nematode *C. elegans* age-associated decline in chemotactic behavior is associated with a decline in sensory neuron excitability due to oxidation-induced enhancement of voltage-gated K^+ currents (Cai and Sesti, 2009; Sesti et al., 2010).

HOW DOES OXIDATIVE STRESS CONTRIBUTE TO DECLINING EXCITABILITY OF AGING NEURONS?

How oxidative stress translates into functional decline and increased vulnerability to disease of the aging brain is only partly understood (Mattson and Magnus, 2006). First it should be noted that in the context of redox modulation of neuronal functions many pro-oxidants have both "good" and a "bad" aspects. For instance, under normal conditions pro-oxidants like RONS play important physiological roles in synaptic plasticity and memory formation (reviewed in Massaad and Klann, 2011; Milton and Sweeney, 2011). Yet, under conditions of oxidative stress the same mechanisms may affect neuronal plasticity and memory function in a negative manner (Hu et al., 2006; Negre-Salvayre et al., 2010; Massaad and Klann, 2011; Niki, 2012).

Second, many of the molecular instruments of neuronal excitability (i.e., membrane ion channels, receptors, vesicle fusion processes) are susceptible to oxidation or sensitive to alterations in cellular redox status (Annunziato et al., 2002; Dukoff et al.,

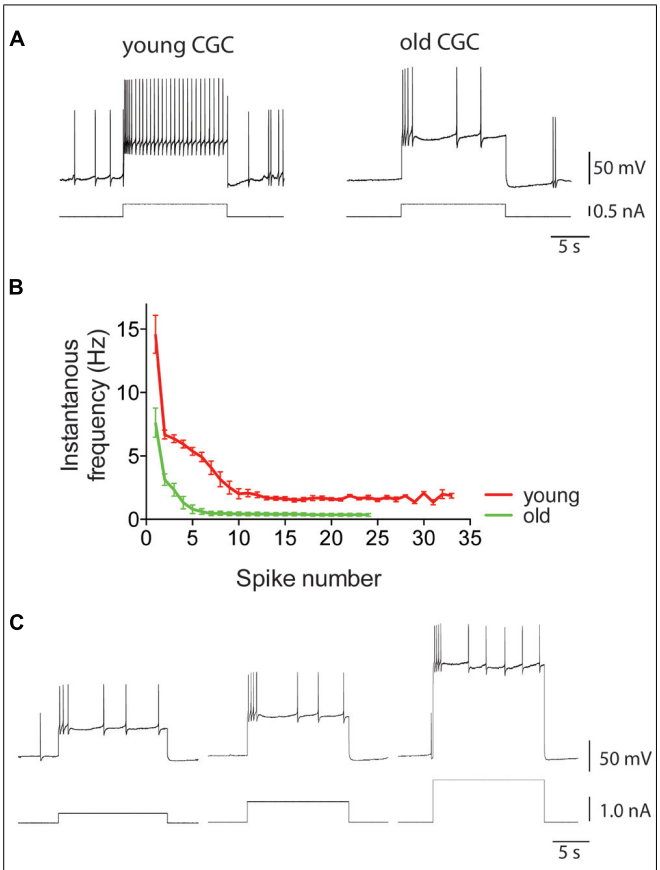

FIGURE 7 | Intrinsic electrical excitability of *Lymnaea* neurons declines with age. Using CGCs as example, this figure illustrates the characteristic age-associated enhancement of spike frequency adaptation that is also observed in other identified *Lymnaea* neurons, including RPeD1 (see for instance Watson et al., 2012b, 2013). **(A)** This figure shows examples of intracellular recordings of spiking activity evoked in young and old CGCs by injection of 0.5 nA depolarizing current. Identical current injection results in a non-accommodating response in young CGCs but a rapidly accommodating response in old CGCs. **(B)** Average instantaneous frequency response of young and old CGCs. Current injected in young CGCs evokes a high-frequency action potential burst followed by a steady non accommodating firing response. In contrast, current injected in old CGCs evokes a few action potentials and a rapidly accommodating response. **(C)** Example of reduced excitability in old CGCs. Increasing stimulus strength fails to evoke a high-frequency non-accommodating response in old CGCs. Adapted from Hermann et al. (2007) with permission from American Psychological Association (APA).

2014). For instance, a particularly salient example concerns the so-called A-current (I_A) family, a family of outward transient voltage-gated K^+ currents (i.e., they are self-inactivating) that plays important roles in the regulation of neuronal excitability (Adams and Galvan, 1986; Bean, 2007). It has long been known that I_A's inactivation properties are modulated by changes in intracellular redox conditions (Ruppersberg et al., 1991; Bähring et al., 2001). That is, under cytosolic reducing conditions the current displays its characteristic fast N-type inactivation behavior whereas under oxidizing conditions this inactivation process is slowed down. In a manner of speaking, this process turns I_A from a "temporary brake" into a "permanent brake" on a neuron's spiking ability. It has been suggested that these mechanisms may operate as a safety switch against metabolic overload by matching a neuron's energy demanding electrical activity to its metabolic capacity (Gulbis, 2002). The I_A family of ion channels is not the only type of ion channels susceptible to changes in cellular redox homeostasis. Many other types of ion channels playing significant roles in the control and execution of neuronal excitability, including voltage-gated Na^+ and Ca^{2+}, are subject to redox modulation (for review see Annunziato et al., 2002).

In addition, over the last number of decades it has become clear that many of the molecular correlates of neuronal excitability and processes are sensitive to the composition and micro-architecture of their membrane environment and/or are modulated by various membrane-derived lipid signaling moieties (for review see Piomelli et al., 2007; Dart, 2010; Rosenhouse-Dantsker et al., 2012). Maintaining lipid membrane homeostasis is therefore a crucial facet of the maintenance of functional integrity of neurons and the brain. Below we illustrate these ideas with some of the most salient examples.

MEMBRANE-ASSOCIATED CORRELATES OF NEURONAL EXCITABILITY CONTROL

There are numerous examples of lipid-mediated signaling in the nervous system. For instance, although there is still some debate whether its actions arise from direct or indirect interactions with ion channel proteins it is evident that AA, one of the most common PUFAs in the nervous system, can modulate a variety of ion channels, including voltage-gated Ca^{2+} channels, mechano-gated K^+ channels like TRAAK, TREK-1, and other members of the four-transmembrane/two-pore domain (4TM2P) family of background K^+ channels (Patel et al., 1998; Maingret et al., 1999; Roberts-Crowley et al., 2009). Alternatively, PUFAs like AA or EPA liberated from their plasma membrane environment can act as metabolic precursors for several signaling pathways active in the nervous system. This includes the eicosanoid pathway that generates a collection of signaling molecules (i.e., prostaglandins, prostacyclins, thromboxanes, lipoxins, and leukotrienes). Many eicosanoids regulate immune functions and inflammation but also double as neuromodulators (for review see Nigam and Schewe, 2000; Liang et al., 2007). Intriguingly, one of those eicosanoid neuromodulators, the 12-lipoxygenase (12-LOX) metabolite 12-HPETE has long been known as an activator of *Aplysia's* S-type K^+-channels (Piomelli et al., 1987). These K^+ channels, later identified to belong to the TREK-1 family, are instrumental in non-synaptic forms of plasticity underlying behavioral modification of *Aplysia*'s gill withdrawal reflex (Hawkins, 1984) Similar PLA_2- and/or LOX-dependent arachidonic-acid modulated background K^+ channels have been described in *Lymnaea* (Kits et al., 1997; Lopes et al., 1998). Opening of these 4TM2P channels generates an outward current. It is, therefore, conceivable that elevated levels of PLA_2-activity associated with inflammation, oxidative stress, and aging induce a decline in neuronal excitability through activation of these 4TM2P background K^+ channels. An alternative route linking membrane oxidative stress to a decline in neuronal excitability involves

aforementioned aldehyde byproducts of lipid peroxidation. As mentioned MDA, 4-HNE and acrolein can cause irreversible damage to many of the cells macromolecules through covalent conjugation (Farooqui and Horrocks, 2006) or engage in various signaling activities including pathways regulating mitochondrial function and other aspects of cell metabolism (Riahi et al., 2010; Ayala et al., 2014).

Yet another intriguing plausible explanation for the effects of lipid peroxidation on neuronal excitability involves structural rather than signaling arguments. Specifically, as noted before peroxidation very likely incites considerable changes in the molecular geometry of affected PUFAs that affect the way they "fit" in the membrane and may introduce considerable changes in the micro-architecture and biophysical properties of bilayer membranes including parameters such as membrane curvature, membrane viscosity, lipid composition, and distribution. These changes may have various implications for neuronal signaling functions, including the organization of transmembrane signaling complexes, vesicle fusion processes, receptor trafficking, and ion channel functions (Yu et al., 1992; Choi and Yu, 1995; Choe et al., 1995; Patel et al., 2001; reviewed in Piomelli et al., 2007). For example, membrane curvature stresses have been found an important factor in the regulation of neuronal electrical activity through the activation of stretch/mechano sensitive background K^+ channels known to contribute to the regulation of neuronal excitability (**Figure 2**; Patel et al., 2001). Also, the lipid architecture of presynaptic terminal membranes does play a vital role in synaptic Ca^{2+}-triggered exocytosis process raising the possibility that PUFA peroxidation interferes with synaptic functions (reviewed in Davletov and Montecucco, 2010).

Taken together, even though there are still a lot of loose ends in the conceptual framework we sketched here it seems evident that proper management of membrane redox status, lipid peroxidation and phospholipid lysis products generated by or with the aid of PLA_2 is likely critical for functional homeostasis of neurons and by extension the nervous system. Below we summarize and discuss evidence from recent work done in our laboratory trying to fit the pieces of this puzzle together.

PLA_2 – A LIPASE AT THE NEXUS OF AGING, OXIDATIVE STRESS, NEURONAL ELECTRICAL EXCITABILITY CONTROL AND FUNCTIONAL DECLINE OF THE AGING NERVOUS SYSTEM?

In the preceding paragraphs we explored the prospect for interplay between oxidative stress, lipid membrane factors, PLA_2, and changes in intrinsic neuronal excitability as a factor in functional decline of normal aging neurons. In this section we review the evidence that lipid peroxidation-induced PLA_2 activity lays at the root of inflammation-, age-, and oxidative stress-associated functional impairment of the nervous system in *Lymnaea* and put our findings in the context of a growing literature suggesting that we are dealing with an evolutionary widely conserved mechanism.

As described above, aging *Lymnaea* display associative LTM impairment in two functionally and anatomically distinct (i.e., appetitive and respiratory) learning paradigms (Hermann et al., 2007, 2013; Watson et al., 2012a, 2013, 2014; Beaulieu et al., 2014). In both instances, LTM failure was associated with reduced spontaneous action potential activity and enhanced spike frequency adaptation of the two key interneurons underlying both execution and plasticity of the respective behaviors (i.e., CGCs and RPeD1). Both behavioral and electrophysiological facets of age-associated respiratory and appetitive LTM impairment could be reproduced in young animals through treatment with 2,2′- azobis-2-methyl-propanimidamide (AAPH), a water-soluble free radical generator commonly used to induce PUFA peroxidation, furthering the notion of plasmamembrane lipid peroxidation as a significant agent of neuronal and behavioral aging in *Lymnaea* (**Figure 8**). Evidence of increased FFA mobilization from brains and other tissues was found in both old and AAPH-treated young animals (Watson et al., 2013; Beaulieu et al., 2014). Moreover, treatment with the lipid-domain anti-oxidant α-tocopherol reversed decline in neuronal excitability in both experimental oxidative stressed and naturally aged neurons (Watson et al., 2012a,b). Remarkably, all these behavioral, electrophysiological, and biochemical symptoms of aging and experimental oxidative stress could be reversed by treatment with aristolochic acid, a broad spectrum PLA_2 inhibitor that inhibits both Ca^{2+}-dependent cPLA2 and Ca^{2+}-independent $iPLA_2$ (**Figure 8**; Vishwanath et al., 1988; Lopes et al., 1998; Carnevale and Cathcart, 2001).

No evidence was found of significant age-associated impairment or experimental oxidative stress-induced repression of transcription-independent short/intermediate term memory (S/ITM) in either of the two behavioral conditioning paradigms, suggesting that transcription-independent forms of memory appear impervious to aging or oxidative stress. As yet, we have no definitive answer to the question why LTM is selectively susceptible. However, preliminary evidence from our laboratory suggests that both aging and AAPH-induced oxidative stress in participation with PLA_2 alters aspects of mitochondrial functions that may affect their ability to contribute to intracellular Ca^{2+}-buffering. The molecular pathways underlying LTM formation in *Lymnaea* involve "traditional" conserved cAMP- and Ca^{2+}-sensitive CREB- and MAPK-dependent pathways (Ribeiro et al., 2003; Wagatsuma et al., 2006; Guo et al., 2010). It is therefore conceivable that dysregulation of intracellular Ca^{2+} homeostasis disrupts transcription-regulation pathways involved in LTM expression. Furthermore, the observation that PLA_2 inhibition rescues LTM is particularly intriguing in view of evidence implicating FFAs and PLA_2 in mitochondrial uncoupling in pancreatic β-cells (Ježek et al., 2010, 2014). Although speculative at this point in time, it is not unreasonable to postulate that a similar mechanism may underlie the PLA_2-associated LTM impairment in *Lymnaea*.

Intriguingly, selective appetitive LTM impairment could also be induced in young *Lymnaea* through systemic activation of their cellular immune system (Hermann et al., 2013). As before in naturally aged and experimentally oxidation-stressed young animals, inflammation-associated LTM impairment could be rescued by means of PLA_2 inhibition (Hermann et al., 2013). Together, our data indicate that the processes underlying *Lymnaea's*

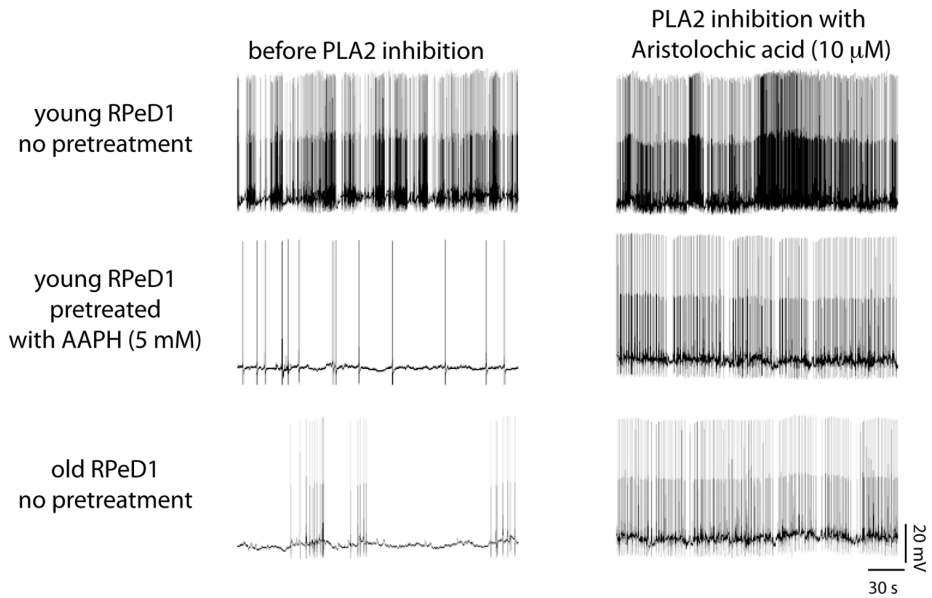

FIGURE 8 | Phospholipase A_2 (PLA_2) contributes to regulation of respiratory neural circuit activity in old and oxidative stressed young brains. Examples of action potential activity recorded from RPeD1 in isolated young central nervous systems (CNSs) pretreated for 30 min with either vehicle-only **(top row)** or the pro-oxidant 2,2-azobis (2-methylpropion-amidine) dihydrochloride (AAPH; **middle row**), and old CNSs maintained in vehicle-only **(bottom row)**, before **(left column)**, and after 30 min of treatment with the PLA_2 inhibitor aristolochic acid **(right column)**. Reprinted from Watson et al. (2013) with permission from Elsevier.

transcription-dependent forms of memory are particularly vulnerable to aging, oxidative stress, and inflammation and that PLA_2 plays a pivotal role in each of these models of associative LTM failure.

PLA_2's AS THERAPEUTIC TARGETS?

PLA_2's and deregulation of lipid metabolism are increasingly implicated in human age-, oxidative stress-, and inflammation-associated conditions including cardiovascular diseases and age-associated neurodegenerative diseases such as Alzheimer's disease (Sun et al., 2004; Farooqui and Horrocks, 2006; Adibhatla and Hatcher, 2008; Sanchez-Mejia et al., 2008; Chalimoniuk et al., 2009; Sanchez-Mejia and Mucke, 2010; Desbene et al., 2012; Gentile et al., 2012; Hui, 2012; reviewed in Sun et al., 2014). They are therefore, not surprisingly, increasingly targeted or considered as targets for therapeutic intervention (see for instance, Garcia-Garcia and Serruys, 2009; Quach et al., 2014). Particularly, it is intriguing that lipoprotein-associated phospholipase A_2 (Lp-PLA_2), also known as platelet-activating factor acetylhydrolase (PAF-AH) was identified as a potential factor in cerebrovascular oxidative stress and inflammation and risk factor for human dementia and subsequently drew considerable interest as a therapeutic target in Alzheimer's disease (Davidson et al., 2012; Acharya et al., 2013; Fitzpatrick et al., 2014). However, there are still a lot of unknowns surrounding the pathways, processes and conditions leading to PLA_2 activation in different cell types that should be resolved before effective and safe therapeutic targets and agents for the treatment and prevention of these increasingly germane age-associated public health concerns (Sun et al., 2014). For instance, whereas many studies implicate PLA_2 hyperactivity as a factor in the etiology of Alzheimer's disease (Sanchez-Mejia and Mucke, 2010; Gentile et al., 2012; Hui, 2012), other studies suggest that hypoactivity of intracellular PLA_2s (cPLA_2 and iPLA_2) are a contributing factor to memory impairment and Alzheimer's neuropathology (reviewed by Schaeffer et al., 2009).

RELEVANCE AND FUTURE DIRECTIONS

The evidence reviewed here infers close ties between inflammation, oxidative stress, lipid-peroxidation, PLA_2-activation, neuronal excitability, and AMI in *Lymnaea* and echo a rapidly growing evidence from basic, clinical, and epidemiological research that PLA2s, inflammation and oxidative stress are important factors in age-related cognitive decline and impairment in humans and other mammals (Sun et al., 2004, 2014; Phillis et al., 2006; Adibhatla and Hatcher, 2008; Lee et al., 2011). Considering this remarkable conservation we believe that *Lymnaea*'s extraordinary suitability for detailed, direct, and tightly integrated molecule-to-behavior dissection of the neurobiological substrates of AMI will in addition to furthering understanding of the biological foundations and parameters of aging potentially also generate valuable insights into mechanisms of and possible solutions for human AMI and other age-associated afflictions of our own nervous system. Before that goal can be achieved many questions need to be answered.

Among the unanswered questions requiring further attention is the identity of the ionic mechanisms through which the products of PLA_2-mediated fatty acyl hydrolysis exert their inhibitory influence on neuronal excitability. As outlined in the preceding pages,

there are many possibilities to test ranging from FA dependent modulation of ion channels to alterations in autacoid modulation of synaptic functions and hypotheses encompassing elements of membrane architecture dependent regulation of neuronal electrical properties.

Another important puzzle to solve is what inhibition of PLA_2, one of the main tools of membrane homeostasis and repair, does to long-term cell/tissues homeostatic integrity. Considering the impact lipid peroxidation is expected to have on membrane structure and function, our finding that PLA_2 inhibition restored rather than aggravated neuro-physiological and behavioral correlates of aging, experimental oxidative stress, and inflammation is puzzling and urges further study.

Yet another intriguing perspective we are currently exploring is mitochondrial involvement in the lipid peroxidation- and PLA_2-dependent declining excitability and plasticity of aging *Lymnaea* neurons. Neurons critically depend on mitochondria to provide the energy to establish and maintain membrane excitability and engage in their quintessential and metabolically costly electrochemical signaling activities (Kann and Kovács, 2007; Mattson et al., 2008). Mitochondria are also a critical partner in intracellular free Ca^{2+} homeostasis and are one of the main sources of RONS and other pro-oxidants. There are many tight reciprocal links between neuronal electrical and mitochondrial activities and many neuronal excitability disorders appear to be associated with mitochondrial aberrancies (Kann and Kovács, 2007; Mattson et al., 2008; Surmeier et al., 2012). Moreover, there is also a substantial body of evidence indicating involvement of mitochondria-associated oxidative stress, lipid signaling, Ca^{2+} dysregulation in normal aging, and the pathogenesis of common age-associated neurological diseases including AD and PD in which lipid peroxidation and PLA_2 may be key partners (reviewed in Thibault et al., 2007; Mattson et al., 2008; Toescu and Vreugdenhil, 2010; Waldbaum and Patel, 2010). The *Lymnaea* model system we have developed provides an excellent platform to investigate these interactions between neuronal energy metabolism, electrical excitability, and plasticity in great detail.

The *Lymnaea* research platform we portrayed here will also be a great tool to investigate fundamental mechanisms of neuronal and behavioral aging and test other existing and emerging aging theories. For example, there is growing evidence that insulin and other insulin-like peptides (ILPs) modulate aspects of plasticity in the *Lymnaea* CNS and enhance learning abilities in older learning-impaired snails (Murakami et al., 2013; Pirger et al., 2014). These findings resonate very well with a rapidly expanding literature that ILPs and insulin resistance are a major endocrine factor in aging in general and aging of the nervous system in particular (Kim and Feldman, 2012; Alcedo et al., 2013). Moreover, because of its identified neurons the model system will allow us to explore many important fundamental scientific questions about aging neurons and brains like: do neurons age according to "plan" or do they fail at random? Are some neuronal physiological phenotypes more vulnerable to aging than others? Can we reverse the effects of aging without repercussion? What are the principles and mechanisms underlying robust and reliable complex brains? Are all changes observed in aging neurons mal-adaptive or are some perhaps compensatory and adaptive? Are neurons and brains life cycles subject to the same resource constraints and trade-offs as other types of cells and tissues in the animal body?

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OPINION ARTICLE
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Oxidative stress and age-related olfactory memory impairment in the honeybee *Apis mellifera*

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The honeybee (*Apis mellifera*) olfactory system is well adapted to detect and discriminate a diverse array of odors. *Apis mellifera* is one of the most important insect species to study olfactory learning and memory because it can be conditioned to respond with feeding movements of the mouthparts (proboscis) to a variety of floral odors by using Proboscis Extension Reflex (PER) conditioning. Honeybee workers switch from in-hive (nursing) to outside-hive (foraging) tasks depending on their age, colony demand, and outside conditions. Workers use their sophisticated cognitive abilities in both foraging, and task performance within the colony (Menzel and Giurfa, 2001; Frasnelli et al., 2014). The honeybee olfactory system possesses olfactory sensory neurons inside the cuticle-covered sensillae along the antennae, which are equivalent to olfactory epithelia within the nasal cavity in vertebrates. From antenna the information is carried via axons of olfactory sensory neurons directly to the antennal lobe (AL) that is equivalent to the olfactory bulb (OB) in vertebrates. This information is processed in the AL then relayed by projection neurons to the mushroom bodies (MB), which contribute to memory consolidation associated with long-term potentiation and synaptic organization (Hourcade et al., 2010). The exhibiting synaptic plasticity in the MB is similar to that of mammalian hippocampal synaptic plasticity (Bliss and Collingridge, 1993).

The neuronal synaptic plasticity can be regulated by important signaling molecules called as "biogenic amines" that modulate synaptic morphology, number of synapses, and receptors, influencing animal behaviors including complex behaviors such as learning and memory formation in both vertebrates and invertebrates. Prominent examples of biogenic amines include epinephrine, norepinephrine, dopamine, serotonin, octopamine, and tyramine. Norepinephrine and epinephrine are preferentially synthesized by vertebrates; whereas octopamine and tyramine are preferentially synthesized by invertebrates. Biogenic amines exert their activity by interacting with specific G-protein coupled receptors, causing changes in the levels of intracellular second messengers (Scheiner et al., 2006). In vertebrates, both the OB and cortex receive heavy inputs from cholinergic, noradrenergic, and serotonergic modulatory systems, exerting profound effects on both odor processing and odor memory by acting on both inhibitory local interneurons γ-amino butyric acid (GABA) and output neurons in both regions (Fletcher and Chen, 2010). The primary sensory afferents from the olfactory neuroepithelium to OB can be modulated by a presynaptic inhibition-mediated by GABA and dopamine released from bulbar interneurons. Increased levels of octopamine in the AL mediate an important role in a reinforcement pathway involving olfactory learning and memory in *Apis mellifera* (Farooqui et al., 2003). Both dopamine and serotonin exert dual roles in appetitive and aversive olfactory memory (Sitaraman et al., 2012; Waddell, 2013); and dopamine and octopamine mediate differential modulation of nicotine-induced calcium in MB kenyon cells (Leyton et al., 2014) in *Drosophila melanogaster*. These findings suggest that biogenic amines play neuromodulatory roles in memory formation in insects like mammals.

Oxidative stress occurs due to a disturbance in the cellular pro-oxidant/antioxidant ratio. It is characterized by increase in reactive oxygen species (ROS) and reactive nitrogen species (RNS), as well as depletion of antioxidant levels. ROS include superoxide anions, hydroxyl and peroxyl radicals, and hydrogen peroxide (H_2O_2), which are generated as by-product of oxidative metabolism (Massaad and Klann, 2011). The major sources of ROS in brain include mitochondrial respiratory chain, uncontrolled arachidonic acid (ARA) cascade, and activation of NADPH oxidase (**Figure 1**). Another mitochondrial source of ROS is the enzyme family of monoamine oxidases (MAO) that catalyze the oxidation of biogenic amines, generating free radicals. Like ROS, RNS are formed during amino acid metabolism with the generation of nitric oxide (NO), which reacts with superoxide radical ($O_2^{\bullet -}$) to form peroxynitrite ($ONOO^-$) that interacts with lipids, DNA, and proteins, disrupting cell signaling to overwhelming oxidative and nitrosative injury (Pacher et al., 2007).

In neural cells, ROS modulate gene expression through phosphorylation, activation and oxidation of transcription factors, adhesion molecules and chemotactic factors, antioxidant enzymes, and vasoactive substances such as Nuclear factor erythroid 2-related factor 2 (Nrf2), activator protein 1 (AP1), nuclear factor-kappaB (NF-kB), FOXO transcription factors (FOXO), hypoxia-inducible

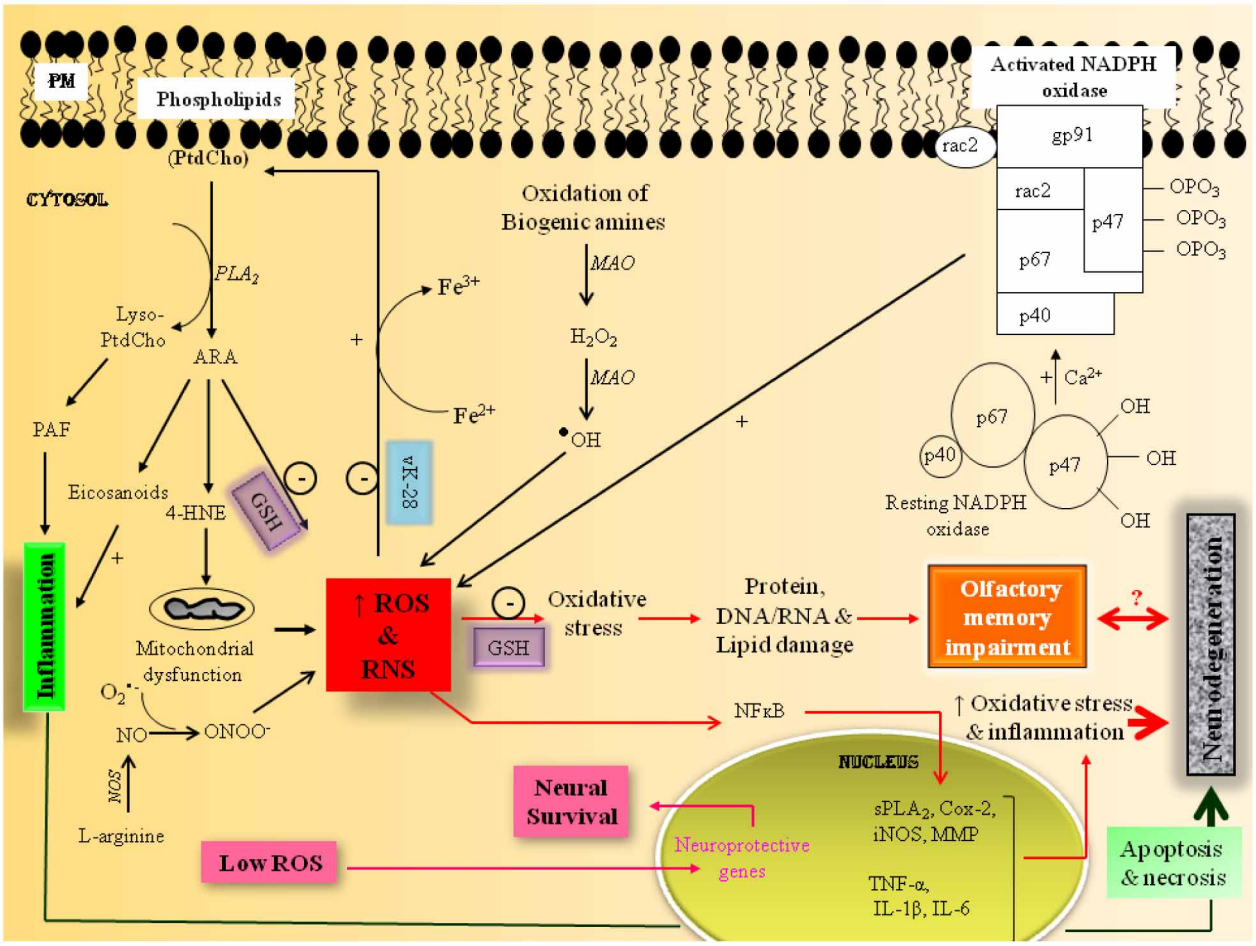

FIGURE 1 | Hypothetical scheme showing the potential molecular mechanisms involved with oxidative stress-mediated impairment of olfactory memory.

factor-1alpha (HIF-1α), p53, and heat shock proteins (Hsps) (Massaad and Klann, 2011). Many of these transcription factors contain redox-sensitive cysteine residues in their DNA binding sites. Oxidative modifications of these residues affect DNA binding and subsequently regulate gene transcription of redox sensitive genes. Low levels of ROS induce Nrf2, a potent transcription factor, which modulates genes for NADPH quinone oxidoreductase (NQO1), glutathione S-transferase, heme oxygenase-1 (HO-1), ferritin, and γ-glutamylcysteine synthetase. Similarly, moderate increase in ROS results in activation of transcription factor AP-1, which modulates immediate early genes (c-fos and c-jun). The activation of Nrf2 and AP1 modulates transcription of neuroprotective genes, contributing to neural cell survival. In contrast, high levels of ROS activate transcription factor, NF-κB, which modulates the transcription of proinflammatory cytokines (TNF-α, IL-1β, IL-6) and enzymes ($cPLA_2$; COX-2, LOX, iNOS, MMP). High levels of ROS accumulation results in opening of membrane permeability transition pore, and release of cytochrome c from the mitochondria, leading to apoptotic cell death (**Figure 1**). Excessive ROS decrease cognitive performance in mammals (Massaad and Klann, 2011) as well as in honeybees (Farooqui, 2008; Williams et al., 2008). Excessive ROS and RNS production is associated with mitochondrial dysfunction-mediated neuronal damage, which involves deficits in olfactory memory in neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD) (Haehner et al., 2007; Bahar-Fuchs et al., 2011). The olfactory nervous systems of insects and mammals exhibit many anatomical and physiological similarities. Therefore, purpose of present opinion is to validate whether honeybee can serve as an excellent tool for studying candidate genes responsible for ROS-mediated olfactory dysfunction in aging and neurodegenerative diseases.

Biogenic amines like octopamine or dopamine modify neural function at multiple levels, modifying insect behavior. Octopamine or dopamine can substitute for the appetitive and aversive reinforcements, respectively. The disruption of octopamine receptor (AmOA1) in the AL of the honeybee brain inhibits olfactory acquisition and recall without

altering odor discrimination, suggesting that octopamine mediates consolidation of a component of olfactory memory at this early processing stage (Farooqui et al., 2003). However, in the MB disruption of AmOA1 does not affect acquisition but significantly inhibits recall, implicating that octopamine is not involved in early stages of immediate memory encoding in the MB (Farooqui et al., unpublished). Age-dependent changes in brain biogenic amine levels are associated with morphological development and behavioral plasticity in honeybees (Harris and Woodring, 1992). A marked deficit in olfactory learning and memory in honeybees can be achieved by the flight activity, environmental and physiological stress (Morimoto et al., 2011), aging (Farooqui, 2007) and presence of excessive iron in the brain (Farooqui, 2008), implicating a link between ROS-mediated oxidative stress and olfactory dysfunction. Similarly, increase in oxidative stress in the olfactory epithelium of AD patients is accompanied by olfactory impairment (Getchell et al., 2003; Perry et al., 2003). The abnormal levels of biogenic amines and disruption in signal transduction also seem to be linked with olfactory memory defecits in PD and AD (Marien et al., 2004). Thus, ROS-mediated mechanisms underlying olfactory dysfunction in mammals and honeybees seem to be analogous.

A variety of model systems are available for aging and neurodegenerative diseases. Fundamental processes in models can be shared with humans; however, models can never capture the full complexity of the human condition. Genetic models fail to phenocopy the human diseases in that they generally lack a behavioral phenotype and/or the characteristic pathological feature of human disease. Moreover, there is a distinct inability of either approach to model sporadic, late-onset neurodegenerative disease, which accounts for over 90% of human cases. In spite of limitations, models have been used to study the molecular mechanism of brain diseases. *Drosophila melanogaster* has provided significant insights into the mechanisms of learning and memory using genetic approaches combined with molecular, anatomical, and behavioral tools (Davis, 2004). However, *Drosophila* is not capable of producing higher order cognitive behaviors like humans. In contrast, *Apis mellifera* has been shown to be a great model in cognitive neuroscience because of its sophisticated cognitive abilities (Menzel and Giurfa, 2001; Frasnelli et al., 2014). The life span of honeybee workers can range from 6 weeks to more than 6 months depending on season, and temporal caste (Omholt and Amdam, 2004). All workers can have similar genotypes due to genetic manipulation; therefore their life span is mainly regulated by environmental factors. *Apis mellifera* genome is evolved more slowly and more similar to vertebrates for circadian rhythm, RNA interference, DNA methylation, and learning and memory genes than other insects. It has fewer genes for innate immunity, detoxification enzymes, cuticle-forming proteins, and gustatory receptors, but more odorant receptor genes compared to fruit fly and mosquito, and novel genes for nectar and pollen utilization (Weinstock et al., 2006). *Apis mellifera* brain contains only one million neurons (five orders of magnitude less than the human brain but four times greater than *Drosophila*). Oxidative stress can be successfully induced in honeybee worker brain. It is also amenabile to molecular, genetic, pharmacological, and social manipulations. These characteristics favor *Apis mellifera* worker for serving as an excellent tool for testing the role of candidate genes for ROS-mediated olfactory dysfunction. This may lead to better understanding of molecular mechanisms involved with olfactory dysfunction in human with aging and neurodegenerative diseases.

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PERSPECTIVE ARTICLE
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Emerging functional similarities and divergences between *Drosophila* Spargel/dPGC-1 and mammalian PGC-1 protein

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Peroxisome Proliferator Activated Receptor Gamma Co-activator-1 (PGC-1) is a well-conserved protein among all chordates. Entire *Drosophila* species subgroup carries a *PGC-1* homolog in their genome called *spargel/dPGC-1* showing very little divergence. Recent studies have reported that significant functional similarities are shared between vertebrate and invertebrate PGC-1's based on their role in mitochondrial functions and biogenesis, gluconeogenesis, and most likely in transcription and RNA processing. With the help of genetic epistasis analysis, we established that *Drosophila* Spargel/dPGC-1 affects cell growth process as a terminal effector in the Insulin-TOR signaling pathway. The association between Spargel/dPGC-1 and Insulin signaling could also explain its role in the aging process. Here we provided a further comparison between Spargel/dPGC-1 and PGC-1 focusing on nuclear localization, oxidative stress resistance, and a possible role of Spargel/dPGC-1 in oogenesis reminiscing the role of Spargel in reproductive aging like many Insulin signaling partners. This led us to hypothesize that the discovery of newer biological functions in *Drosophila* Spargel/dPGC-1 will pave the way to uncover novel functional equivalents in mammals.

Keywords: *Drosophila*, PGC-1, mitochondria, oogenesis, RNA processing

Homeothermic mammals utilize the Peroxisome Proliferator Activated Receptor Gamma Co-activator 1 (PGC-1) as a thermogenic regulator (maintains body temperature) to protect against excessive cold or excess calorie intake (Puigserver et al., 1998). Thus PGC-1 is expressed in tissues with high metabolic requirement and it is linked to multiple metabolic pathways such as gluconeogenesis, adipogenesis, myogenesis, and mitogenesis (Handschin and Spiegelman, 2006). Apart from metabolism, PGC-1 might play a central role in maintaining oxidative homeostasis (Austin and St-Pierre, 2012). Homologs of *PGC-1* were found in all chordates including the fish genomes (Lin et al., 2005) where body temperature regulation isn't necessary (with the exception of a few marine species). Among invertebrate models, only *Drosophila* carries a single *PGC-1* homolog in its genome (Gershman et al., 2007) whereas other invertebrate models such as yeast and *C. elegans* do not carry any *PGC-1* homologous sequence (Lin et al., 2005). *Drosophila PGC-1*, designated as *spargel/dPGC-1* (Tiefenbock et al., 2010), is well conserved in distantly related *Drosophila* species subgroups with its C-terminal RS and RRM domains (**Figure 1A**). Two notable differences between PGC-1 and Spargel/dPGC-1 are: the C-terminal FDSLL domain of PGC-1 is replaced with FEALL in all *Drosophila* species (Gershman et al., 2007; Tiefenbock et al., 2010; Bugger et al., 2011), and the larger size of Spargel/dPGC-1 protein as it carries ∼300 more amino acids than PGC-1 (**Figure 1A**). Although nuclear receptors are generally known to interact with the leucine rich motifs (LXXLL) (Matsuda et al., 2004), the FEALLL variant of this motif in *Drosophila* is able to interact with the nuclear receptors as well (Wang et al., 2007). In light of the fact that significant functional overlap exists between the three PGC-1 homologs in mice PGC-1α, PGC-1β and PRC, which makes it difficult to tease apart their relative roles *in vivo*, we propose that the presence of a single *Drosophila PGC-1* homolog will provide an enormous advantage to study the function of this essential transcriptional coactivator in an alternate model. Within the last few years, significant functional homologies have surfaced between mammalian PGC-1 and *Drosophila* Spargel/dPGC-1, which called for a discussion of this topic in greater detail.

ENERGY METABOLISM

As a transcriptional coactivator PGC-1 activates many nuclear receptor, which in turn regulate the transcription activity of variety of nuclear genes (Puigserver and Spiegelman, 2003). Similarly, Spargel/dPGC-1 regulates the expression of mitochondrial oxidative phosphorylation (OXPHOS) genes through *NRF1* (Nuclear Respiratory Factor) homolog *delg* (Tiefenbock et al., 2010). *Spargel/dPGC-1* gain of function (overexpression) correlates with an increased rate of mitochondrial oxygen consumption (Rera et al., 2011) and ATP production (Mukherjee and Duttaroy, 2013), enhanced mitochondrial DNA content, increased enzyme activity and protein production in the mitochondrial matrix (Rera et al., 2011). These observations are reminiscent of the effect of PGC-1α on mitochondrial biogenesis, functional capacity and energy metabolism (Liu and Lin, 2011). Thus, regulation of

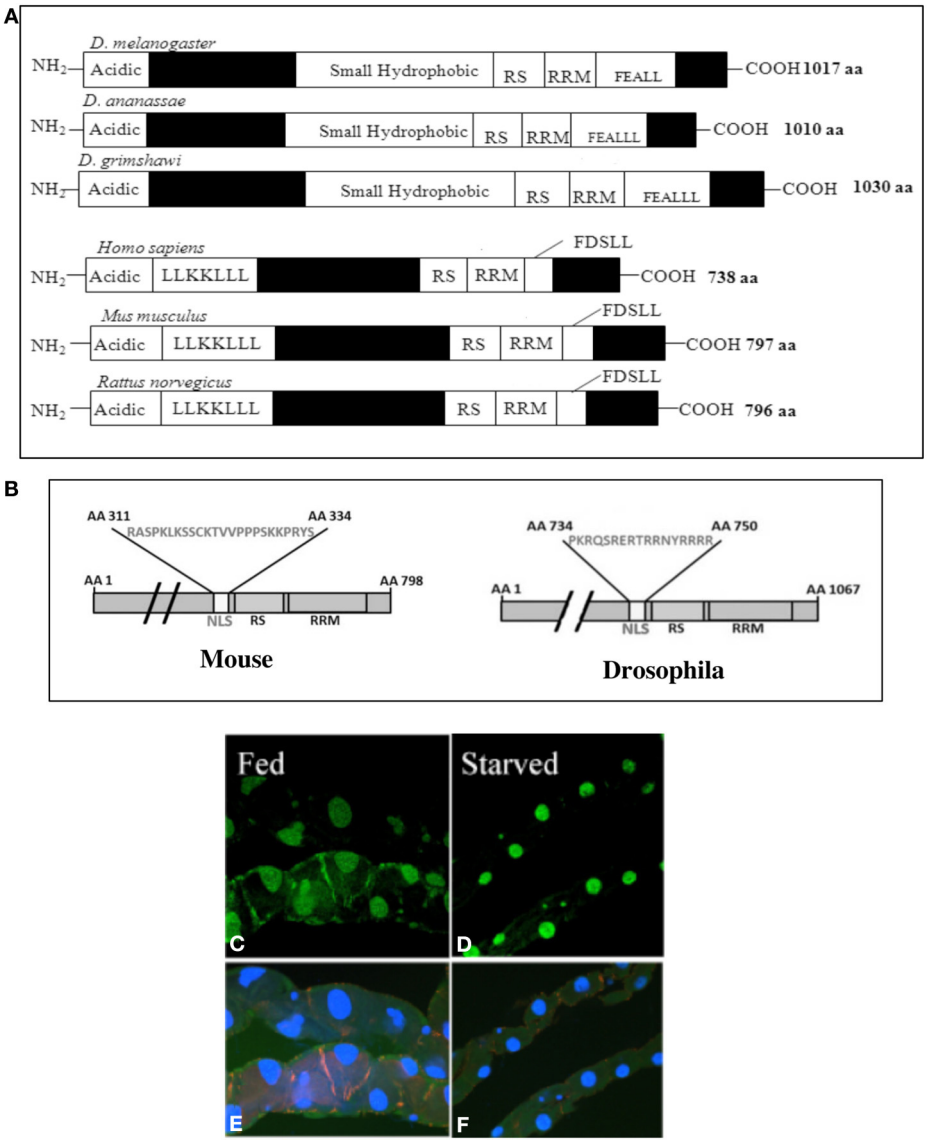

FIGURE 1 | (A) Comparison of the Spargel/dPGC-1 protein structure in 3 widely divergent subgroups of *Drosophila melanogaster*, *D. ananassae*, and *D. grimshawi* (van der Linde and Houle, 2008). Vertebrate PGC-1 is about 300 amino acids shorter in length than the Spargel/dPGC-1. Filled boxes represent regions of non-homology. **(B)** An authentic Nuclear Localization Signal (NLS) has been found in mammalian PGC-1 with the help of NLS Predict Software. **(C)** Nuclear localization of Spargel is documented in the gut tissue with a Spargel-GFP protein (green). Following 48 h of starvation, the gut turns thinner and Insulin signaling is reduced, but it imposes no effect on nuclear localization of the Spargel-GFP protein **(D)**. **(E,F)** DAPI staining of the same gut tissue.

mitochondrial function is truly an ancestral function for PGC-1 group of proteins.

INTRACELLULAR LOCALIZATION

Serine-Arginine (SR) repeats and RNA Recognition Motifs (RRM) are classical hallmarks of "RNA processing domains." Furthermore, localization of PGC-1 in the nuclear compartment where it is concentrated in nuclear speckles along with splicing factor α-U1 and SR splicing factor SC-35 are irrefutable evidence that PGC-1 is involved in the splicing complex (Monsalve et al., 2000). We recently demonstrated that a Spargel-GFP fusion protein also localizes itself in the nucleus forming distinct punctate structures (Mukherjee and Duttaroy, 2013). An authentic Nuclear Localization Signal (NLS) was uncovered in Spargel/dPGC-1 with the help of a NLS predict software (Mukherjee and Duttaroy, 2013) that most likely assists in Spargel/dPGC-1 localization into the nucleus. With the help of the same software we now located a NLS between the amino acids 311–334 in mammalian PGC-1 (score 0.93) (**Figure 1B**), which should also justify the presence of PGC-1 protein in the nucleus (Monsalve et al., 2000).

An earlier study claimed that activation of Insulin signaling is important for transport of Spargel/dPGC-1 protein from cytoplasm to the nucleus (Tiefenbock et al., 2010) and Spargel/dPGC-1 is now established as a member of the

Insulin-TOR signaling pathway (Mukherjee and Duttaroy, 2013). Nutrient availability controls the Insulin/TOR signaling pathway from TOR downstream (Takano et al., 2001) therefore, under starvation condition, Insulin mediated signal transduction is reduced. We rationalized that if Insulin signaling is essential for nuclear localization of Spargel/dPGC-1 then reduction of this signal should sequester the protein to the cytosol. To test this prediction, gut malphigian tubule preparations were obtained from flies following two days of starvation (only water given to prevent dehydration) and analyzed for Spargel expression. Since the localization of Spargel/dPGC-1 is still restricted to the nucleus following starvation (**Figures 1C–F**) this supports that cellular localization of Spargel/dPGC-1 occurs independent of Insulin signaling.

Spargel/dPGC-1 DOESN'T INFLUENCE ANTIOXIDANT ENZYMES

The apparent involvement of PGC-1 in oxidative metabolism has been established from the following: PGC-1 activates Nuclear Respiratory Factor1 (*NRF1*); muscle specific overexpression of PGC-1α induces specific antioxidants like *Sod2* and *GpX* transcription activity (St-Pierre et al., 2006) whereas ablation of PGC-1α in cultured cell cause down regulation of a whole panel of antioxidants including *SOD1, SOD2, GpX*, *UCP1*, and *UCP2,* resulting in hypersensitivity to hydrogen peroxide induced oxidative stress (St-Pierre et al., 2006). Another interesting observation is that cells from patients with Friedrich's Ataxia show coordinated suppression of PGC-1 and antioxidant enzymes (Coppola et al., 2009). It was inferred from these observations that PGC-1 controls mitochondrial reactive oxygen species (ROS) by regulating the antioxidant defense system (Austin and St-Pierre, 2012).

We attempted to validate this prediction in a whole animals model by utilizing the *Drosophila spargel* mutant hypomorph, srl^1 (Tiefenbock et al., 2010) and a *spargel* transgenic line that is capable of overexpressing Spargel/dPGC-1 (Mukherjee and Duttaroy, 2013). For systemic oxidative stress generation, we used methyl viologen (commercially known as "paraquat") that is stable at room temperature. Male and female flies overexpressing Spargel were exposed to 20 mM paraquat and both survived better than the control (**Figures 2A,B**). After 48 h of paraquat treatment, Spargel/dPGC-1 overexpression helped 50% males to remain viable, where as in control flies 50% survival was attained within 24 h. Female flies appear slightly more sensitive to paraquat treatment although a significant difference still persists with respect to the control (**Figures 2A,B**). This increased resistance to oxidative stress in Spargel/dPGC-1 overexpressing flies makes it tempting to conclude that Spargel/dPGC-1 is also involved in oxidative stress resistance and imposes the same effect on antioxidant defense enzymes like PGC-1. However, this expectation may not be true since the expression of two front line antioxidant defense enzymes SOD2 and SOD1 remain unchanged when Spargel/dPGC-1 is overexpressed (gain of function) or reduced in srl^1 hypomorphs (**Figure 2C**). Since these experiments were performed on whole animals as opposed to cultured cells, it might be necessary to check the status of these antioxidant enzymes in *PGC-1KO* mice. Secondly,

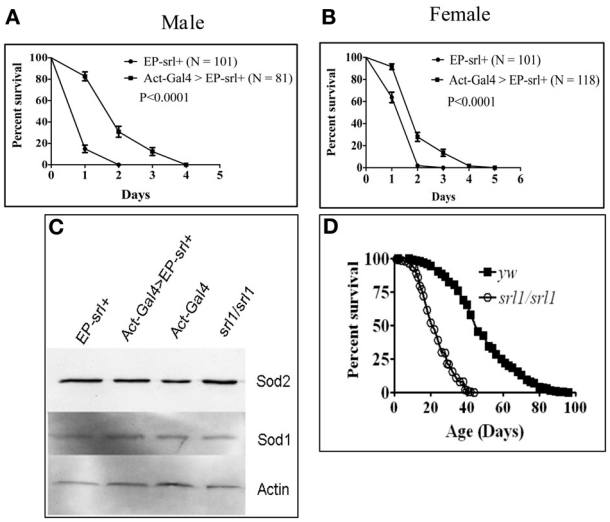

FIGURE 2 | Spargel gain of function was achieved by activating an *EP-srl*$^+$ transgene ubiquitously with the help of *Actin-GAL4* driver (see Kapahi et al., 2004, for detail). Percent survival was measured following exposure to 20 mM paraquat made in 1% sucrose solution. **(A,B)** Male and female flies overexpressing Spargel/dPGC-1 survive longer in paraquat than the control indicating that Spargel/dPGC-1 overexpression cause increased resistance to oxidative stress, **(C)** Spargel/dPGC-1 overexpression or its ablation in srl^1 does not alter the expression of SOD2 and SOD1 antioxidant enzymes, **(D)** Reduced Spargel/dPGC-1 in srl^1 cause significant reduction in life span.

since it is involved in the Insulin-TOR signaling (Mukherjee and Duttaroy, 2013), Spargel/dPGC-1 might utilize other stress resistance pathways (which remain to be tested) such as FoxO or Jnk as opposed to antioxidant enzymes. Incidentally, a recent study in *Drosophila* showed that *Wolbachea* induced metabolic stress promotes mitogenesis through activation of Spargel/dPGC-1 (Chen et al., 2012). Clearly, more needs to be done to understand the relationship between Spargel/dPGC-1 and stress resistance.

GROWTH, LONGEVITY, AND AGING

Insulin-TOR signaling pathway is involved in systemic regulation of growth, longevity and aging in all taxa (Hietakangas and Cohen, 2009; Partridge et al., 2011). In the absence of Insulin, PGC-1 expression is elevated in liver and gluconeogenesis is initiated (Puigserver et al., 2003). Therefore liver-specific knock down of PGC-1 shows higher insulin sensitivity in mice (Koo et al., 2004). On the contrary, absence of PGC-1 in skeletal muscles imposes no effect on insulin sensitivity (Zechner et al., 2010). Thus one can assume that the metabolic role of PGC-1 and its relationship with Insulin/TOR signaling became more tissue specific during the course of evolution.

The requirement of Spargel/dPGC-1 in the Insulin-TOR signaling pathway (Tiefenbock et al., 2010; Mukherjee and Duttaroy, 2013) automatically connects it to the cell growth process. Spargel/dPGC-1 is a terminal effector of this pathway hence reduced *spargel* expression results in growth retardation with smaller body size and developmental delays,

though Spargel/dPGC-1 overexpression has no immediate effect on growth (Rera et al., 2011; Mukherjee and Duttaroy, 2013).

With respect to longevity, members of the Insulin-TOR signaling pathway extend life span in *C. elegans* (Vellai et al., 2003; Kenyon, 2010; Partridge et al., 2011), *Drosophila* (Clancy et al., 2001; Tatar et al., 2001; Kapahi et al., 2004) and mice (Fontana et al., 2010; Kenyon, 2010) by delaying the aging process. Mutant forms of *Insulin receptor (InR)*, the Insulin receptor substrate *chico* and TOR all extend longevity, whereas for *FoxO* lifespan extension happens through its overexpression. Spargel/dPGC-1's action on longevity is apparent as reduced Spargel/dPGC-1 expression cause significant shortening of life span (**Figure 2D**). Reduced ROS production and stem cell over proliferation were cited as two major reasons for Spargel/dPGC-1 mediated extension of lifespan (Rera et al., 2011), though the gain of function effects are specific to the intestinal stem cells as opposed to the Insulin and TOR mediated lifespan extensions, which are largely ubiquitous. So, research on *Drosophila* is pioneering for understanding the aging aspects of the PGC-1 group of proteins, and we are anxiously awaiting studies on how the mammalian PGC-1 protein influences aging.

To summarize, the effects of Spargel/dPGC-1 on growth and survival are positive, like all the other members of the Insulin/TOR signaling pathway (Mukherjee and Duttaroy, 2013). So, *spargel/dPGC-1* loss of function is lethal (Duttaroy et al., in preparation), *spargel/dPGC-1* hypomorphs have a much shorter life span (**Figure 2D**) where as ubiquitous gain of Spargel/dPGC-1 function does not extend lifespan (Rera et al., 2011). Thus, Spargel/dPGC-1 completes the function of Insulin-TOR pathway leading to survival.

IS Spargel/dPGC-1 ESSENTIAL FOR FEMALE FERTILITY?

Reduced Spargel/dPGC-1 expression causes only a few viable adults to appear from srl^1 homozygous mothers, indicating that fecundity is seriously compromised in srl^1 hypomorphic females though the fertility of srl^1 males remain unchanged (**Table 1**). The growth retardation effect of srl^1 could be the reason for the appearance of dysgenic ovaries, which carry about half the number of ovarioles compared to the wild type (**Figures 3A,B**). A simple time course analysis of oogenesis revealed that srl^1 ovaries develop slowly. In 24 h post eclosion (PE) wild type ovarioles reach up to stage 10/11 whereas ovarioles in srl^1 are lagging behind in stage 6/7. By 48 h mature oocytes appear in the wild type ovarioles, whereas most srl^1 ovarioles are observed around stage 10 during this time (**Figure 3C**). Efforts are underway to pin down the requirement of Spargel/dPGC-1 during oogenesis through its selective ablation in the ovaries. Interestingly, decreased female fecundity results from oogenesis defects in *InR* and *chico* mutants (Tatar et al., 2001; Partridge et al., 2011). These observations suggested a correlation between Insulin signaling and reproductive aging. Incidentally, the effect of srl^1 mutant on

Table 1 | Fertility of srl^1 females.

Genotype	Fecundity (# of adults)
y w	361
y w; srl^1 female X *y w* male	8
y w; srl^1 male X *y w* female	296
y w; srl^1 female X *y w,* srl^1 male	0

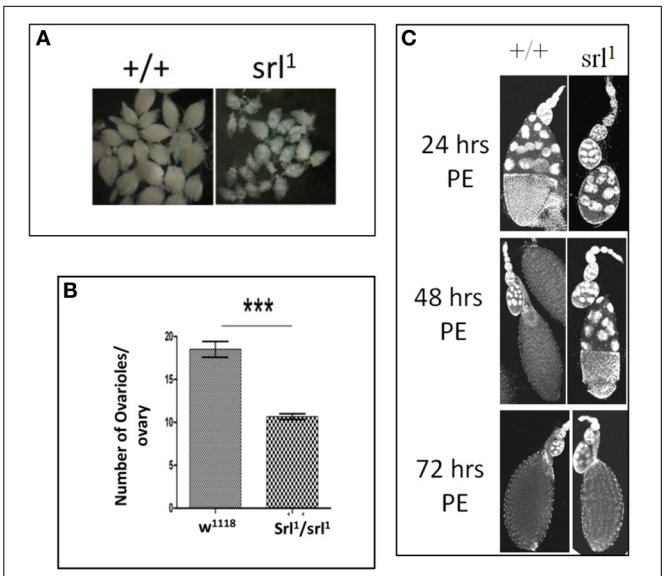

FIGURE 3 | Possible requirement of Spargel/dPGC-1 during oogenesis. **(A)** Mature ovaries from *spargel* hypomorph srl^1 females are much smaller in size with respect to the control ovaries of same age, **(B)** Quantification of number of ovarioles suggest srl^1 ovaries carry about 40% less number of ovarioles with respect to the wild type ovaries. **(C)** Time course analysis of oogenesis shows srl^1 ovaries matures more slowly in comparison to the wild type ovaries (PE, post eclosion). $^{***}P < 0.001$.

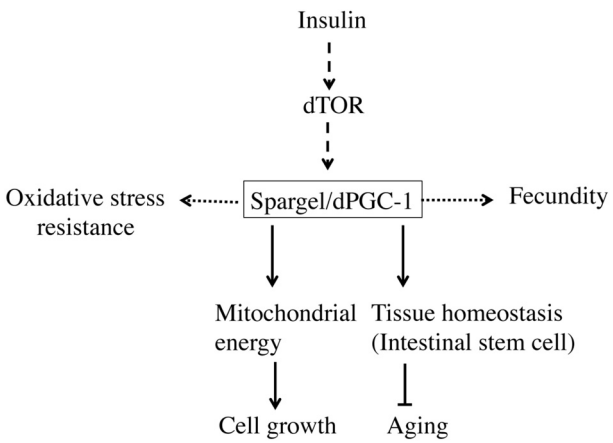

FIGURE 4 | Spargel/dPGC-1 plays a central role in maintaining homeostasis. Spargel/dPGC-1 is acting downstream of Insulin/TOR signaling pathway. At this position, Spargel/dPGC-1 is regulating the mitochondrial energy flow into the system leading to cell growth. In addition, Spargel/dPGC-1 provides oxidative stress resistance. Though unlike mammals, this stress resistance does not confer SOD1 and SOD2 protein expression. Spargel/dPGC-1 also affects *Drosophila* fecundity possibly through oogenesis. Moreover, Spargel/dPGC-1 regulates tissue homeostasis by controlling over proliferation of intestinal stem cells in older flies.

female fecundity and oogenesis appears to suggest that Spargel could be important for reproductive aging.

In summary, the PGC-1 group of proteins retained many important biological functions between vertebrates and invertebrates, though many are still unknown. The overarching hypothesis of this article is that Spargel/dPGC-1 can pave the way to uncover newer biological functions, which can be tested in mammalian PGC-1 (**Figure 4**). Given the amount of functional overlaps already existing between PGC-1 and Spargel/dPGC-1, some of these similarities may be of immediate interest, including the role of the PGC-1 group of proteins on transcription and RNA processing and finding PGC-1 interacting proteins involved in growth and metabolism. Available genetic tools and genomic reagents in *Drosophila* should come in handy for exploring the functionality of this omnipotent transcription co-activator.

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Use of *Caenorhabditis elegans* as a model to study Alzheimer's disease and other neurodegenerative diseases

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Advances in research and technology has increased our quality of life, allowed us to combat diseases, and achieve increased longevity. Unfortunately, increased longevity is accompanied by a rise in the incidences of age-related diseases such as Alzheimer's disease (AD). AD is the sixth leading cause of death, and one of the leading causes of dementia amongst the aged population in the USA. It is a progressive neurodegenerative disorder, characterized by the prevalence of extracellular Aβ plaques and intracellular neurofibrillary tangles, derived from the proteolysis of the amyloid precursor protein (APP) and the hyperphosphorylation of microtubule-associated protein tau, respectively. Despite years of extensive research, the molecular mechanisms that underlie the pathology of AD remain unclear. Model organisms, such as the nematode, *Caenorhabditis elegans*, present a complementary approach to addressing these questions. *C. elegans* has many advantages as a model system to study AD and other neurodegenerative diseases. Like their mammalian counterparts, they have complex biochemical pathways, most of which are conserved. Genes in which mutations are correlated with AD have counterparts in *C. elegans*, including an *APP*-related gene, *apl-1*, a tau homolog, *ptl-1*, and presenilin homologs, such as *sel-12* and *hop-1*. Since the neuronal connectivity in *C. elegans* has already been established, *C. elegans* is also advantageous in modeling learning and memory impairments seen during AD. This article addresses the insights *C. elegans* provide in studying AD and other neurodegenerative diseases. Additionally, we explore the advantages and drawbacks associated with using this model.

Keywords: ***apl-1, C. elegans,* Alzheimer's disease, ALS, Parkinson disease, model systems**

INTRODUCTION TO ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the 6th leading cause of death in the US and affects more than 35 million people worldwide (Alzheimer's Disease International, 2014). AD is a neurodegenerative disease characterized by a progressive loss of memory. Most cases of AD occur sporadically in aged people (>60 years, late-onset AD) without a clear inheritance pattern. However, in 5% of the cases (familial or early onset AD) AD symptoms appear earlier and are linked with gene mutations. Both forms of AD have two main neuropathologic features: the presence of extra-neuronal amyloid plaques, often referred to as senile plaques, and intraneuronal neurofibrillary tangles (Kidd, 1964; Luse and Smith, 1964; Terry et al., 1964; Krigman et al., 1965). Amyloid plaques are aggregates of the beta-amyloid peptide (Aβ), a cleavage product of the amyloid precursor protein (APP; Glenner and Wong, 1984; Masters et al., 1985; Kang et al., 1987). Hyperphosphorylation of the microtubule associated protein tau causes its polymerization into paired helical filaments (PHFs) and, presumably, its formation into neurofibrillary tangles (Goedert et al., 1989a).

Mutations in the *APP* gene and/or the enzymes involved in APP processing (γ-secretase components presenilins, PSEN1 and PSEN2; Chartier-Harlin et al., 1991; Goate et al., 1991; Murrell et al., 1991; Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995) are correlated with early onset AD. These mutations increase the levels of toxic Aβ species and promote neurodegeneration. By contrast, a recently identified mutation in *APP* affects cleavage of APP, causing less Aβ production and conferring neuroprotective benefits (Jonsson et al., 2012). Despite the significant advances made using *APP* transgenic and knock-out models in mammals, unraveling the cellular role of APP has been difficult. Alternative animal models provide complementary approaches to dissecting the function of APP and tau. In this review, we discuss the latest uses of the nematode *Caenorhabditis elegans* as a model system for the study of AD. We also include a brief review of a few representative examples of how *C. elegans* is being utilized to model other neurodegenerative diseases.

C. elegans AS A MODEL FOR ALZHEIMER'S DISEASE

Caenorhabditis elegans is a free-living, non-parasitic nematode that was first introduced as a model organism by Sydney Brenner in 1963 (Brenner, 1974). It is a small (1 mm in length), transparent roundworm, which makes it easy for manipulation, and has a short life cycle of 3 days from egg to adult at 25°C (Brenner, 1974). Under suitable growing conditions, hatched animals develop through four larval stages (L1–L4), each punctuated by a molt, to arise as an adult hermaphrodite with 959 somatic cells (Sulston and Horvitz, 1977). Its life span is between 2 and

3 weeks, which facilitates the study of its biology. Completion of the *C. elegans* genome sequence in 1998 (*C. elegans* Sequencing Consortium, 1998) demonstrated that roughly 38% of worm genes have a human ortholog, such as *APP* and *tau* (Shaye and Greenwald, 2011). Hence, *C. elegans* has many excellent advantages as an *in vivo* model for the study of AD and other neurodegenerative diseases.

MOLECULAR PATHWAYS OF APP. SIMILARITIES AND DIFFERENCES BETWEEN MAMMALS AND *C. elegans*

FUNCTION AND PROCESSING OF APP: NON-AMYLOIDOGENIC AND AMYLOIDOGENIC Aβ PATHWAY

The APP family of proteins contains three members, APP, APLP1, and APLP2 (Wasco et al., 1992, 1993; Sprecher et al., 1993; Sandbrink et al., 1994; Slunt et al., 1994), which are characterized by a large extracellular region containing conserved E1 and E2 domains, a single transmembrane domain, and a small cytosolic domain (Kang et al., 1987). APLP1 and APLP2 do not contain the Aβ sequence and, hence, do not produce Aβ (**Figure 1A**; Wasco et al., 1992, 1993). The APP gene family is required for viability and brain development. *APP* mouse knockouts were viable, but had behavioral and cognitive defects (Ring et al., 2007). While knockout of APLP1 resulted in postnatal growth defects (Heber et al., 2000), mice in which APLP2 was inactivated appeared wild type (von Koch et al., 1997). Double knockouts of APLP2 and either APP or APLP1, however, resulted in postnatal lethality (von Koch et al., 1997; Heber et al., 2000); the lethality of *APP/APLP2* double knockouts could be rescued by knock-in of an APP extracellular fragment, sAPPα (Weyer et al., 2011), suggesting that sAPPα is sufficient for viability. The triple knockout caused lethality and a type II lissencephaly and cortical disorganization (Herms et al., 2004). Collectively, these results suggest that APP family members have essential and redundant functions during development, including proper brain development, and these functions do not require Aβ.

In mammals, APP is processed through two proteolytic pathways, only one of which produces Aβ (**Figure 1B**; Haass et al., 1992, 1994a,b). In the non-amyloidogenic pathway, APP is first cleaved by an α-secretase within the Aβ sequence to release an extracellular fragment, sAPPα (**Figure 1B**). The remaining APP fragment (known as APP-CTFα or C83) is then cleaved by the γ-secretase complex to release the APP intracellular domain (AICD) to the cytosol. By contrast, in the amyloidogenic pathway, after cleavage by the β-secretase (BACE) to release sAPPβ, the remaining APP fragment (known as APP-CTFβ or C99) is then cleaved by the γ-secretase complex, liberating Aβ to the lumen and AICD to the cytosol (Gu et al., 2001; Sastre et al., 2001; Weidemann et al., 2002). This latter pathway is likely favored in AD patients.

Mammalian γ-secretase is a protease complex consisting of several components: presenilins 1 and 2 (PSEN1 and PSEN2), nicastrin (NCT), anterior pharynx defective (APH-1), and the presenilin enhancer (PEN-2; Yu et al., 2000; Francis et al., 2002). PSEN1 and PSEN2 are the catalytic components of the γ-secretase complex. NCT works as a stabilizing cofactor required for γ-secretase complex assembly and trafficking (Li et al., 2003; Zhang et al., 2005) and PEN-2 and APH-1 have a role in the maturation process of PSEN1 and PSEN2 (Luo et al., 2003). Besides APP, the γ-secretase complex is also involved in the proteolysis of Notch receptors, and the first identification of any PEN-2 or APH-1 ortholog was in *C. elegans* as the result of a genetic screen for modifiers of the Notch pathway (Francis et al., 2002; Goutte et al., 2002). Within the γ-secretase complex, only mutations in PSEN1 and PSEN2 have been associated with early onset AD (Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995).

PROCESSING OF *C. elegans* APL-1

In *C. elegans* there is only one APP-related gene, *apl-1*. Like human APP (Kang et al., 1987), APL-1 contains a large extracellular region whose conserved E1 and E2 domains share 46 and 49% sequence similarity to human APP, respectively, a transmembrane domain, and a relatively small cytosolic domain, which shares 71% sequence similarity to human APP (**Figure 1A**; Daigle and Li, 1993). Notably, unlike APP but similar to APLP1 and APLP2, APL-1 does not contain the Aβ sequence (Daigle and Li, 1993).

Two α-secretase proteins are present in *C. elegans*, SUP-17 and ADM-4 (Jarriault and Greenwald, 2005). They work redundantly in the cleavage of the *C. elegans* Notch homologs, LIN-12 and GLP-1 (Jarriault and Greenwald, 2005). However, no experiments thus far have tested whether SUP-17 or ADM-4 cleaves APL-1. No BACE ortholog has been identified by bioinformatic searches and no β-secretase activity that cleaves human APP has been detected in *C. elegans*, suggesting that APL-1 is only processed by the α/γ-secretase processing pathway (Link, 2006). α-secretase cleavage of APL-1 releases the extracellular fragment, sAPL-1; subsequent cleavage of APL-1-CTFα by the γ-secretase complex liberates the intracellular domain (AICD; **Figure 1B**).

The initial characterizations of human PSEN1 (then called S182) and PSEN2 (first named E5-1) described them as novel proteins with multiple transmembrane domains (Rogaev et al., 1995; Sherrington et al., 1995). The cellular functions of the presenilins were determined by their homology to the *C. elegans* protein, SEL-12 (Levitan and Greenwald, 1995). The two *C. elegans* Notch genes, *lin-12* and *glp-1*, are involved in many cell fate decisions during development, including vulval cell specification and germline development (Greenwald et al., 1983; Lambie and Kimble, 1991; Newman et al., 1995; Levitan et al., 1996) *sel-12*/PSEN was identified in a genetic screen to isolate suppressors of a dominant Lin-12/Notch multivulva phenotype (Levitan and Greenwald, 1995). Loss of *sel-12*/PSEN suppressed the Lin-12/Notch multivulva phenotype and produced a defect in egg laying that was rescued by introducing human PSEN1 or PSEN2, suggesting a conserved function between human and *C. elegans* presenilins (Levitan and Greenwald, 1995; Levitan et al., 1996). Like human PSENs (Thinakaran et al., 1996), SEL-12/PSEN is cleaved to attain its final topology (Li and Greenwald, 1996). A *C. elegans* PSEN gene family was identified and includes *sel-12*, *hop-1*, and *spe-4* (L'Hernault and Arduengo, 1992; Levitan and Greenwald, 1995; Li and Greenwald, 1997; Westlund et al., 1999); *spe-4* is exclusively expressed in male gonadal cells and will not be further discussed (Arduengo et al., 1998). Knockdown of *hop-1*/PSEN in *sel-12*/PSEN mutants showed maternal effect lethality, germline defects, and missing anterior pharynx, defects associated with loss of *glp-1*/Notch function, suggesting

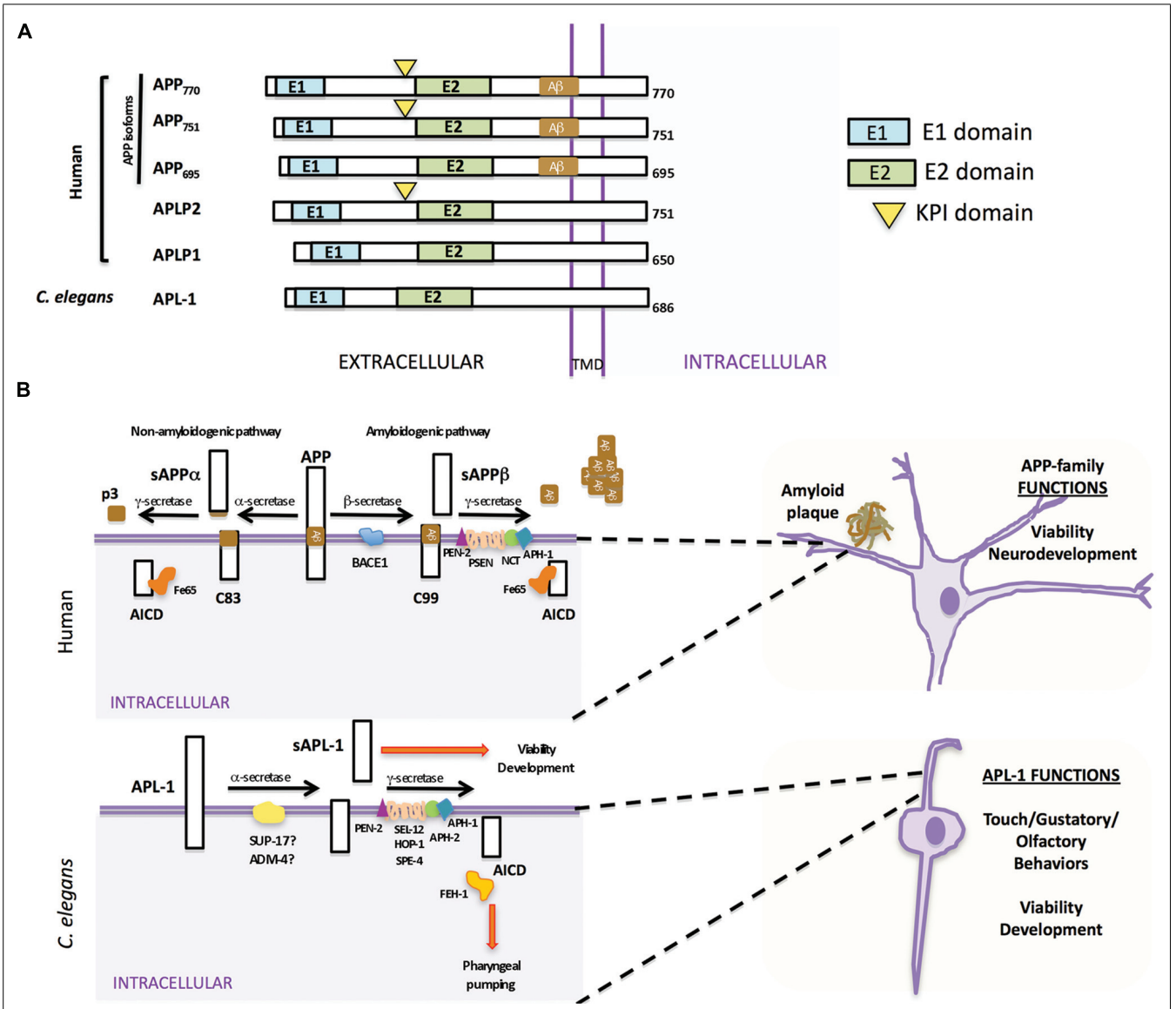

FIGURE 1 | Similarities and differences between human APP and *Caenorhabditis elegans* APL-1. (A) Schematic representation of human APP isoforms, other members of the APP family (APLP1 and APLP2), and *C. elegans* APL-1. **(B)** Comparison between human APP proteolytic pathways (top) and *C. elegans* APL-1 proteolytic pathway (bottom). *Top.* APP can be cleaved by two different pathways. In the anti-amyloidogenic pathway, APP is first cleaved by the α-secretase to release an extracellular fragment sAPPα. The remaining APP fragment (APP-CTFα or C83) is then cleaved by the γ-secretase complex to release p3 extracellularly and the APP intracellular domain (AICD) to the cytosol. In the amyloidogenic pathway, β-secretase first cleaves APP, releasing the sAPPβ fragment. The APP-CTFβ (C99) fragment is subsequently cleaved by the γ-secretase complex, liberating the AICD to the cytosol and Aβ to the lumen. Aβ will aggregate to form amyloid plaques. *Bottom.* In *C. elegans*, APL-1 is first cleaved by the α-secretase homologs SUP-17/ADM-4, liberating the extracellular sAPL-1 that is known to regulate worm viability and development. The γ-secretase complex will then cleave the remaining APL-1-CTF to release AICD into the cytosol. General functions of the APP family and APL-1 are indicated.

that *sel-12* and *hop-1* function redundantly in the LIN-12 and GLP-1/Notch pathways (Westlund et al., 1999). Similarly, mice carrying a null mutation in *PSEN1* showed embryonic lethality, skeletal defects, and disrupted somite boundaries (Shen et al., 1997), similar to the phenotypes seen in Notch1 knockouts (Krebs et al., 2000, 2003, 2004; Duarte et al., 2004; Gale et al., 2004).

In screening for novel mutants showing the *glp-1*/Notch phenotype of defective anterior pharynx, Goutte et al. (2000, 2002) identified two genes, *aph-1* and *aph-2*, which encodes the *C. elegans* NCT ortholog. Independently, Francis et al. (2002) screened for enhancers of *sel-12*/PSEN activity and identified *pen-2*. *aph-2*/NCT, *pen-2*, and *aph-1* are all required for proper Notch signaling. Human PSEN, NCT, Aph1α2, and PEN-2 were subsequently shown to physically associate and cooperatively regulate the maturation of individual components to form a proteolytically active γ-secretase complex (Kimberly et al., 2003).

FUNCTION AND REGULATION OF APL-1

Like the mammalian APP family (Slunt et al., 1994; Lorent et al., 1995; Thinakaran et al., 1995), *apl-1* is expressed in multiple tissue types. *apl-1* expression is observed in neurons, supporting cells, and head muscles throughout development, while expression in vulval muscles, vulval cells, and hypodermal seam cells is not detected until the L4 stage to adult (Hornsten et al., 2007; Niwa et al., 2008).

Inactivation of *apl-1*, such as with the *yn10* null allele, results in a completely penetrant lethality during the first to second larval (L1–L2) transition due to a molting defect (Hornsten et al., 2007). *apl-1* activity is also necessary for later larval transitions, as RNAi knockdown of *apl-1* in an RNAi-sensitized background showed animals with molting defects during the L3–L4 and L4 to adult transitions (Wiese et al., 2010). This lethality was rescued by microinjection of an *apl-1* genomic fragment or cDNA. Hence, similar to the mammalian APP family, *apl-1* has an essential function. High levels of *apl-1* expression caused an incompletely penetrant L1 lethality (70% lethality), shortened body length, and morphogenetic, reproductive, and locomotory defects (Hornsten et al., 2007; Ewald et al., 2012b). These results indicate that levels of APL-1 must be tightly regulated as loss of APL-1 as well as high levels of APL-1 result in lethality. When *sel-12*/PSEN activity was reduced in transgenic animals with APL-1 overexpression, the 70% lethality was partially rescued, suggesting that SEL-12/PSEN regulates APL-1 cleavage and/or trafficking (Hornsten et al., 2007). The underlying basis of the loss- and gain-of-function *apl-1* lethality is still unclear, but is not dependent on activation of CED-3/caspase or necrotic cell death pathways (Hornsten et al., 2007). Characterization of *apl-1* function may provide insights into the general function and pathways of APP, of which much is still unknown.

The *apl-1(yn5)* mutant, which contains a deletion of the region encoding the APL-1 transmembrane and cytosolic domains, produces only the extracellular domain of APL-1 (APL-1EXT) and is viable. Because APL-1EXT is not further cleaved by α-secretase, APL-1EXT is slightly larger than sAPL-1 and is expressed at high levels in *apl-1(yn5)* mutants (Hornsten et al., 2007). Hence, the APL-1 extracellular domain is sufficient for viability, similar to the rescue of APP/APLP2 double mutants by the knock-in of sAPPα (Weyer et al., 2011). However, although *apl-1(yn5)* mutants are viable, they display several phenotypes, including a slower developmental progression, decreased body length, reproductive defects, and temperature-sensitive lethality (Hornsten et al., 2007; Ewald et al., 2012b). Because these defects can be phenocopied by microinjection of APL-1EXT transgenes into wild-type animals, the phenotypes are due to overexpression of APL-1EXT and not due to loss of APL-1 signaling through its cytoplasmic domain (Ewald et al., 2012b). Interestingly, pan-neuronal expression of APL-1EXT, but not expression from muscle or hypodermal cells, is sufficient to rescue the lethality observed in *apl-1* null mutants (Hornsten et al., 2007), suggesting that the cells (i.e., neurons) from which sAPL-1 is released as well as the extracellular milieu in which sAPL-1 travels is functionally relevant. We suggest that high levels of sAPP may also contribute to the pathology seen in AD patients. Down's syndrome patients, whose chromosome 21 trisomy includes trisomy of APP, display a high incidence of AD and intellectual disability (Zigman, 2013), perhaps contributed in small part by the high levels of APP expression.

Decreasing *apl-1* activity by RNAi resulted in hypersensitivity to aldicarb, an acetylcholinesterase inhibitor (Wiese et al., 2010). Using *apl-1* knockouts to test different *apl-1* deletion constructs, Wiese et al. (2010) determined that lack of sAPL-1 is responsible for the aldicarb hypersensitivity. These findings are consistent with mammalian studies, which show that a lack of APP and APLP2 impairs synaptic function at cholinergic neuromuscular junctions (Wang et al., 2005).

Heterochronic genes, whose differential spatiotemporal expression ensures proper progression through larval stages and transition into adulthood (Chalfie et al., 1981; Ambros and Horvitz, 1984), regulate expression of *apl-1* in hypodermal seam cells (Niwa et al., 2008). Loss of *let-7* microRNA (miRNA) function caused precocious seam cell development and vulval bursting at the adult stage, leading to death (Reinhart et al., 2000). These *let-7* phenotypes can be rescued by knockdown of *apl-1* (Niwa et al., 2008). *apl-1*, however, is not a direct target of *let-7* miRNA. NHR-25/Ftz-F1, which is a nuclear hormone receptor (NHR) that is required for completion of larval molts (Asahina et al., 2000; Gissendanner and Sluder, 2000), binds an enhancer element in the promoter of *apl-1* to regulate *apl-1* expression in seam cells (Niwa and Hada, 2010). *nhr-25*/Ftz-F1 transcripts are possible targets of the *let-7* family of miRNAs for downregulation (Hayes et al., 2006). Regulation of continued *apl-1* expression in adult seam cells and other cell types is unknown.

Pathways through which apl-1 functions

The *apl-1(yn5)* phenotypes require activity of the DAF-16/FOXO transcription factor, which is negatively regulated by the insulin pathway. *C. elegans* has only one insulin/IGF-1 receptor, DAF-2 (Kimura et al., 1997). Under favorable environmental conditions, such as when adequate food is present, signaling through the insulin pathway activates a conserved PI 3-kinase/AKT cascade (Morris et al., 1996; Kimura et al., 1997; Paradis and Ruvkun, 1998; Paradis et al., 1999), which causes phosphorylation of DAF-16/FOXO, thereby allowing reproductive development (Larsen et al., 1995; Gems et al., 1998; Henderson and Johnson, 2001). Phosphorylation of DAF-16/FOXO causes its sequestration in the cytoplasm (Lin et al., 2001), thereby preventing it from entering the nucleus to activate its target genes (Henderson and Johnson, 2001; Lee et al., 2001), which regulate longevity (Lin et al., 1997; Ogg et al., 1997), stress resistance (McElwee et al., 2003, 2004; Murphy et al., 2003), and dauer formation (Riddle et al., 1981; Vowels and Thomas, 1992). Environmental conditions also affect other metabolic functions, such as reproductive behavior, which is inhibited under starvation conditions (Seidel and Kimble, 2011), and body size. Starvation survival behavior is regulated by DAF-16/FOXO activity (Lee and Ashrafi, 2008) and the insulin (So et al., 2011) and DAF-7/TGFβ (Savage-Dunn et al., 2003) pathways work in parallel to regulate body length via *daf-16*/FOXO activity.

The slowed development, decreased body size, and decreased reproductive rates of *apl-1(yn5)* mutants are dependent on *daf-16*/FOXO activity. At 20°C, mutants with decreased insulin

signaling or *apl-1(yn5)* mutants showed a delayed developmental progression and shorter body length, which were enhanced when insulin signaling was decreased in *apl-1(yn5)* mutants [i.e., *daf-2(e1370); apl-1(yn5)* double mutants]; at 25°C, the *apl-1(yn5)* mutants with decreased insulin signaling went into L1 arrest (Ewald et al., 2012b). By contrast, when *daf-16*/FOXO activity was removed from *apl-1(yn5)* mutants, the delayed developmental progression, decreased reproductive rate, and smaller body length of *apl-1(yn5)* single mutants were suppressed. Furthermore, loss of *daf-16*/FOXO activity in *apl-1(yn5)* mutants with decreased insulin signaling rescued the short body length and L1 arrest phenotypes (Ewald et al., 2012b). These results suggest that sAPL-1 signals in a parallel pathway to the insulin pathway or modulates the DAF-2/insulin/IGF-1 pathway to activate *daf-16*/FOXO activity to affect developmental progression, reproductive rates, and body length (**Figure 2**). Mammalian sAPP may have similar roles in development.

Activity of the *daf-12*/NHR signals in multiple pathways to integrate environmental stimuli with metabolic needs and can modulate the insulin pathway as well as function in an independent pathway (Gerisch et al., 2001; Dumas et al., 2010). Decreasing *daf-12*/NHR activity in *apl-1(yn5)* mutants rescued the slow development, low reproductive rate, and decreased body length phenotypes (Ewald et al., 2012b). Hence, decreased insulin signaling and signaling through a parallel *daf-12*/NHR pathway converge to activate *daf-16*/FOXO for the phenotypes seen in *apl-1(yn5)* mutants. Noteworthy, levels of insulin/IGF-1 receptors are decreased in AD brains (Steen et al., 2005), and APP processing and Aβ production *in vitro* was modulated by insulin signaling (Gasparini et al., 2001). Analogous to *C. elegans*, sAPP may also act to modulate the insulin pathway.

Pan-neuronal APL-1 expression affects learning

In transgenic mice expressing human or mouse APP, animals showed an increased lethality and learning defects that were not correlated with Aβ deposition (Hsiao et al., 1995); similarly, doubly transgenic mice carrying transgenes with APP and PSEN1 mutations showed learning defects that were not correlated with the number of Aβ plaques (Holcomb et al., 1999). The mechanisms underlying these defects are unclear. Use of the *C. elegans* model could be informative. *C. elegans* has many sensory modalities, including smell and taste. They respond to volatile and water soluble chemicals by moving toward or away from chemoattractive or chemorepulsive stimuli, respectively. Many chemoattractants and chemorepellants have been identified and the neural circuits mediating the chemosensory response identified (Bargmann and Horvitz, 1991). For instance, when *C. elegans* is given the choice between a neutral compound and a chemoattractant, such as benzaldehyde, animals will move toward benzaldehyde; this response is mediated by the AWC neurons (Bargmann et al., 1993); similarly, ASEL, a gustatory neuron, mediates chemoattraction to sodium acetate (Bargmann and Horvitz, 1991; Pierce-Shimomura et al., 2001).

Although *apl-1* is not expressed in AWC neurons and the morphology of sensory neurons appears wild type with GFP markers, the overall chemoattractive response to benzaldyhyde and sodium acetate was decreased in *apl-1(yn10/+)* heterozygotes and transgenic animals that overexpress APL-1 [e.g., *ynIs79* (P*apl-1::apl-1::GFP*)] (Ewald et al., 2012a). The chemotaxis response was restored in APL-1 overexpression lines [e.g., *ynIs79* (P*apl-1::apl-1::GFP*)] when insulin signaling was decreased, but not when *daf-16*/FOXO activity was decreased, suggesting that *daf-16*/FOXO activity is needed for normal chemotaxis in transgenic lines overexpressing APL-1 (**Figure 2**). Pan-neuronal expression of APL-1 or targeted overexpression of APL-1 in the AWC or ASEL neurons resulted in wild-type chemotaxis responses (Ewald et al., 2012a). By contrast, ectopic expression of *apl-1* with the *snb-1* promoter, which drives pan-neuronal and multi-cell type expression, resulted in no chemotaxis response to benzaldehyde or sodium acetate (Ewald et al., 2012a). When signaling through the DAF-2/insulin/IGF-1 receptor, DAF-12/NHR, or DAF-7/TGFβ was decreased, the chemotaxis response toward benzaldehyde and sodium acetate in these transgenic lines was restored, indicating that the loss of the chemotaxis response due to ectopic *apl-1* signaling in cells outside the nervous system is dependent on insulin and TGFβ signaling.

In addition to chemosensory responses, *C. elegans* is also capable of associative chemosensory plasticity (Wen et al., 1997). For example, when benzaldehyde was paired with starvation for as short as 30 min, *C. elegans* showed a significant reduction in preference for benzaldehyde; persistence of this plasticity was positively correlated with the length of pairing time (Colbert and Bargmann, 1995; Tomioka et al., 2006; Lin et al., 2010), suggestive of stable memory formation. Both chemotaxis and associative plasticity are dependent on insulin signaling (Tomioka et al., 2006; Lin et al., 2010). Little associative plasticity, however, was observed after pairing benzaldehyde with starvation for 60 min in animals with pan-neuronal APL-1 expression (Ewald et al., 2012a). The plasticity could be restored when *daf-16*/FOXO, *daf-12*/NHR, or *daf-7*/TGFβ activity was decreased, indicating that the impaired associative plasticity with pan-neuronal APL-1 expression requires *daf-16*/FOXO, *daf-12*/NHR, and *daf-7*/TGFβ activity (Ewald et al., 2012a).

Touch habituation is another sensory characteristic affected by pan-neuronal APL-1 expression. When a gentle touch is applied to the head of the animal, *C. elegans* responds by moving backward; conversely, when touched on the tail, the animal moves forward (Chalfie and Sulston, 1981). This response to gentle body touch is mediated by six mechanosensory touch neurons (Chalfie et al., 1985). After six cycles of alternating head/tail touches, wild-type animals habituated and became unresponsive (Ewald et al., 2012a). *apl-1(yn10/+)* heterozygotes or transgenic animals that overexpress APL-1 showed touch habituation. By contrast, animals with pan-neuronal APL-1 expression were slow to habituate and required more alternating head/tail touch cycles before becoming habituated (Ewald et al., 2012a). Collectively, these results indicate that pan-neuronal overexpression of APL-1 causes learning defects. These results parallel those seen in mammalian models in which overexpression of APP leads to cognitive defects, independent of Aβ aggregates (Hsiao et al., 1995; Simón et al., 2009), thereby suggesting that sAPP activity, in addition to Aβ aggregates, contributes to cognitive

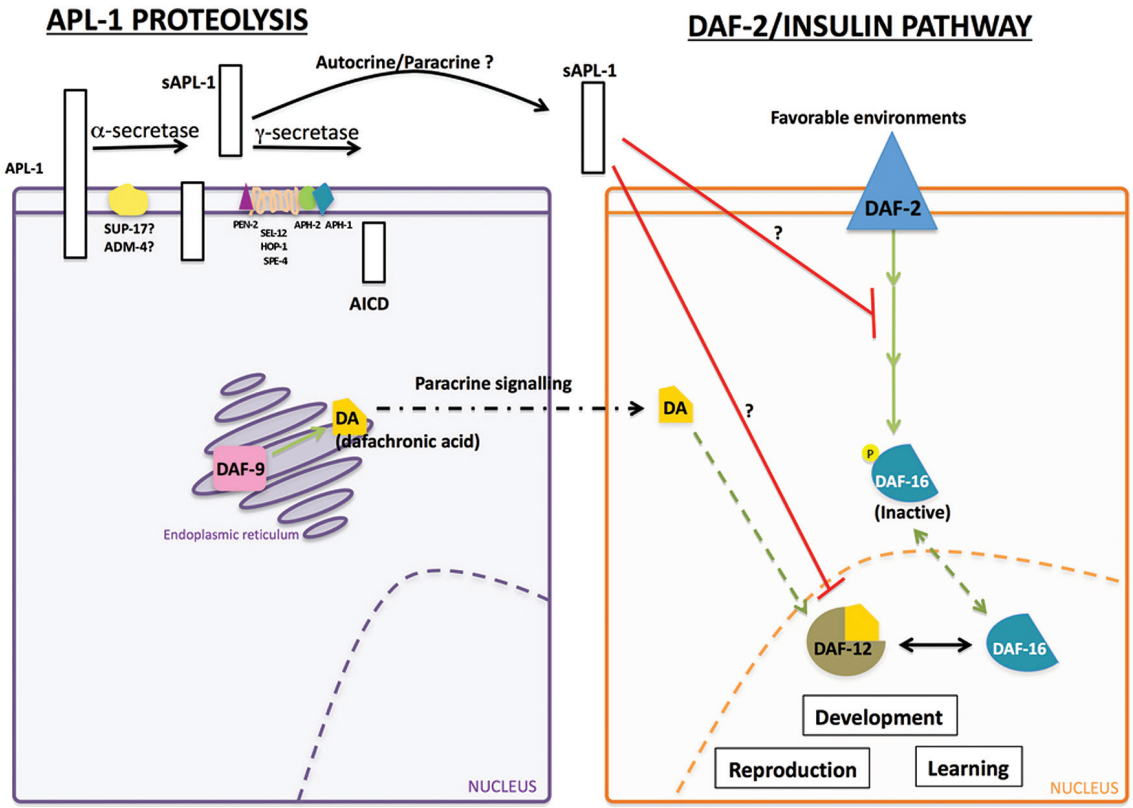

FIGURE 2 | Interaction between sAPL-1 and DAF-2 insulin/IGF-1 receptor and DAF-12/NHR pathways. Schematic representation of APL-1 proteolytic pathway and how sAPL-1 may modulate DAF-2 insulin/IGF-1 receptor and DAF-12/NHR signaling pathways. APL-1 is cleaved by the α/γ-secretase pathway in *C. elegans*. Released sAPL-1 could act as a signaling molecule in the same cell (autocrine regulation) or in neighboring cells (paracrine regulation) to inhibit *daf-2* insulin/IGF-1 receptor and *daf-12*/NHR pathways to affect worm viability and development. The exact mechanism by which sAPL-1inhibits *daf-2* insulin/IGF-1 receptor and *daf-12*/NHR is still unknown and labeled as a question mark (see Ewald et al., 2012b).

defects. Whether these cognitive defects depend on the DAF-16/FOXO transcription factor and/or TGFβ signaling remains to be tested.

APL-1 TRAFFICKING IS IMPORTANT FOR SYNAPTIC TRANSMISSION

APL-1, like APP (Koo et al., 1990), is transported from the cell body to synapses (Wiese et al., 2010). UNC-108, which is a neuronally expressed GTPase and localizes to the Golgi complex and early endosomes (Mangahas et al., 2008; Edwards et al., 2009), is involved in the maturation of dense core vesicles (Borgonovo et al., 2006; Mangahas et al., 2008; Edwards et al., 2009; Sumakovic et al., 2009) and the packaging of APL-1 into mature dense core vesicles (Wiese et al., 2010). Both UNC-116/kinesin-1 and UNC-104/KIF1A/kinesin-3 are involved in the anterograde transport of APL-1 (Wiese et al., 2010; Arimoto et al., 2011), but only UNC-116/kinesin-1 and dynein motors are responsible for the retrograde transport of APL-1 back to the cell body (Arimoto et al., 2011). The rates of anterograde and retrograde transport of APL-1 vesicles are 1.1 μm/s and 1.6 μm/s, respectively (Arimoto et al., 2011). Hence, APL-1 is transported similarly as in mammalian models where kinesin-1 is responsible for APP axonal transport (Koo et al., 1990). Surprisingly, mutations in *unc-116*/kinesin-1 and *unc-104*/KIF1A/kinesin-3 both caused decreased levels of APL-1 expression without affecting transcript levels, suggesting that without transport motors, APL-1 does not accumulate in cell bodies because of protein degradation (Wiese et al., 2010; Arimoto et al., 2011). APL-1 is also internalized from the cell surface of neurons via a RAB-5-dependent endocytosis (Wiese et al., 2010).

AICD INTRACELLULAR TRAFFICKING

The Fe65 family of proteins binds the cytoplasmic YENPTY sequence of APP, APLP1, and APLP2, via their PTB2 domain (Guénette et al., 1996; Zambrano et al., 1997; Duilio et al., 1998; Russo et al., 1998). Likewise, the sole family member ortholog in *C. elegans*, FEH-1, has a WW domain and PTB1 and PTB2 domains, which closely resemble those of the Fe65 family, and the PTB2 domain of FEH-1 interacts with APL-1 (Zambrano et al., 2002).

FEH-1 is expressed in pharyngeal muscle and neuronal processes and is necessary for survival. Inactivation of *feh-1* caused an incompletely penetrant embryonic lethality. Survivors showed little pharyngeal pumping and were unable to feed, thereby resulting in L1 arrest (Zambrano et al., 2002). Decreasing *feh-1* activity or decreasing *feh-1* dosage caused pharyngeal pumping rates to increase, suggesting that the rate of pharyngeal pumping is *feh-1*

dosage dependent. However, the functional significance of FEH-1 and APL-1 interactions is unclear as *apl-1(yn5)* mutants, which do not have an AICD domain, and *apl-1(yn10/+)* heterozygotes do not have defective pumping rates (Ewald et al., 2012b).

INVESTIGATING THE AMYLOID HYPOTHESIS OF AD IN *C. elegans*

Aβ peptide, the cleavage product of APP believed to underlie the pathology of AD (Glenner and Wong, 1984; Masters et al., 1985; Gorevic et al., 1986; Selkoe et al., 1986), is not present in APL-1 (Daigle and Li, 1993) nor does *C. elegans* possess β-secretase activity to produce Aβ (Link, 2006). Nevertheless, *C. elegans* provides a powerful *in vivo* genetic system to study the effects of neurotoxic Aβ through transgene analysis (Shankar et al., 2008). Many transgenic strains have been generated in which a signal sequence followed by the human Aβ sequence is expressed in all cells, in all neurons, in specific subsets of neurons, or in muscle cells (**Figure 4**). These strains produce either $A\beta_{1-42}$ or $A\beta_{3-42}$.

AD is a late onset neurodegenerative disease. *C. elegans* expressing human $A\beta_{3-42}$ in muscle tissue (Link, 1995; Link et al., 2001) showed an age-dependent paralysis at 20°C (Cohen et al., 2006; McColl et al., 2009); paralysis occurred more rapidly and more severely when $A\beta_{1-42}$ was produced at 25°C (McColl et al., 2009). The level of muscle paralysis was significantly decreased when insulin signaling was decreased (Cohen et al., 2006). Furthermore, inhibiting *daf-16*/FOXO and *hsf-1,* which encodes a heat shock protein transcription factor (Hsu et al., 2003; Morley and Morimoto, 2004), reversed the effects of decreased insulin signaling (Cohen et al., 2006). Hence, the paralysis effects of Aβ correlates with age and is dependent on insulin signaling.

Since aggregated Aβ is toxic to neurons (Glenner and Wong, 1984; Masters et al., 1985; Gorevic et al., 1986; Selkoe et al., 1986) and causes muscle paralysis in *C. elegans*, molecules and pathways that can prevent the formation or promote the disassembly of Aβ aggregates can be screened for in *C. elegans.* For instance, when *C. elegans* extracts are incubated with aggregated human $A\beta_{3-42}$ in the presence or absence of protease inhibitors, disaggregation occurred, but disaggregation did not occur when extracts were either heated to denature proteins or incubated with proteinases (Bieschke et al., 2009). Hence, an unidentified protein or protein complex in *C. elegans* extracts can disaggregate $A\beta_{3-42}$ aggregates.

Several orthologs to human heat shock (HSP) chaperone proteins were found to interact directly with $A\beta_{3-42}$ in *C. elegans. C. elegans* HSP-16 proteins, HSP-16-1, HSP-16-2, and HSP-16–48, orthologs of αB-crystallin, bound intracellular $A\beta_{3-42}$ monomers and soluble $A\beta_{3-42}$ oligomers, but not fibrillar $A\beta_{3-42}$ (Fonte et al., 2002). Moreover, *hsp-16* transcript levels were upregulated in $A\beta_{3-42}$ transgenic lines, but whether these chaperone proteins protect against or promote Aβ paralysis is unclear (Fonte et al., 2002). By contrast, increased expression of the HSP70 chaperones had a protective role by suppressing paralysis (Fonte et al., 2008). These results are consistent with human studies showing that HSP70 and αB-crystallin were upregulated in AD brains (Hamos et al., 1991; Perez et al., 1991; Shinohara et al., 1993; Renkawek et al., 1994; Yoo et al., 2001) and binds Aβ (Liang, 2000).

Transgenic lines in which Aβ is expressed in glutamatergic neurons showed age-dependent neurodegeneration, whereby 7-day adults showed 75% glutamatergic neurodegeneration (Treusch et al., 2011). This degeneration was suppressed when genes involved in clathrin-mediated endocytosis, such as *unc-11*, *unc-26*, *Y44E3A.4*, *C. elegans RTS1* ortholog, *C. elegans ADE12* ortholog, and human *CRMI*, were co-expressed with Aβ, and enhanced when a *PBS2/MAP2K4* mitogen-activated protein kinase transgene was co-expressed with Aβ in glutamatergic neurons (Treusch et al., 2011). Interestingly, mutations in the *C. elegans* human REST ortholog *spr-4*, which suppressed the *sel-12*/PSEN egg-laying defect (Lakowski et al., 2003), also enhanced the degeneration seen in the transgenic animals expressing Aβ in glutamatergic neurons (Lu et al., 2014). Modifying clathrin-mediated endocytosis in rat cortical neurons was similarly neuroprotective against Aβ aggregates (Treusch et al., 2011). In addition, early stage AD brains showed higher expression of REST target genes, while late stage AD and frontotemporal dementia (FTD) brains showed lower expression (Lu et al., 2014). Hence, REST may confer neuroprotective benefits in *C. elegans* and in humans (Lu et al., 2014).

The *C. elegans* Aβ model also proves useful in screens to identify drugs that disaggregate Aβ. The drug PBT2, an 8-hydroxy quinoline analog, reversed AD phenotypes in mice within days (Adlard et al., 2008). Similarly, *C. elegans* expressing inducible $A\beta_{1-42}$, which become paralyzed within 48 h after induction, were protected against paralysis when exposed to PBT2 (McColl et al., 2012).

C. elegans lrp-1 FUNCTIONS SIMILARLY TO LRP2/MEGALIN, AN LDL RECEPTOR FAMILY MEMBER

In mammals, the LDL receptor family is responsible for many functions, including binding ligands for internalization and degradation and cholesterol metabolism (Brown and Goldstein, 1986; Mahley, 1988; Herz et al., 1992; Willnow et al., 1994). Binding of LRP1 to sAPP770 or full-length APP770, one of the APP isoforms (**Figure 1**), causes its internalization and degradation (Kounnas et al., 1995; Knauer et al., 1996); disrupting cell surface APP internalization with an LRP-antagonist increases sAPPα processing and full-length APP at the cell surface and decreases Aβ formation, suggesting that LRP1-APP interactions favor APP processing through the amyloidogenic pathway (Ulery et al., 2000). Apolipoprotein E and LRP2/megalin have also been implicated in Aβ clearance (Zlokovic et al., 1996; Deane et al., 2004; Carro et al., 2005).

C. elegans LRP-1 most closely resembles mammalian LRP2/megalin (Yochem and Greenwald, 1993; Yochem et al., 1999). *C. elegans* does not have the ability to synthesize cholesterol and, therefore, must rely on dietary sources (Hieb and Rothstein, 1968). Inactivation of *lrp-1* resulted in late larval lethality due to molting defects during the L3–L4 transition (Yochem et al., 1999). When wild-type *C. elegans* were grown in the absence of cholesterol, the molting defects of the *lrp-1* knockouts were phenocopied (Yochem et al., 1998; Wiese et al., 2012), suggesting that LRP-1 is involved in cholesterol uptake from the environment. LRP-1 is expressed in the epithelial hypodermal cells, hyp6 and hyp7, where it localizes to their apical surface (Yochem et al., 1999) and where *apl-1* is also expressed in adults (Niwa et al., 2008). Similarly, its

mammalian counterpart, LRP2/megalin, is mainly expressed at the apical surface of epithelial cells (Cui et al., 1996; Morales et al., 1996; Willnow et al., 1996; Nielsen et al., 1998; Zheng et al., 1998; Hermo et al., 1999; Mizuta et al., 1999).

LRP2/megalin interacts with different domains of APP (Zlokovic et al., 1996; Pietrzik et al., 2004; Carro et al., 2005; Yoon et al., 2005; Cam and Bu, 2006). A physical interaction between APL-1 and LRP-1 has not been determined. Expression of LRP-1 with an N-terminal domain truncation did not rescue the lethality of *apl-1* null mutants, suggesting that sAPL-1 is not activating an *lrp-1* pathway (Hornsten et al., 2007). When *lrp-1* expression is decreased or when wild-type animals are deprived of dietary cholesterol, neurotransmission is affected (Wiese et al., 2012). However, *apl-1* null mutants die at an earlier stage in development than *lrp-1* null mutants, suggesting that *apl-1* functions in earlier developmental pathways that are necessary for survival.

C. elegans AS A MODEL FOR OTHER NEURODEGENERATIVE DISEASES

PTL-1 AS A TAU MODEL

Accumulation of neurofibrillary tangles in cell bodies is another hallmark characteristic of AD and other neurodegenerative disorders. The major component of these tangles is tau, which belongs to the family of microtubule-associated proteins (MAPs) that includes MAP2 and MAP4 (Lee et al., 1988; Lewis et al., 1988; Goedert et al., 1989b; Chapin and Bulinski, 1991). MAPs share characteristic homology domains, including a proline-rich domain and a region of a variable number of tandem amino acid repeats (**Figure 3A**; Goedert et al., 1988, 1989a,b, 1992a; Lee et al., 1988; Lewis et al., 1988; Aizawa et al., 1990). Tau is the predominant MAP expressed in axons, while MAP2 is expressed in dendrites (Matus et al., 1981; Binder et al., 1985) and MAP4 is expressed in dividing cells (Bulinski and Borisy, 1980). MAPs bind microtubules and are responsible for promoting microtubule assembly and stability (reviewed in Amos and Schlieper, 2005). MAP family members appear to have redundant functions; mice in which tau was knocked out were viable, but showed increased levels of MAP1A (Harada et al., 1994), suggesting that upregulation of MAP1A can compensate for the lack of tau *in vivo*. Tau phosphorylation affects its ability to bind microtubules and can cause a conformational change that favors tubulin assembly (**Figure 3B**; Feijoo et al., 2005). Aberrant hyperphosphorylation of tau, however, impairs its ability to bind microtubules, thus resulting in their disassembly (Lindwall and Cole, 1984; Biernat et al., 1993; Bramblett et al., 1993). In addition, phosphorylated tau self-aggregates into PHFs and presumably generates the intracellular neurofibrillary tangles characteristic of AD patients (**Figure 3B**; Goedert et al., 1992b; Alonso et al., 1996, 1997, 2001; Billingsley and Kincaid, 1997).

Because of the functional redundancy of MAPs, their specific functions have been difficult to determine. *C. elegans* has only one tau homolog protein with tau-like repeats 1 (PTL-1; Goedert et al., 1996; McDermott et al., 1996). PTL-1 exists as two isoforms, PTL-1A and PTL-1B, with five or four tandem repeats, respectively (Goedert et al., 1996; **Figure 3A**). They have a high level of sequence homology with mammalian tau, especially in the C-terminal microtubule binding region (Goedert et al., 1996; McDermott et al., 1996). Both PTL-1A and PTL-1B bound microtubules *in vitro* and induced tubulin polymerization (Goedert et al., 1996; McDermott et al., 1996). PTL-1 is initially expressed embryonically in the epidermis of elongating embryos and in head neurons; in larval and adult animals PTL-1 is expressed mainly in the mechanosensory neurons mediating gentle body touch (**Figure 3B**; Goedert et al., 1996; Gordon et al., 2008), although transcriptional fusions show a wider expression pattern in neurons and stomatointestinal cells (Gordon et al., 2008).

Loss of *ptl-1*/tau results in an incompletely penetrant lethality at the same stage of embryogenesis (Gordon et al., 2008) at which PTL-1/tau expression is first observed (Goedert et al., 1996). *ptl-1*/tau mutants that escaped lethality showed normal development, but had a shortened lifespan, and although the overall integrity of microtubule structure appeared unaffected at the light microscopic level, there was a significant reduction in gentle touch sensitivity as compared to wild-type (Gordon et al., 2008; Chew et al., 2013). These touch defects were enhanced in mutants with defects in β- and α-tubulin, indicating that the absence of full-length PTL-1/tau disrupts mechanosensation, but it does so independently of tubulin (Gordon et al., 2008). In mutants in which only the C-terminal microtubule binding repeats of PTL-1/tau are deleted, touch sensitivity was identical to that of wild-type (Chew et al., 2013), suggesting that the N-terminal domain of PTL-1/tau is sufficient for gentle touch responses.

As *C. elegans* ages, mechanosensory touch neurons exhibit age-related morphological changes: cell bodies initially elaborate branches and axons subsequently display blebbing and branching (Pan et al., 2011; Tank et al., 2011; Toth et al., 2012). Strikingly, mechanosensory touch neurons in *ptl-1*/tau mutants displayed these aging characteristics at higher incidences and at an earlier stage than wild-type animals; GABAergic neurons also showed age-related phenotypes, such as ectopic branching (Chew et al., 2013). Expression of a human tau isoform (htau40) in *ptl-1*/tau mutants rescued the touch insensitivity, but not the morphological aging defects, indicating that htau40 shares some functional conservation with PTL-1/tau (Chew et al., 2013). Interestingly, when *ptl-1*/tau was expressed in Sf9 cells, cells projected neurite-like processes that were positive for PTL-1/tau immunoreactivity (Goedert et al., 1996) and that were indistinguishable from those visualized when htau40 was expressed in Sf9 cells (Knops et al., 1991; Chen et al., 1992). Although wild-type PTL-1/tau has not been reported to aggregate into fibrils, PTL-1/tau, like human tau, clearly has an essential role in maintaining neuronal integrity, controlling neuronal aging, and affecting lifespan (Goedert et al., 1996; Gordon et al., 2008). While there are no known mutations in tau that are associated with AD, tau mutations are associated with FTD with parkinsonism (FTPD-17), another form of dementia (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998; see Section "Frontotemporal Dementia").

FRONTOTEMPORAL DEMENTIA

Frontotemporal dementia (FTD) is a group of neurodegenerative disorders characterized by severe brain frontotemporal lobar degeneration (reviewed in Rabinovici and Miller, 2010). In some cases it may be hard to distinguish between FTD and AD; however,

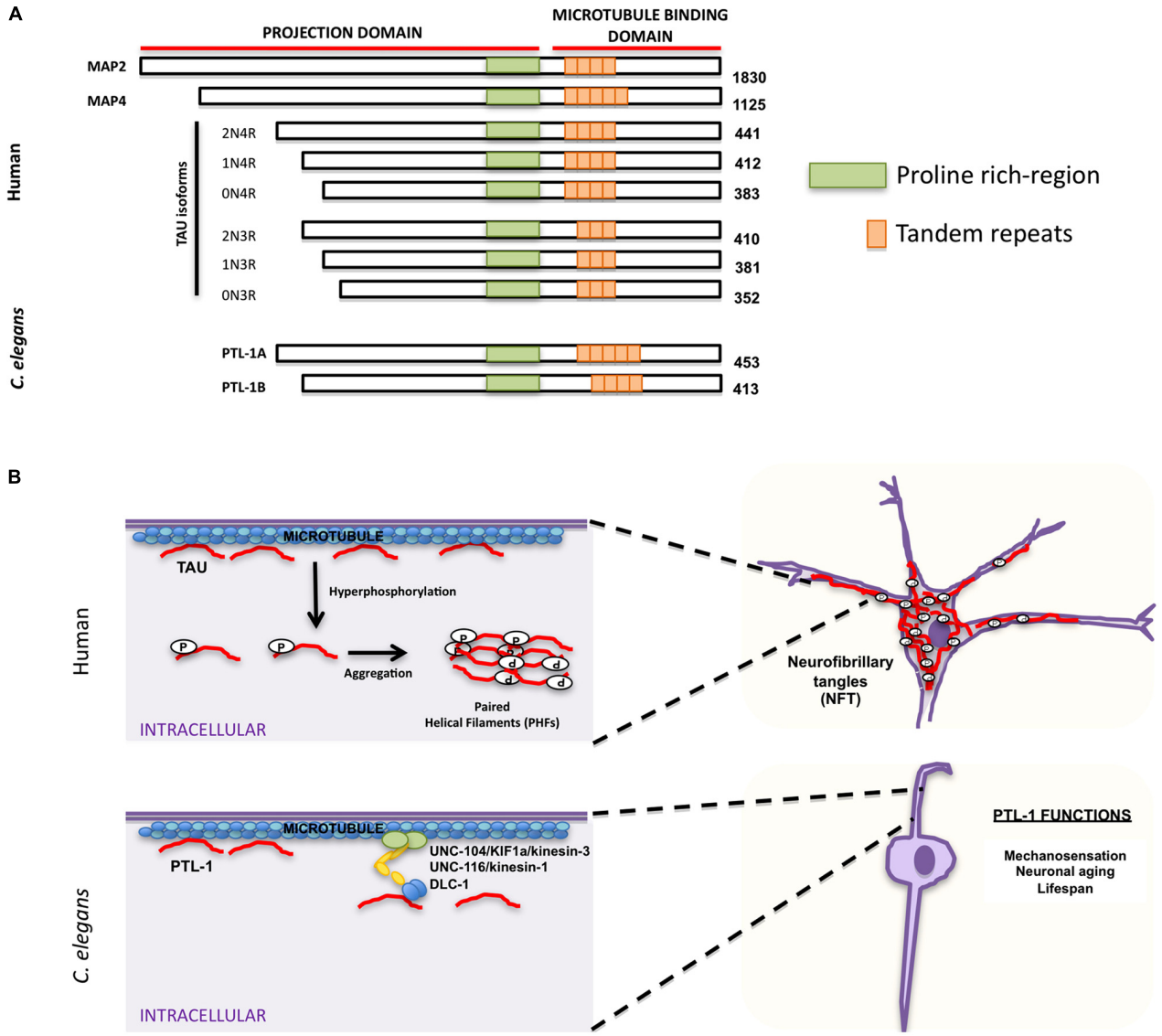

FIGURE 3 | Similarities and differences between human tau and *Caenorhabditis elegans* PTL-1. (A) Schematic representation of human microtubule binding proteins (MAPs) family, including tau isoforms and *C. elegans* PTL-1 isoforms. **(B)** Comparison between tau functions in humans (top) and C. *elegans* PTL-1/tau functions (bottom). *Top.* Tau has a physiological role in promoting and maintaining microtubule stability. In pathological conditions tau is hyperphosphorylated and self-aggregates into paired helical filaments (PHFs) that can form intracellular neurofibrillary tangles (NFT). *Bottom. C. elegans* PTL-1/tau binds microtubules and induces microtubule assembly. It also affects synaptic transport through motor proteins UNC-104/KIF1a/kinesin-3, UNC-116/kinesin-1, and DLC-1/dynein. PTL-1/tau is also important for *C. elegans* mechanosensation and aging.

FTD usually develops earlier in life and is more likely to have a genetic component (Lindau et al., 2000; Pasquier, 2005). Many mutations can cause FTD with or without motor neuron disease (Cruts et al., 2012). Two mutations have been well characterized and are associated with specific types of FTD: tau-positive FTD linked to chromosome 17 (FTD-17) and FTD caused by TDP43 proteinopathy (FTD-TDP43). Patients with FTD-17 suffer behavioral changes and often Parkinson-like motor problems. While mutations in the tau gene *MAPT* are not described in familial or sporadic AD, *MAPT* tau mutations are linked with FTD-17 (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998).

Several transgenic lines expressing human tau harboring FTD-17 mutations (htau-FTD-17) have been generated in *C. elegans* (**Figure 4B**) (see also Section "PTL-1 as a Tau Model"; Kraemer et al., 2003; Miyasaka et al., 2005; Brandt et al., 2009; Fatouros et al., 2012). Pan-neuronal transgene expression of wild type or htau-FTD-17 caused an uncoordinated phenotype that progressively worsened with age, an accumulation of insoluble tau, and neurodegeneration (Kraemer et al., 2003). Similarly, expression of htau-FTD-17 in touch neurons resulted in a decrease in the touch response due to neuritic abnormalities and tau accumulation (Miyasaka et al., 2005). Using these *C. elegans* models of

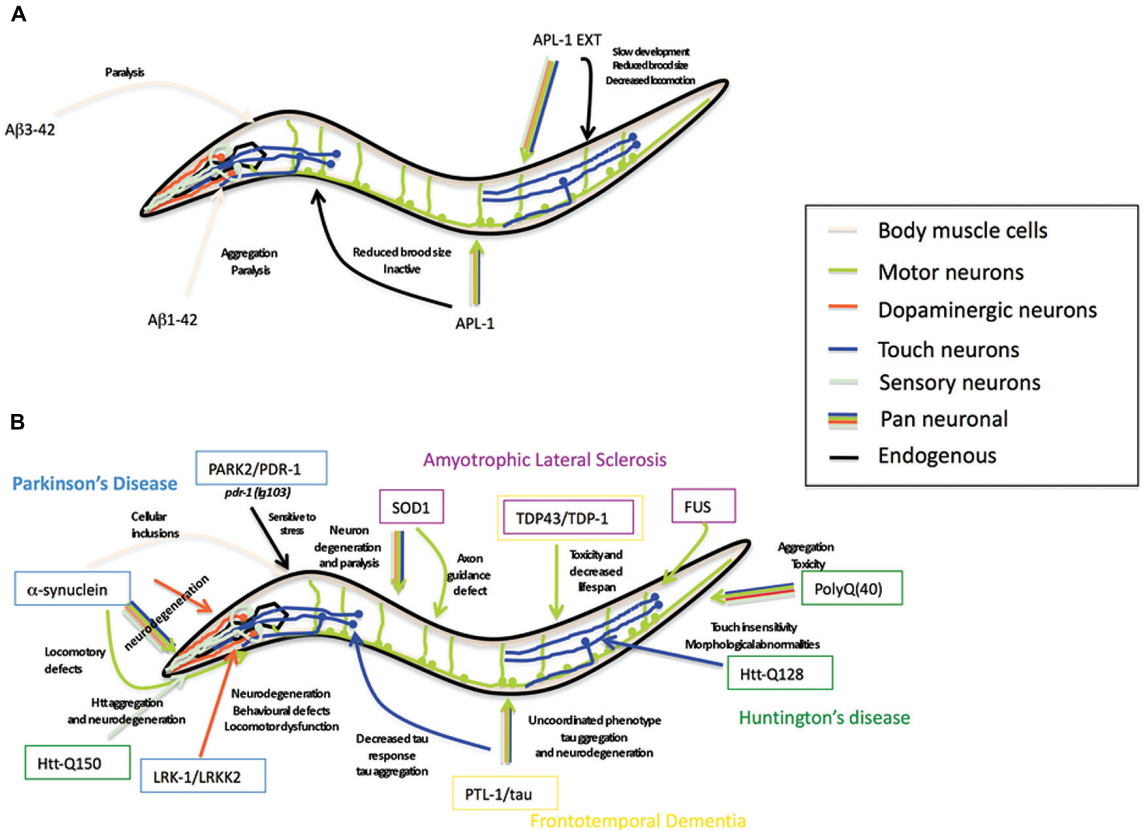

FIGURE 4 | *Caenorhabditis elegans* as a transgenic model for AD and other neurodegenerative diseases. (A) Summary of the Alzheimer's disease models in *C. elegans* expressing human Aβ peptide or *C. elegans* full-length APL-1 or APL-1 extracellular domain (APL-1 EXT). Arrow color represents the tissues where transgenes were expressed. Phenotypes observed are next to the arrow. **(B)** Summary of the *C. elegans* models for Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), and Huntington's disease (HD). Genes modeling PD are shown in blue boxes, ALS in pink boxes, FTD in yellow boxes, and HD in green boxes. Arrow color represents tissues where transgenes were expressed. Phenotypes observed are written close to the arrow.

tauopathy in forward genetic screens, Kraemer and co-workers identified two new factors, SUT-1 and SUT-2, that may participate in the pathological pathway activated by tau (Kraemer et al., 2003; Kraemer and Schellenberg, 2007; Guthrie et al., 2009). Moreover, down-regulation of the human SUT-2 homolog (MSUT-2) in mammalian cell lines caused a marked decrease in tau aggregation, suggesting that MSUT-2 may be a good candidate target for FTD therapies (Wheeler et al., 2010; Guthrie et al., 2011). More recently, Fatouros et al. (2012) generated two htau-FTD-17 transgenic models: one with a pro-aggregant mutated form of human tau (deletion of K280) and a second with mutated forms of human tau (I277P and I308P) that prevented tau aggregation. The tau (ΔK280) transgenic line had high levels of tau aggregation, which caused uncoordinated movement in adults, axonal defects, and alterations in presynaptic structures (Fatouros et al., 2012); the locomotory defects could be partially suppressed by a compound of the aminothioenopyridazine (ATPZ) class cmp16, suggesting that this compound may be neuroprotective (Fatouros et al., 2012). The tau (I277P and I308P) transgenic lines had low levels of tau aggregates and displayed only mild phenotypes with significantly less morphological abnormalities.

Accumulation of TDP-43 [transactive response (TAR) DNA-binding protein] is found in ∼50% of the cases of FTD (The Association for Frontotemporal Degeneration, 2014) and has numerous genetic causes. However, only one case has been reported with mutations in the TDP-43 gene (Borroni et al., 2009). *C. elegans* models overexpressing human TDP-43 or its *C. elegans* ortholog TDP-1 recapitulates some of the FTD phenotypes, including neurotoxicity and protein aggregation (see also Section "Amyotrophic Lateral Sclerosis").

C9orf72 encodes a protein that regulates endosomal trafficking and autophagy in primary neurons and neuronal cells (Farg et al., 2014). It is expressed in multiple tissue types, including cerebellar cortex and spinal cord (DeJesus-Hernandez et al., 2011). Hexanucleotide (GGGGCC) repeat expansions in a non-coding region of C9orf72 are found in patients with amyotrophic lateral sclerosis (ALS) and FTD (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Majounie et al., 2012), providing the first genetic link between the two diseases, although it remains unclear how C9orf72 hexanucleotide expansion triggers ALS and FTD pathology. Mutations in the *C. elegans* C9ORF72 ortholog *alfa-1* caused age-dependent motility

defects, leading to paralysis and degeneration of GABAergic motoneurons (Therrien et al., 2013), suggesting that a loss-of-function mechanism is involved in the C9ORF72-dependent pathogenesis.

PARKINSON'S DISEASE

Parkinson's disease (PD) is a progressive neurodegenerative disorder that affects the control of body movements. The impaired motor control in PD is the result of the death of dopaminergic (DA) neurons (Hughes et al., 1992; Fahn and Sulzer, 2004). The disease is characterized by the accumulation of α-synuclein into neuronal inclusions called Lewy bodies (Lewy, 1912; Tretiakoff, 1919). Most PD cases are of unknown cause. However, ∼5–10% of PD cases are familial (Wood-Kaczmar et al., 2006) and include mutations in the following genes: α-synuclein, parkin (PRKN), leucine-rich repeat kinase 2 (LRRK2), PTEN-induced putative kinase 1 (PINK1), and ATP13A2 (Hardy, 2010).

α-synuclein is a presynaptic neuronal protein whose cellular function is not well understood, but may include controlling the supply of synaptic vesicles in neuronal terminals and regulating dopamine release. It is a small acidic protein (14 kDa) whose sequence can be divided into three domains: the N-terminal α-helical domain (amino acids 1–65), the central hydrophobic domain (residues 66–95), and the acidic carboxyl-terminal domain (residues 96–140; Recchia et al., 2004). Three mutations in the α-helical domain (A53T, A30P, E46K) are linked with autosomal dominant early onset PD, suggesting that these mutations can predispose to oligomer and fibril formation (Polymeropoulos et al., 1997; Krüger et al., 1998; Conway et al., 2000a,b,c; Zarranz et al., 2004). Because *C. elegans* has no α-synuclein ortholog, *C. elegans* models are based on transgenic worms overexpressing wild-type or mutant forms of human α-synuclein (**Figure 4B**). Although different transgenic lines showed some differences, most lines with pan-neuronal or DA neuronal expression of wild-type or mutated α-synuclein (A53T and/or A30P mutations) displayed locomotory defects and degeneration of dopamine neurons (Lakso et al., 2003; Kuwahara et al., 2006; Cao et al., 2010). Furthermore, downregulating the activity of the nuclease EndoG decreased α-synuclein toxicity in DA neurons (Büttner et al., 2013). EndoG is a mitochondria-specific endonuclease that mediates cellular death by apoptosis (Li et al., 2001). When cell death is induced, EndoG translocates from the mitochondria to the nucleus to fragment DNA (Li et al., 2001). Approximately 50% of the dopamine neurons expressing α-synuclein degenerate, whereas a mutation in *cps-6*, which encodes the *C. elegans* EndoG ortholog, rescues this degeneration (Büttner et al., 2013). Similar results were found in yeast, flies, and human cells suggesting that EndoG is a conserved requirement for α-synuclein toxicity (Büttner et al., 2013).

C. elegans models of α-synuclein overexpression-induced toxicity have also been examined by whole genome RNAi knockdown and microarray screenings (Vartiainen et al., 2006; Hamamichi et al., 2008; Kuwahara et al., 2008; van Ham et al., 2008). These screens highlighted the importance of endocytosis for ameliorating α-synuclein-dependent neurotoxicity (Kuwahara et al., 2008). Transgenic lines expressing α-synuclein specifically in body wall muscle cells produced inclusions as animals aged, resembling a feature of neurons in patients with PD; the number of inclusions decreased when genes affecting different biological processes, such as vesicle and lysosomal trafficking (W08D2.5/ATP13A2), lipid metabolism, and lifespan control (*sir-2.1*, *lagr-1*), were knocked down (van Ham et al., 2008). In a genome-wide microarray analysis to identify genes that were modulated in *C. elegans* overexpressing wild-type or A53T human α-synuclein, seven genes encoding components of the ubiquitin-proteasome machinery and 35 mitochondrial function genes were found to be upregulated, while nine genes encoding histones H1, H2B, and H4 were down regulated (Vartiainen et al., 2006). These data provide support for the role of the proteasome complex and mitochondrial proteins in mediating neurotoxicity.

Parkin (human PARK2) is a component of an ubiquitin E3 ligase that is part of the proteasome complex (Shimura et al., 2000). Mutations in PARK2 have been associated with early onset recessive forms of PD (Kitada et al., 1998; Poorkaj et al., 2004). *C. elegans* has a parkin ortholog, PDR-1. A truncated form of PDR-1(Δaa24–247) encoded by the in-frame deletion null allele *pdr-1*(*lg103*) had altered solubility and propensity to aggregate when expressed in cell lines, resembling parkin mutant proteins in PD (Springer et al., 2005). Furthermore, *pdr-1* mutants were hypersensitive to different proteotoxic stress conditions, suggesting that PDR-1/PARK2 mutations act to block the proteostasis machinery, thereby making it easier for proteins to abnormally fold and aggregate (Springer et al., 2005).

Mutations in LRRK2/leucine-rich repeat kinase 2 are the most common known cause of late-onset PD. LRRK2 belongs to the LRRK family; gain-of-function LRRK2 mutations interfere with chaperone-mediated autophagic functions and presumably decrease levels of α-synuclein degradation (Orenstein et al., 2013). Transgenic worms overexpressing pathogenic mutant forms of LRRK2 in DA neurons caused DA neurodegeneration (Liu et al., 2011; Yao et al., 2013). Interestingly, treatment of the transgenic worms with kinase inhibitors resulted in arrested neurodegeneration, suggesting that LRRK2 kinase activity is important for its pathogenesis (Liu et al., 2011; Yao et al., 2013).

Many studies have reported a link between toxin exposure and increased risk of PD. *C. elegans* has been used to test different toxins and help elucidate the mechanism by which they produce neurotoxicity. Administration of the 6-hydroxydopamine (6-OHDA) neurotoxin to *C. elegans* produced specific degeneration of dopamine neurons (Nass et al., 2002). By performing forward genetic and high-throughput chemical screens, mutations within the dopamine transporter *dat-1* were found to suppress 6-OHDA sensitivity (Nass et al., 2005) and bromocriptine, quinpirole, and acetaminophen, and plant extracts from *Bacopa monnieri* and *Uncaria tomentosa* were found to be neuroprotective (Marvanova and Nichols, 2007; Locke et al., 2008; Ruan et al., 2010; Jadiya et al., 2011; Shi et al., 2013). These data demonstrate that pathological characteristics of PD can be recapitulated in *C. elegans* models and used to investigate the mechanism by which α-synuclein and other PD proteins produce neurotoxicity and cause motor defects.

AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis is a neurodegenerative disease characterized by the death of motor neurons in brain and spinal cord and progressive paralysis of the body (Hardiman et al., 2011). Approximately 10% of ALS cases are familial and associated with mutations in several genes. The most common mutation in familial ALS is found in the superoxide dismutase enzyme (SOD1) with more than 160 different mutations identified (Wroe et al., 2008). SOD1 is a ubiquitously expressed protein that converts the toxic radical superoxide anion to hydrogen peroxide. Although it is not clear yet how SOD1 mutations causes motor neuron degeneration, toxicity is likely generated by a gain-of-function mechanism (Valentine et al., 2005) and associated with misfolding and aggregation of the enzyme (Pasinelli and Brown, 2006).

Transgenic lines expressing mutant human SOD1 proteins have been successfully generated in *C. elegans* and recapitulate the motor neuron degeneration and paralysis characteristic of ALS patients (**Figure 4B**) (Witan et al., 2008; Gidalevitz et al., 2009; Wang et al., 2009; Li et al., 2014). The locomotion defect caused by pan-neuronal expression of the SOD1(G85R) mutant isoform was reduced when insulin signaling was decreased (Boccitto et al., 2012), suggesting that decreased insulin signaling increases the capacity of cells to prevent the accumulation of toxic non-soluble proteins and opening the possibility of finding new therapeutic targets. Similarly, when wild-type or mutant SOD1(G93A) was expressed exclusively in GABAergic motor neurons, animals showed an age-dependent paralysis and accumulation of wild-type and mutant SOD1(G93A), although defects were more severe in the mutant lines; interestingly, the SOD1 aggregates were soluble in the wild-type SOD1 lines and insoluble in the SOD1(G93A) lines (Li et al., 2014). In addition, motor neurons showed axonal guidance defects during development and caspase-independent cell death in adulthood in the wild-type and SOD1(G93A) lines (Li et al., 2014).

Other genes associated with ALS have also been modeled using *C. elegans*. TDP-43 [TAR DNA-binding protein] is a 43 kDa RNA binding protein identified as the main component of ubiquitinated protein aggregates (Tsuda et al., 2008; Murakami et al., 2012; Vaccaro et al., 2012a,b; Han et al., 2013; Therrien et al., 2013) found in patients with sporadic ALS (Neumann et al., 2006) and also in some cases of FTD (see Section "Frontotemporal Dementia"). TDP-43 is normally located in the nucleus of neurons, but dominant mutations in TDP-43 cause aberrant localization of TDP-43 in the cytoplasm, thereby preventing it from functioning in the nucleus (Gitcho et al., 2008; Kabashi et al., 2008; Sreedharan et al., 2008; Van Deerlin et al., 2008; Yokoseki et al., 2008; Ash et al., 2010). *C. elegans* has one TDP-43 ortholog, TDP-1. TDP-1 controls longevity and oxidative stress in the worm by regulating the insulin pathway (Vaccaro et al., 2012b). Overexpression of *tdp-1*/TDP-43 resulted in toxicity and decreased lifespan, analogous to the phenotypes found in ALS patients (Vaccaro et al., 2012b). In transgenic worms expressing TDP-43 harboring ALS-associated mutations, proteotoxicity affecting neuronal functions was induced. Similar results were found when the RNA binding protein FUS with ALS-related mutations was expressed in the nematode (Murakami et al., 2012; Vaccaro et al., 2012b).

Excess exposure to some pesticides and chemicals, such as the metalloid selenium, have been implicated in the etiology of ALS (Vinceti et al., 2009; Kamel et al., 2012; Malek et al., 2012). Exposure to high levels of sodium selenite in the worm induced neurodegeneration and resulted in paralysis (Estevez et al., 2012, 2014). When insulin pathway activity was reduced, the adverse effects of environmental selenium exposure was altered (Estevez et al., 2014). Overall, the *C. elegans* models have highlighted the possible importance of the insulin and autophagy pathways in the generation of ALS.

HUNTINGTON'S DISEASE

Huntington's disease (HD) is a progressive neurodegenerative disorder inherited through autosomal dominant mutations of the *IT15* gene. *ITI5* encodes the huntingtin protein, whose functions remain unknown (The Huntington's Disease Collaborative Research Group, 1993). The mutations result in an N-terminal polyglutamine (polyQ) expansion (Goldberg et al., 1996; Mangiarini et al., 1996). In normal individuals, up to 34 repeats have been reported, whereas in HD afflicted individuals, up to 100 polyQ repeats have been recorded (The Huntington's Disease Collaborative Research Group, 1993). The huntingtin-polyQ (HdhQ) proteins form aggregates, whose toxicity is determined by the length of the polyQ expansion and which cause swollen, disorganized, and ribosome-deficient endoplasmic reticulum and chromatin irregularities (Martindale et al., 1998). Eventually, cellular defects caused by the aggregates culminate in HD symptoms, which include involuntary movement, cognitive impairment, and loss of neurons in the striatum and deep layers of the frontal cortex (Martin and Gusella, 1986).

Although *C. elegans* does not have a huntingtin homolog, transgenic *C. elegans* models that express an N-terminal human huntingtin (htt) fragment with different numbers of CAG repeats have been used to model HD and identify genes that prevent polyQ aggregates (**Figure 4B**). The models generally express the repeats in specific neurons, such as the ASH sensory neurons, which are multi-modal sensory neurons that mediate avoidance to chemo- and mechanosensory stimuli. In transgenic animals expressing htt171 with 150 CAG repeats (htt171-Q150), 13% of the ASH neurons began to lose function after 8 days, suggesting an age-dependent degeneration (Faber et al., 1999). This loss of ASH function was reversed in a *ced-3*/caspase (Faber et al., 1999) or *hda-3*/HDAC (Bates et al., 2006) mutant background, suggesting that processes characteristic of apoptotic cell death and histone deacetylases play a role in HD (Dragunow et al., 1995). By contrast, the number of htt171-Q150 aggregates and neurodegeneration were enhanced when genes mediating autophagy, CREB, CREB binding proteins, and *pqe-1* were disrupted, suggesting that autophagy and activation of CREB target genes decreases htt171-Q150 aggregation and are neuroprotective (Faber et al., 2002; Bates et al., 2006; Jia et al., 2007). Transgenic animals expressing fewer CAG repeats (2, 23, and 95 polyQ) showed normal ASH function (Faber et al., 1999). The onset of behavioral defects are consistent with most cases of HD, in which symptoms usually appear during midlife (Vonsattel et al., 1985; Martin and Gusella, 1986; Strong et al., 1993; The Huntington's Disease Collaborative Research Group, 1993; Gusella and MacDonald, 1995) and

fewer than 10% of reported cases occur before the age of 21 (Farrer and Conneally, 1985; van Dijk et al., 1986; Nance, 1997; Siesling et al., 1997).

In a different HD model, htt57-Q128 was expressed in the touch mechanosensory neurons (Parker et al., 2001). These transgenic animals did not show neurodegeneration, but had a significantly reduced response to posterior touch and a milder defect in anterior touch response (Parker et al., 2001). The touch neurons contained polyQ aggregates and morphological abnormalities primarily along axonal processes (Parker et al., 2001). The touch insensitivity could be rescued by activating Sir2 sirtuins (Parker et al., 2005), which act through the DAF-16/FOXO transcription factor to promote longevity (Tissenbaum and Guarente, 2001). Similarly, in neuronal cell lines derived from knockin HdhQ111 mice, activation of sirtuins reduced the level of cell death (Parker et al., 2005). Additionally, in an RNAi based screen for genes that suppressed htt57-Q128 defects, identified *C. elegans* genes were also upregulated in the striatum of mouse HD models (Lejeune et al., 2012). Thus, *C. elegans* is a useful model to identify additional genes that may protect against or contribute to defects caused by polyQ expansions.

RNAi knockdown of *dnj-27*/ERdj5, an ER luminal protein upregulated in response to ER stress, exacerbated the impaired mobility observed when a Q40 transgene is expressed in body wall muscles, suggesting that *dnj-27* interacts with polyQ and protects against polyQ induced paralysis (Muñoz-Lobato et al., 2014).

C. elegans has the advantage that it is transparent, allowing visualization of the formation of that aggregates, including aggregates made by shorter polyQ tracts, whereas only longer tracts are visible in mammals (Brignull et al., 2006). To determine the threshold number of polyQ repeats needed to elicit a morphological and behavioral response, varying lengths of polyQ repeats were tested in *C. elegans*. Pan-neuronal expression of more than 40 polyQ led to variable protein aggregation and paralysis (Brignull et al., 2006). These data suggest that 40 polyQ may be the critical number of repeats to elicit HD symptoms and are consistent with unaffected humans who have up to 34 polyQ repeats and HD patients who have as few as 42 repeats (The Huntington's Disease Collaborative Research Group, 1993).

Overall, *C. elegans* HD models illustrate that human huntingtin polyQs disrupt the morphology and function of sensory neurons. The genetic and RNAi screens highlight candidate genes that may be involved in HD pathogenesis in mammalian models and provide insights into genes that may serve a protective role against polyQ toxicity. In addition to HD, other diseases caused by polyQ repeats include spinocerebellar ataxias and spinal and bulbar muscular atrophy (Orr and Zoghbi, 2007). Hence, using *C. elegans* provides another approach toward determining how polyQ pathogenicity contributes to neurodegeneration.

ADVANTAGES AND LIMITATIONS OF THE *C. elegans* MODEL

The use of *C. elegans* to study AD and other neurodegenerative diseases has, as many other models, many advantages as well as some drawbacks. Major advantages of *C. elegans* include the ability to perform forward genetic, RNAi, and high throughput chemical screens and the ease of generating transgenic lines. These benefits have been effective in informing the role of APP and the presenilins and identifying components of the γ-secretase complex. The function of APP and the pathways in which it acts are still unclear. *C. elegans* presents a complementary system to understand the function and pathways of an APP-related protein, APL-1. Furthermore, overexpression of APL-1 by mutation or by transgene induces phenotypes that converge on the insulin/DAF-16/FOXO pathways, similar to what has been found in mammals. Although APL-1 does not contain the Aβ sequence and *C. elegans* does not have β-secretase activity, transgenic lines that produce Aβ expression pan-neuronally or in muscle are being used to identify pathways that detoxify the Aβ aggregates, some of which also involve the insulin/DAF-16/FOXO pathways. Whether these models are relevant to human pathology or whether the pathways will be conserved in humans are unknown; however, they present alternative approaches to understanding neurodegenerative diseases for which there are currently few effective therapies. Human tau, as well as mutant tau isoforms, have also been expressed in the worm to recapitulate AD and FTD phenotypes. Recent findings have shown that PTL-1 regulates neuronal aging in the worm. These findings may be important to link aging and tau pathology in AD and FTD patients. Although *C. elegans* transgene models have many advantages, they also have several disadvantages. In *C. elegans*, transgenes are present as extrachromosomal arrays and are not integrated into the genome as they are in other systems; a few copies to several hundred copies of the transgene are present in the arrays, so the level of overexpression can be much higher than what is found *in vivo*. Fortunately, methods for single copy insertions have now been developed (Frøkjaer-Jensen et al., 2008).

AD is considered a multifactorial disease in which other risk factors, such as neuroinflammation, head trauma, and diabetes, may be important in the development of the disease. The *C. elegans* nervous system is simple compared to the human nervous system. This simplicity allows researchers to study neuronal function and neural circuits in a tractable system. However, the complex network of connections and cell interactions found in humans is not mimicked in *C. elegans* and this complexity may underlie some of the pathology of neurodegenerative diseases. Nevertheless, most of the pathways and signaling molecules in *C. elegans* are conserved between worms and mammals. The goal is to translate some of the *C. elegans* insights into understanding the pathology of AD and other neurodegenerative diseases and designing effective strategies to treat the diseases.

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Insulin signaling in the aging of healthy and proteotoxically stressed mechanosensory neurons

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Insulin signaling is central to cellular metabolism and organismal aging. However, the role of insulin signaling in natural and proteotoxically stressed aging neurons has yet to be fully described. We studied aging of *Caenorbaditis elegans* mechanosensory neurons expressing a neurotoxic expanded polyglutamine transgene (polyQ128), or lacking this proteotoxicity stressor (polyQ0), under conditions in which the insulin signaling pathway was disrupted by RNA interference (RNAi). We describe specific changes in lifespan, mechanosensory neuronal morphologies, and mechansensory function following RNAi treatment targeting the insulin signaling pathway. Overall, we confirmed that transcription factor DAF-16 is neuroprotective in the proteotoxically stressed model, though not strikingly in the naturally aging model. Decreased insulin signaling through *daf-2* RNAi improved mechanosensory function in both models and decreased protein aggregation load in polyQ128, yet showed opposing effects on accumulation of neuronal aberrations in both strains. Decreased *daf-2* signaling slightly enhanced mechanosensation while greatly enhancing branching of the mechanosensory neuron axons and dendrites in polyQ0 animals, suggesting that branching is an adaptive response in natural aging. These effects in polyQ0 did not appear to involve DAF-16, suggesting the existence of a non-canonical DAF-2 pathway for the modulation of morphological adaptation. However, in polyQ128 animals, decreased *daf-2* signaling significantly enhanced mechanosensation while decreasing neuronal aberrations. Unlike other interventions that reduce the strength of insulin signaling, *daf-2* RNAi dramatically redistributed large polyQ128 aggregates to the cell body, away from neuronal processes. Our results suggest that insulin signaling strength can differentially affect specific neurons aging naturally or under proteotoxic stress.

Keywords: neuronal aging, insulin signaling, proteotoxicity, Huntington's Disease, *Caenorhabditis elegans*

INTRODUCTION

The insulin/insulin-like growth factor (IGF) signaling pathway is involved in longevity and stress response across species (Broughton and Partridge, 2009). Signaling through this evolutionarily conserved pathway can promote longevity through increased expression of cellular stress and metabolism genes, including those encoding stress-response, chaperone, and antioxidant proteins (Kenyon, 1993; Hsu et al., 2003; Taguchi et al., 2007; Cohen et al., 2009). The progression of neurodegenerative disorders, such as Huntington's, Alzheimer's, and Parkinson's diseases, has been linked to insulin signaling in both invertebrate and mammalian model systems (Dillin and Cohen, 2011). In addition, decreased insulin signaling has protective effects against neurodegenerative-associated proteotoxicity across species (Freude et al., 2009; Killick et al., 2009).

The *Caenorhabditis elegans* insulin signaling pathway is regulated by insulin-like signaling ligands, INS-1 through INS-39, that modulate the activity of the DAF-2 tyrosine kinase receptor (**Figure 1**). DAF-2 is orthologous to the mammalian insulin/IGF receptor. This receptor activates a protein kinase signaling pathway, which, through phosphorylation of downstream transcription factor DAF-16 by AKT protein kinase, regulates functions similar to receptor kinases in the insulin signaling pathway in humans. Under high signaling conditions, DAF-16 is phosphorylated to prevent nuclear entry and hence transcription. Under low signaling conditions, DAF-16 is free from inhibitory phosphorylation and can regulate the expression of many different genes contributing to metabolism and physiological defense and homeostasis responses (Mukhopadhyay et al., 2006). This insulin signaling pathway has many branch points, including AKT, and

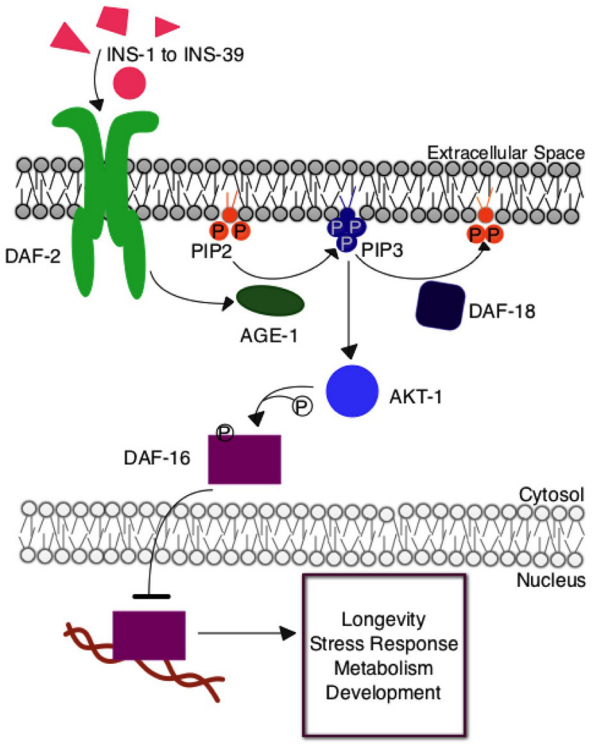

FIGURE 1 | The insulin signaling pathway in *Caenorhabditis elegans*. Upon insulin ligand binding to the DAF-2 insulin receptor in *C. elegans*, AGE-1 (homolog to mammalian PI3-K) is activated and converts PIP_2 to PIP_3 at the cellular membrane. PIP_3 activates the central kinase AKT-1 (ortholog to mammalian PKB), which phosphorylates DAF-16, the *C. elegans* FOXO transcription factor, preventing its entry into the nucleus where it would otherwise regulate the expression of genes contributing to longevity, stress response, and metabolism. The DAF-18 (homolog to mammalian PTEN) phosphatase negatively regulates the system by decreasing the amount of PIP_3 present at the membrane by reconverting it to PIP_2.

there is some variation in the identities of proteins involved in nematodes, flies, and mice, three organisms in which the role of insulin signaling in aging has been investigated. Nevertheless, this central, conserved insulin signaling pathway is critical for appropriate cellular metabolism and maintenance of overall organismal health.

Various mutations in genes modulating the *C. elegans* insulin signaling pathway have been shown to directly regulate lifespan. For example, *daf-2* insulin receptor mutants live twice as long as wildtype animals (Kenyon, 1993), *age-1* PI3K mutants live longer than wildtype animals (Friedman and Johnson, 1988), and the lack of the *daf-16* FOXO transcription factor gene shortens lifespan (Oh et al., 2006). Longevity effects can result from limiting insulin signaling in only the neurons or intestinal cells (Libina et al., 2003). In mammals, the effect of reduced insulin signaling on overall health and lifespan is complex, but combined evidence from many studies points to the potential of insulin signaling reduction to extend lifespan (Taguchi and White, 2008). In mice, reduced neuronal or whole animal expression of IRS2, a kinase activated by the insulin receptor, increases lifespan (Taguchi et al., 2007). In humans, various studies have detected an association between longevity and single nucleotide polymorphisms in genes involved in insulin signaling, including the DAF-16-related transcription factor inhibited by insulin signaling (FOXO3A), the insulin receptor (IGF1R), and a central protein kinase (AKT-1) (Newman and Murabito, 2013). Excellent reviews comparing insulin signaling in various model systems have been published (Taguchi and White, 2008; Broughton and Partridge, 2009; Neri, 2012).

C. elegans exhibit many important neuronal components found in humans, including, but not limited to, neurotransmitters, ligand receptors, and ion channels; thus, these animals are a powerful model for studying neuronal aging and neurodegeneration *in vivo*. Neurodegenerative disorders, such as Huntington's, Alzheimer's, and Parkinson's diseases, result in the progressive loss of structure and function of neurons with age. Of these protein aggregation-associated neurodegenerative diseases, Huntington's disease is caused by the expansion of CAG trinucleotide repeats in the huntingtin gene, which results in an expansion of the length of polyglutamine residues at the N-terminus of the huntingtin protein. Expanded polyglutamine repeats in mutated huntingtin lead to neuronal protein aggregation, impairments in movement and cognitive function, and psychological disorders. Multiple Huntington's disease model strains of *C. elegans* have been developed (Faber et al., 1999; Parker et al., 2001, 2005; Morley et al., 2002). In the model strain used in this study, touch-receptor neuron-specific expression of a transgene encoding the first 57 amino acids of human huntingtin with 128 polyglutamine repeats impairs function, without neuronal death (Parker et al., 2001, 2005). Thus, this model may feature conserved events associated with dysfunction that typify early disease stages in humans.

Aging is the primary risk factor for multiple neurodegenerative diseases, yet the intersection of natural neuronal aging and neurodegenerative states is not well understood. As a consequence of sensing and responding to the environment, the nervous system is known to play a role in physiological aging (Alcedo et al., 2013). In normal, healthy aging, *C. elegans* mechanosensory and other neuron classes develop morphological aberrations, including new outgrowths from the soma, novel process branching, and dendritic restructuring (Pan et al., 2011; Tank et al., 2011; Toth et al., 2012). Neuronal insulin signaling appears to be involved in this natural aging process; the link between normal aging and decline under disease conditions is relatively unexplored. To address this relationship, we studied and compared morphological features of aging mechanosensory neurons with and without a neurotoxic expanded polyglutamine transgene, under conditions in which genes of the canonical insulin signaling pathway were disrupted by neuron-targeted RNA interference (RNAi). Our findings suggest that insulin signaling strength can differentially affect specific neurons aging naturally or under conditions of disrupted proteostasis. Under conditions of polyglutamine expansion stress, insulin receptor DAF-2 appears to act through DAF-16/FOXO. However, under conditions of normal aging, DAF-2 activates a non-canonical pathway that acts independently to induce neuroprotection.

MATERIALS AND METHODS

STRAINS

The *C. elegans* Huntington's disease model strain used was derived from two previously engineered strains. Strain ID1 (*igls1* [$P_{mec\text{-}7}$*yfp,* $P_{mec\text{-}3}$*htt57Q128::cfp, lin-15(+)*]) (polyQ128) contains the first 57 amino acids of human huntingtin fused to CFP-labeled expanded polyglutamine tract (Q128) expressed in the 6 mechanosensory neurons as well as in PVD and FLP neurons (Parker et al., 2001). These polyQ128 animals show functional deficiencies in touch response and accumulation of huntingtin protein aggregates in mechanosensory neurons, without cell death (Parker et al., 2001). Strain TU3270 (*uIs57* [$P_{unc\text{-}119}$*SID-1,* $P_{unc\text{-}119}$*yfp,* $P_{mec\text{-}6}$*mec-6*]) overexpresses the transmembrane channel SID-1 pan-neuronally, allowing the dsRNA from RNAi treatment to enter all neurons (Calixto et al., 2010). TU3270 and ID1 were crossed to generate ZB4062 *igIs1*[$P_{mec\text{-}7}$*yfp,* $P_{mec\text{-}3}$*htt57Q128::cfp, lin-15(+)*]; *uIs57* [$P_{unc\text{-}119}$*SID-1,* $P_{unc\text{-}119}$*yfp,* $P_{mec\text{-}6}$*mec-6*], a polyQ128-expressing strain with neurons susceptible to RNAi. We also crossed a healthy transgenic model, ZB154 (*zdIs5* [$P_{mec\text{-}4}$*GFP, lin-15(+)*]), to TU3270 to generate ZB4064 *zdIs5* [$P_{mec\text{-}4}$*GFP, lin-15(+)*]; *uIs57* [$P_{unc\text{-}119}$*SID-1,* $P_{unc\text{-}119}$*yfp,* $P_{mec\text{-}6}$*mec-6*] to render mechanosensory neurons susceptible to RNAi treatment (polyQ0).

WORM MAINTENANCE

Standard methods were used for strain maintenance, bacterial culturing, and animal manipulation (Brenner, 1974). Stock animals were cultured at room temperature (about 22°C) on nematode growth media (NGM) agar plates seeded with live bacteria (*E. coli* strain OP50-1).

RNA INTERFERENCE TREATMENTS

We prepared RNAi plates using 4× concentrated live HT115 *E. coli* bacteria from the Ahringer Library induced at room temperature for 2 days on agar plates. For each batch of RNAi experiments, we performed a control experiment comparing the amount of nerve ring fluorescence knockdown following GFP treatment to empty vector (L4440) in age-matched animals to confirm neuronal RNAi sensitivity of the strain used (Supplemental Figure 1). Only experiments that showed a significant (unpaired *t*-test, $p < 0.05$) knockdown of GFP were used for further RNAi studies. We performed RNAi treatments at 25°C protected from light with age-synchronous populations created using timed egg lay. To perform each egg lay, adult worms laid eggs on each of the described RNAi treatment plates for 4 h. Animals in all RNAi experiments were transferred by hand each day of adulthood to fresh RNAi plates. We performed and analyzed RNAi experiments "blinded" to the intervention so that the experimenter was not aware of the genetic identity of the RNAi treatment given to each population of animals. We repeated each experiment at least 3 times. We selected day 5 of adulthood as a time point for analysis in the following experiments based on Toth et al. (2012) who reported a significant difference in mechanosensory neuronal morphology between day 1 and day 5 of adulthood.

LIFESPAN ANALYSIS

Following the production of age-synchronous populations, we transferred approximately 50 animals from each RNAi treatment group everyday of adulthood to fresh, seeded RNAi treatment small plates and checked for survival by visual observation or gentle prodding with a platinum wire. Animals with protruding intestines, those that bore live young, or that crawled off the plates were censored. Survival experiments always included all 6 RNAi treatment groups (L4440 empty vector, *daf-2, age-1, daf-18, akt-1, daf-16*) at once for, usually, one strain at a time, and were repeated at least twice. We used Kaplan–Meier log-rank survival statistics to analyze differences in mean survival between RNAi treatment groups and $p < 0.05$ was noted as significant.

MECHANOSENSORY RESPONSE ASSAY

We generated synchronous populations as described above, maintained cultures at 25°C, and transferred each day of adulthood to fresh RNAi treatment plates. On day 5 of adulthood we scored individuals for motility class. Individuals were grouped into 3 classes: A class indicates normal, voluntary sinusoidal movement, B class indicates locomotion following gentle prodding, and C class indicates inability to locomote. We then tested individuals for their ability to respond to touch by gently touching alternatively on the anterior and posterior end with an eyelash pick, 5 times each (**Figure 2B**). Animals responded either by moving (or attempting to move) in the opposite direction of the touch or by showing no movement. We scored animals (0–5) based on the number of positive responses to touch out of 5 touches at the anterior and 5 touches at the posterior. We also recorded the mobility of each animal. We then imaged the mechanosensory neurons of the tested individuals as described below.

NEURONAL MORPHOLOGY IMAGING

Following the mechanosensory response assay on day 5 of adulthood, we mounted animals between 2 cover slips using 2 μL of 36% w/v Pluronic™ solution dissolved in water and imaged using the 20× objective of an Axiovert S100 inverted fluorescent microscope. Using constant microscope settings, we collected images of the 6 mechanosensory neurons of each individual and their associated huntingtin protein aggregates, if present, using FITC and CFP filters, respectively. We discarded animals after imaging, so data presented is cross-sectional rather than longitudinal. We repeated mechanosensory response assays and neuronal morphology imaging for each strain and RNAi treatment group at least 3 times. We examined the images of 6 mechanosensory neurons of each individual for morphological aberrations, namely cell body outgrowths, cell body guidance errors, and process branching (Toth et al., 2012).

PROTEIN AGGREGATE QUANTIFICATION AND DATA ANALYSIS

For polyQ128 experiments, we analyzed huntingtin aggregates (fused with CFP) with ImageJ, using a custom macroinstruction that includes quantification of the total area of aggregates seen in each cell. For comparisons of mechanosensory response

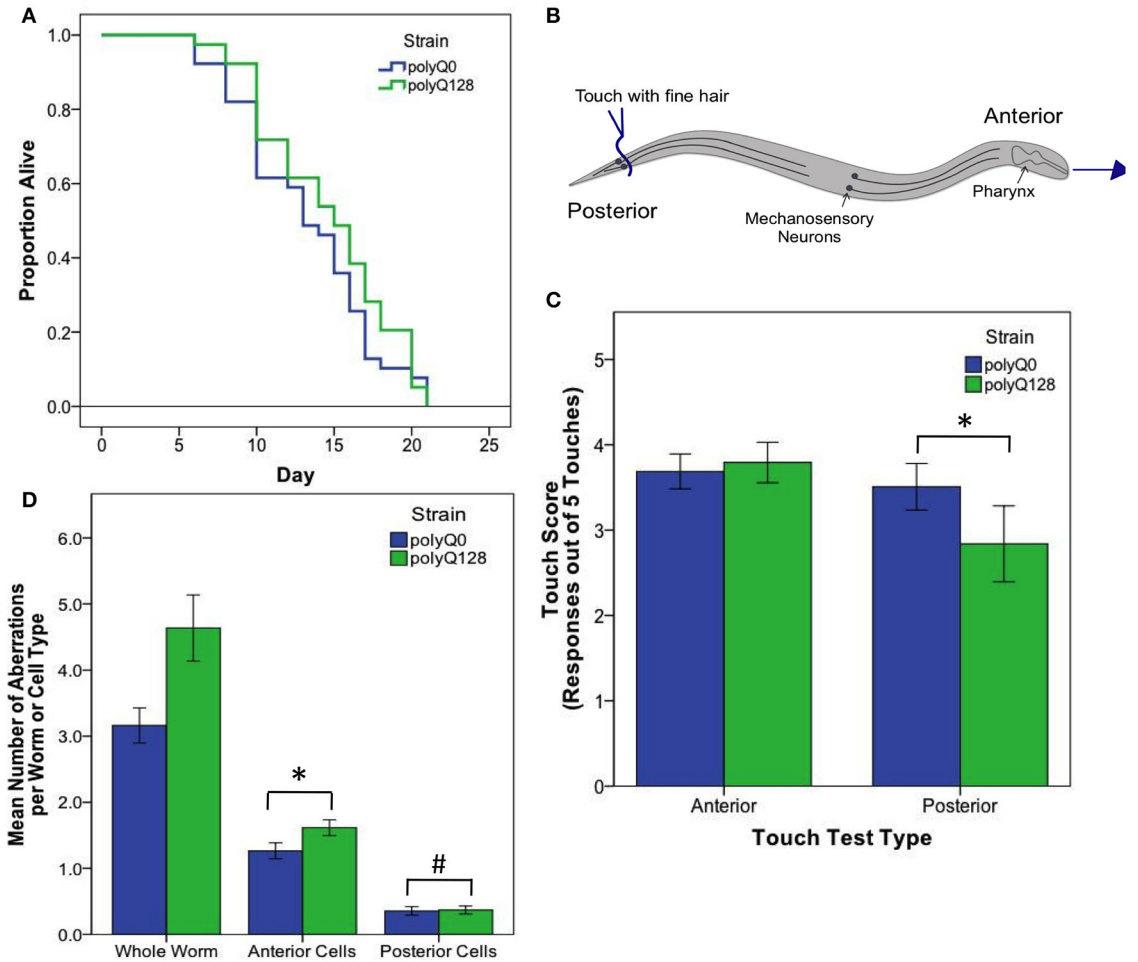

FIGURE 2 | PolyQ0 and polyQ128 exhibit basal differences in mechanosensory neuron morphology and function without altered lifespan. (A) Kaplan–Meier survival curves for one representative survival experiment shown for empty vector (L4440) treated polyQ0 and polyQ128 animals. Mean and median lifespan do not differ between the two strains. **(B)** Schematic of posterior touch test; an animal is touched at the tail and normally moves forward, gaining 1 positive response to touch (out of 5). For anterior touch test, animals are touched at the head and scored for backwards movement. Mechanosensory neurons (PLMs and ALMs) and pharynx shown. **(C)** Anterior and posterior touch test scores for empty vector control individuals. Each bar represents the mean number of positive responses out of 5 to soft touch. **(D)** Total number of aberrations (e.g., number of outgrowths and branches and presence of abnormal cell body and punctae) per worm and cell type for each empty vector control group. Whole worm bars represent the mean values of the sum of all 4 neurons scored (ALML, ALMR, PLML, and PLMR) for each individual. Anterior and posterior bars represent the mean values for individual anterior (ALML or ALMR) or posterior (PLML or PLMR) neurons. *Denotes significance of $p < 0.01$ and #denotes significance of $p < 0.10$ following Mann–Whitney U comparison. Each bar shows mean ± SE for $N = 69$ (polyQ0) and $N = 84$ (polyQ128) animals.

and neuronal morphology between empty vector control polyQ0 and polyQ128 strains, we used a Mann–Whitney U comparison. For the mechanosensory response assays, neuronal morphology imaging, and protein aggregate counts following RNAi treatments, we used a generalized linear model with a log link function (Poisson regression) and Wald tests for significance of treatment effects. For total aggregate area measurements, we used a One-Way ANOVA with Tukey's pairwise comparisons. SPSS (Version 20) statistical software was used to perform, the analyses. A *p*-value of less than 0.05 was considered statistically significant. Values presented in the text represent mean ± standard error.

RESULTS

NEURON AND SYSTEMIC RNAi KNOCKDOWN OF INSULIN SIGNALING PROTEINS ALTERS polyQ0 AND polyQ128 *C. ELEGANS* LIFESPAN

To initiate analysis of the influence of key insulin signaling pathway genes on normal aging and polyQ128-induced neuronal deficits in mid-adult life, we constructed *C. elegans* strains by genetic crosses with fluorescent mechanosensory (or touch) neuron reporters that express the *sid-1* double stranded (ds) RNA transporter pan-neuronally. This *sid-1* compensates for the lack of a neuronal dsRNA transporter, enabling genes expressed in neurons to be targeted by RNAi (Calixto et al., 2010). Moreover, *sid-1* overexpression in neurons can diminish non-neuronal RNAi

effects, such that the *sid-1(+)* neurons act as a sink for double stranded RNA (Calixto et al., 2010). We studied one strain that was free of proteotoxic stress (hereafter referred to as polyQ0), and one that expresses the first 57 amino acids of human huntingtin protein with expanded polyglutamines fluorescently labeled with CFP (polyQ128) (Parker et al., 2001). In these strains, we can measure neuronal function via mechanosensory touch response assays, visualize neuronal morphology structures, and directly observe polyQ128 aggregates. We first confirmed that polyQ0 and Huntington's disease model (polyQ128) strain neurons were sensitive to RNAi treatment by feeding as detected by GFP knockdown in the nerve ring (Supplemental Figure 1). Indeed, GFP knockdown was efficient in both strains, supporting that our intended studies on insulin signaling pathway genes could effectively target mechanosensory neurons.

Interestingly, healthy polyQ0 and proteotoxically stressed polyQ128 empty vector (L4440) treated animals exhibited similar, not significantly different, mean lifespan (**Figure 2A**). Thus, increased polyglutamine load in mechanosensory neurons does not confer decreased lifespan, consistent with previous work showing mechanosensory neurons are dispensable for viability and lifespan (Chalfie et al., 1985). However, empty vector treated control polyQ128 animals do exhibit signs of abnormal function as measured by motility, touch response, and accumulation of mechanosensory neuronal aberrations (**Table 1**, **Figures 2C,D**), agreeing with previous work (Parker et al., 2001). Specifically, polyQ128 posterior touch response is significantly decreased (Mann–Whitney U, $p < 0.01$) and neuronal aberrations are increased at the whole worm (Mann–Whitney U, $p < 0.01$), anterior cells (Mann–Whitney U, $p < 0.01$), and posterior cells level (Mann–Whitney U, $p = 0.04$) when compared to age-matched polyQ0 individuals. This suggests that increased polyglutamine load in the mechanosensory neurons negatively affects the function and healthspan of polyQ128 animals.

Table 1 | Effect of RNAi treatment on polyQ0 and polyQ128 motility.

Strain	RNAi treatment	Number per motility class			Total *N*
		A	***B***	***C***	
polyQ0	Empty vector	65	4	0	69
	daf-2	53	0	0	53
	age-1	67	4	0	71
	daf-18	47	2	0	49
	akt-1	50	1	0	51
	daf-16	48	3	0	51
polyQ128	Empty vector	69	15	0	84
	daf-2	87	6	0	93
	age-1	77	18	0	95
	daf-18	47	0	0	47
	akt-1	31	15	1	47
	daf-16	51	20	1	72

Number of individuals falling into A, B, or C class motility for each treatment group is shown. A class indicates normal, voluntary sinusoidal movement, B class indicates locomotion following gentle prodding, and C class indicates inability to locomote. Overall, polyQ128 animals have higher incidence of B class motility than polyQ0. Only polyQ128 daf-18(RNAi) results in a significant difference in motility compared to control.

Because several previously published mutant and RNAi experiments did not utilize *sid-1* enhanced neuronal RNAi targeting, and thus would not have assayed neuronal knockdown effects, we also confirmed RNAi effects on longevity under the conditions we used for our studies. We found that knockdown of insulin signaling pathway genes in neurons and other tissues altered healthy polyQ0 life as previously reported, with *daf-2*, *age-1*, and *akt-1* RNAi interventions lengthening lifespan, and *daf-16* RNAi shortening it (**Figure 3A** and **Table 2**). Insulin signaling pathway interventions in the polyQ128 strain (*daf-2, age-1*) similarly increased mean lifespan, while *daf-16* RNAi treatment decreased mean lifespan (**Figure 3B** and **Table 2**). In the 3 biological replicates of *akt-1* RNAi in polyQ128 animals, lifespan impact was variable.

To further address whether there might be differences in general viability between polyQ0 and polyQ128 strains, we compared percent change in mean lifespan between polyQ0 and polyQ128 animals for each RNAi treatment group. We found no significant differences in relative percent changes between healthy transgenic and polyQ128 animals for the same RNAi treatment (*t*-tests, $p > 0.05$), except for *daf-16* RNAi, which approached significance in the polyQ128 background (**Figure 3C**; $p = 0.07$). We also measured endogenous reactive oxygen species (ROS) as detected by the membrane permeable 2′, 7′-Dichlorofluorescin diacetate (DCF-DA, Sigma) following RNAi treatments and found that RNAi treatment targeted at insulin signaling pathway proteins from embryo did not consistently significantly affect young adult (day 1) endogenous ROS levels in both healthy transgenic and polyQ128 strains (data not shown). However, *daf-16* RNAi treatment through mid-life in polyQ128 worms significantly increased endogenous ROS levels across all biological replicates.

We conclude that RNAi interventions in our polyQ0 and polyQ128 strains are efficacious and exert similar general influences on aging biology in polyQ0 and polyQ128 strains. Disruption of a small set of sensory neurons by the proteotoxic stress of polyQ128 is not sufficient to grossly impair whole animal function, although there are some functional impairment and increased morphological aberrations. However, we note that in middle-aged adults, *daf-16* RNAi is associated with elevated ROS levels and decreased mid-life viability specifically in the polyQ128 strain, raising the possibility that the combination of small scale neuronal proteostasis disruption can influence entire organism decline when DAF-16-dependent defenses are impaired.

DECREASED INSULIN SIGNALING THROUGH RNAi KNOCKDOWN INFLUENCES HEALTHY MECHANOSENSORY NEURON MORPHOLOGY AND FUNCTION

Previous studies on morphological aging of mechanosensory neurons indicated that manipulation of the insulin signaling pathway can change accumulation of neuronal aberrations (Pan et al., 2011; Tank et al., 2011; Toth et al., 2012). These studies differed in details of methods and outcomes, and did not address *akt-1* or *daf-18* activities. Moreover, these studies left open the

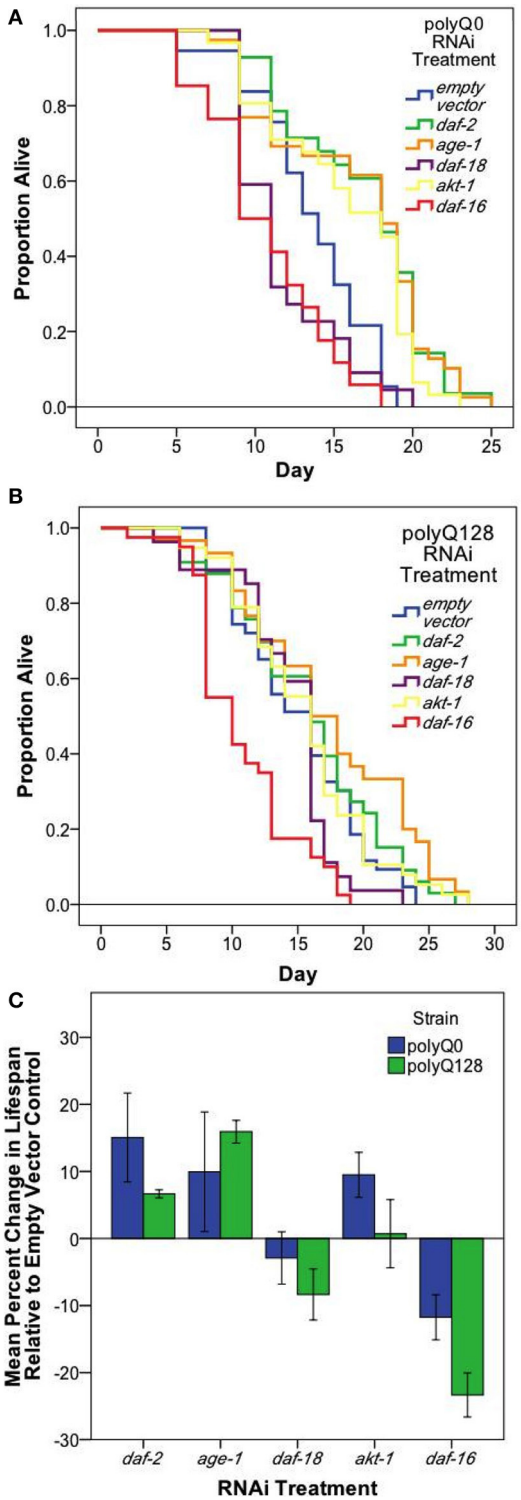

FIGURE 3 | Neuronal and systemic insulin signaling mediates lifespan of both healthy (polyQ0) and proteotoxically stressed (polyQ128) animals. Kaplan–Meier survival curves for one representative survival experiment shown for polyQ0 **(A)** and polyQ128 **(B)** animals with RNAi knockdown of the indicated insulin signaling pathway gene. Mean lifespan, sample size, and significance reported as Replicate 1 for each strain in

(Continued)

FIGURE 3 | Continued

Table 2. **(C)** Mean percent change in lifespan with RNAi treatment for polyQ0 and polyQ128 animals pooled across biological replicates. Only *daf-16* RNAi showed a significant impact on lifespan for polyQ128 animals when compared to polyQ128 empty vector controls (23.3 ± 4.1% for 3 replicates of 45–50 animals). Each bar represents mean ± SE.

question of how morphological features relate to function. To address these gaps and discrepancies, we measured neuronal morphology and touch response as a surrogate for mechanosensory function when components of the insulin signaling pathway were knocked down in polyQ0 animals. We performed touch response tests (**Figure 4A**) and then imaged mechanosensory neurons of characterized animals for changes in morphology (**Figures 4B,C**) at mid-life (day 5 of adulthood; described in detail in Materials and Methods). Total neuronal aberrations include all morphological aberrations observed in an individual or cell type, including soma outgrowths, abnormal cell somas, process branching, and process punctae (**Figure 4B**).

Our analysis of touch sensory function revealed that both anterior and posterior touch responses in *daf-2* RNAi were better than empty vector at day 5 of adult life (**Figure 4A**; Wald test, $p < 0.01$ for anterior and posterior scores). *daf-16* RNAi preferentially decreased posterior touch ($p < 0.01$), revealing an interesting difference between anterior and posterior mechanosensory neurons. We found that RNAi knockdown of *age-1*, *daf-18*, and *akt-1* did not alter touch responses as compared to empty vector.

A striking result from our analysis of morphological aberrations at day 5 of adulthood is that *daf-2* RNAi increased the occurrence of total neuronal aberrations as compared to control (from 3.2 ± 0.23 to 4.7 ± 0.26 per individual) in both anterior and posterior neurons (**Figure 4C**). We found that this increase was driven exclusively by process branching, which was the only specific neuronal aberration observed to change with *daf-2* RNAi (**Figure 4D**; from 12.8 ± 3.40 to 32.7 ± 3.80% in anterior cells and 14.5 ± 3.20 to 39.4 ± 3.70% in posterior cells). *age-1* RNAi also increased total aberrations (from 3.2 ± 0.23 to 4.4 ± 0.23 per individual) (**Figure 4C**) and novel branching in anterior neurons (**Figure 4D**; from 12.8 ± 3.40 to 25.3 ± 3.40% in anterior cells and 14.5 ± 3.20 to 26.7 ± 3.30% in posterior cells), whereas *daf-18*, *akt-1*, and *daf-16* interventions did not induce statistically significant changes in overall aberrations or in the hyper-branching phenotype. Our findings suggest that *daf-2* disruption, and lowered insulin signaling could differentially effect different morphologies, and raise the question as to whether branching might be an indication of a neuroprotective response (see Discussion).

TOUCH RESPONSE, MECHANOSENSORY NEURONAL MORPHOLOGY, AND PROTEIN AGGREGATE ACCUMULATION ARE AFFECTED BY INSULIN SIGNALING IN A MODEL STRAIN OF HUNTINGTON'S DISEASE PATHOGENESIS

We next examined functionality and morphological aberrations in the polyQ128 strain in which mechanosensory neurons are exposed to a chronic proteotoxic stress that promotes early dysfunction (Parker et al., 2001). As previously noted,

Table 2 | Effects of RNAi treatment targeting insulin signaling on polyQ0 and polyQ128 animals' mean lifespan.

Strain	RNAi treatment	Replicate 1		Replicate 2		Replicate 3	
		N	**Mean lifespan days ± *SE***	***N***	**Mean lifespan days ± *SE***	***N***	**Mean lifespan days ± *SE***
polyQ0	Empty vector	50	13.7 ± 0.56	60	14.3 ± 0.58	55	13.5 ± 0.66
	daf-2	40	16.8 ± 0.83*	*N/A*	*N/A*	48	16.3 ± 1.04*
	age-1	51	16.3 ± 0.82*	59	16.9 ± 0.61*	*N/A*	*N/A*
	daf-18	30	11.7 ± 0.70	60	14.8 ± 0.57	50	13.5 ± 0.62
	akt-1	40	15.8 ± 0.79*	60	16.2 ± 0.52*	48	13.4 ± 0.50
	daf-16	46	11.0 ± 0.61*	60	12.1 ± 0.53*	51	12.9 ± 0.68
polyQ128	Empty vector	50	15.2 ± 0.71	50	15.1 ± 0.61	56	13.4 ± 0.55
	daf-2	51	16.1 ± 0.94	50	16.2 ± 0.89*	*N/A*	*N/A*
	age-1	40	17.9 ± 1.01*	*N/A*	*N/A*	59	15.0 ± 0.89*
	daf-18	38	15.6 ± 0.79	50	14.5 ± 0.66	59	11.4 ± 0.42*
	akt-1	47	15.8 ± 0.79	25	14.4 ± 1.15	56	11.4 ± 0.44*
	daf-16	48	10.9 ± 0.60*	50	12.8 ± 0.63*	61	10.4 ± 0.39*

*Mean lifespan in days ± SE of each RNAi treatment replicate for the polyQ0 and polyQ128 strains are shown. Each replicate of 6 treatments (empty vector control, DAF-2, AGE-1, DAF-18, AKT-1, and DAF-16) was run one strain at a time. Entries with * are significantly different ($p < 0.05$) from the strain- and replicate-matched empty vector control following Kaplan–Meier log-rank survival statistics. Replicate with missing data (N/A) was censored from experiment.*

empty vector treated polyQ128 animals exhibit increased aberrant neuronal morphology and decreased touch response compared to age-matched, empty vector treated wildtype animals (**Figures 3C,D**).

We tested touch response in polyQ128 animals subjected to RNAi for insulin signaling pathway components (**Figure 5A**). *daf-2* RNAi had a neuroprotective effect on both anterior (Wald test, $p < 0.001$) and posterior touch sensitivity (Wald test, $p < 0.001$). Interestingly, however, all other RNAi knockdown interventions (*age-1*, *daf-18*, *akt-1*, and *daf-16*) had generally deleterious effects on touch sensitivity in the polyQ128 background, with a particularly significant change in posterior touch response in polyQ128 animals compared with empty vector (L4440).

We then examined neuronal morphology in the polyQ128 strain following RNAi of the insulin signaling pathway genes (**Figure 5B**). We found that numbers of aberrations in the polyQ128 strain were reduced upon *daf-2* RNAi when compared to empty vector (from 4.78 ± 0.21 to 2.94 ± 0.15 per individual, Wald test, $p < 0.001$). In contrast, *age-1* RNAi, modestly increased aberrations (from 4.3 ± 0.2 to 5.4 ± 0.1 per individual), as did *akt-1* and *daf-16* interventions in anterior mechanosensory neurons. Thus for polyQ128-expressing neurons, morphological aberrations generally inversely correlate with function: low abnormality abundance corresponds to enhanced mechanosensory function.

In polyQ128 animals, the huntingtin:polyQ128 protein is fused with CFP and can be visualized as aggregates in our strain (**Figure 5C**). We therefore also examined the number and size of fluorescent aggregates in mechanosensory neurons following RNAi knockdown of insulin signaling components (**Figure 5**D). Under conditions of *daf-2* RNAi we found that mean numbers of aggregates were lowered compared to wildtype (Wald test, $p < 0.01$ for whole individuals, anterior cells, and posterior cells). Mean aggregate area was unchanged for *daf-2* RNAi (Supplemental Figure 2), suggesting that less aggregated protein persists in mid-life mechanosensory neurons when DAF-2 signals are reduced. Conversely, we found modest increases in aggregate number and size for *age-1*, *akt-1*, and *daf-16* knockdown (**Figure 5D** and Supplemental Figure 2). Importantly, Parker et al. (2005) showed no difference in huntingtin expression of the polyQ128 animals in *age-1* and *daf-16* genetic mutants. This suggests that our findings of altered protein aggregation in the polyQ128 strain is likely not due to changed huntingtin expression, but rather is more likely attributed to cellular responses of the expanded polyglutamine protein (**Figure 5**).

Interestingly, we noted a striking difference in the localization of polyQ128 protein aggregates in posterior and anterior cells for *daf-2* RNAi (**Figures 5E,F**). In *daf-2* RNAi treated animals, 80–90% of the detected aggregates localized in the cell body of anterior and posterior cells whereas other insulin signaling pathway knockdowns were associated with a majority (50–60%) of aggregates localized within the process of these cells, similar to empty vector controls. We also note that aggregates were never observed in outgrowths without also being present in the cell body. This dramatic difference of aggregate localization in *daf-2* RNAi animals suggests that subcellular distribution of protein aggregates is regulated by a DAF-2 non-canonical pathway.

RNAi KNOCKDOWN OF *daf-2* RETURNS polyQ128 MECHANOSENSORY NEURON MORPHOLOGY TO HEALTHY LEVELS

At the whole worm and anterior and posterior cell levels, *daf-2* RNAi knockdown in the neurons of polyQ128 worms lowers the occurrence of total neuronal aberrations to levels observed in polyQ0 empty vector treated animals (Wald test, $p < 0.01$). However, *daf-2* RNAi polyQ128 touch response is still significantly lower than polyQ0 empty vector treated animals (Wald test, $p < 0.01$).

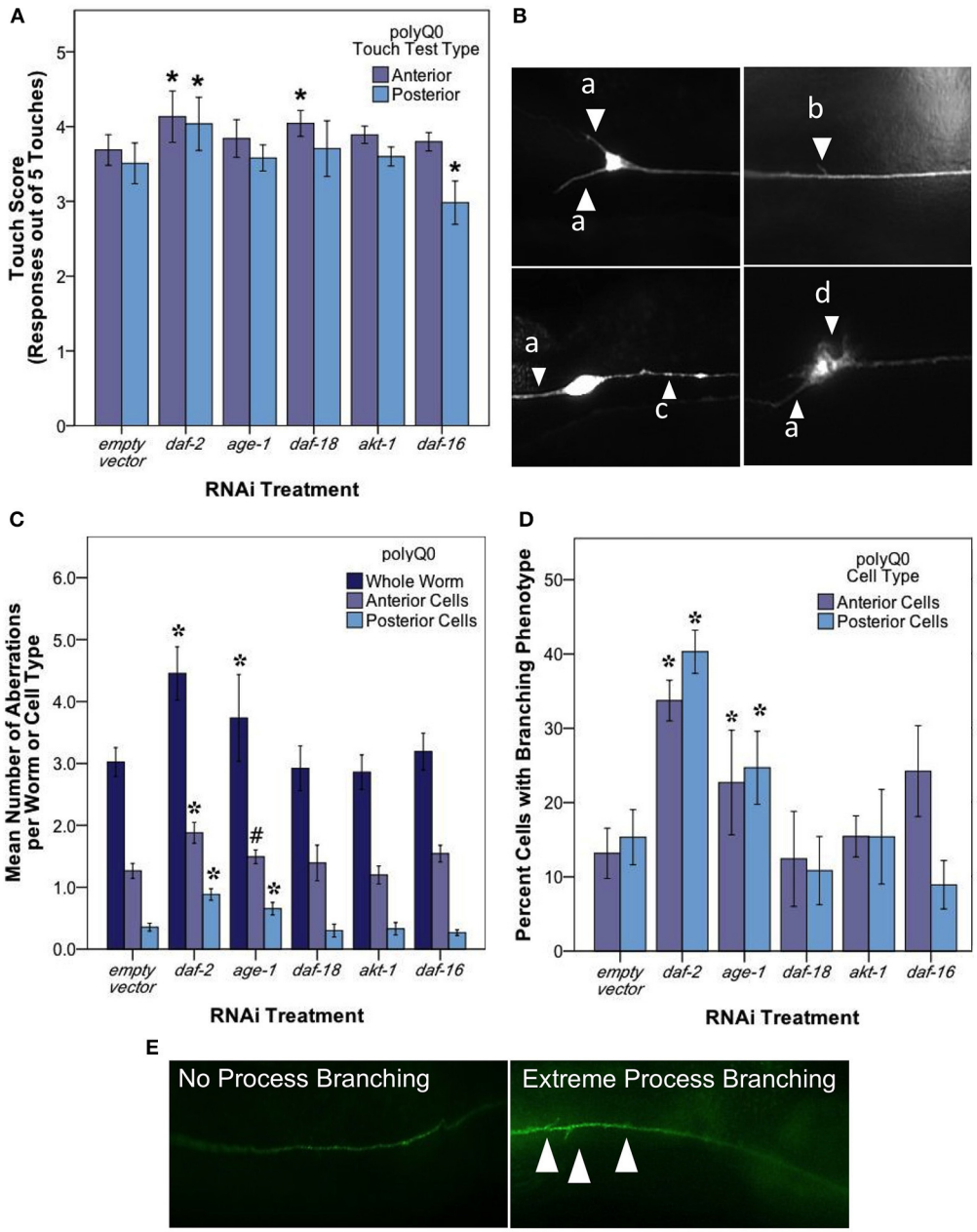

FIGURE 4 | *daf-2* RNAi mediates mechanosensory neuron morphology and function in polyQ0 animals. (A) Anterior and posterior touch test scores of polyQ0 animals following RNAi treatment. Each bar represents the mean number of positive responses to 5 soft touches. **(B)** Representative examples of outgrowths (a), branching (b), punctae (c), and abnormal cell body (d) phenotypes seen in aging mechanosensory neurons of polyQ0 and polyQ128 individuals. **(C)** Mean number of neuronal aberrations per whole animal (sum of all 4 neurons scored in an individual) and individual cell type (anterior [ALML or ALMR] and posterior [PLML or PLMR]). **(D)** Percentage of neurons with branching phenotype for each RNAi treatment. Neurons with this phenotype may have more than one branch (data not shown). **(E)** Representative images of mechanosensory neuron processes and process branching (white arrows) in polyQ0 animals. *Denotes significance of $p < 0.01$ and #denotes significance of $p < 0.10$ relative to appropriate empty vector control following a generalized linear model with a log link function (Poisson regression) and Wald tests for significance of treatment effects. Each bar represents mean ± SE for $N = 49$–70 animals.

DISCUSSION

SUMMARY OF FINDINGS

To begin to address the relationship between natural and proteotoxically challenged neuronal aging in a physiological context, we took advantage of high resolution *in vivo* analyses of *C. elegans* mechanosensory neuron morphology and function. We compared mid-life morphological and functional features of mechanosensory neurons that aged without, or

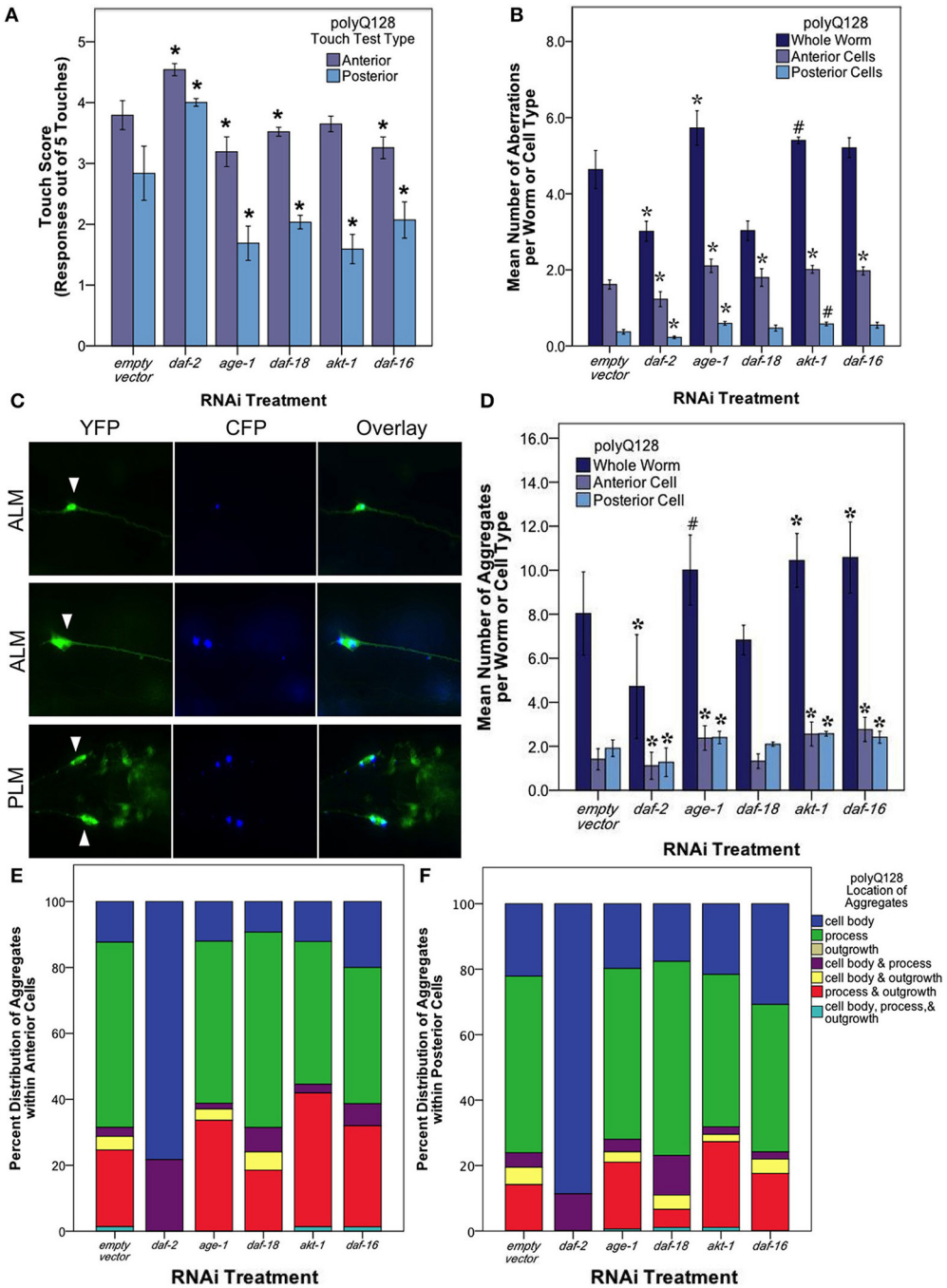

FIGURE 5 | The insulin signaling pathway mediates mechanosensory neuron morphology and function in polyQ128 animals. **(A)** Anterior and posterior touch test scores, representing the number of positive responses to 5 soft touches, following RNAi treatment for indicated insulin signaling pathway gene of polyQ128 animals. **(B)** Mean number of neuronal aberrations per whole animal (sum of all 4 neurons scored in an individual) and individual cell type (anterior [ALML or ALMR] and posterior [PLML or PLMR]). **(C)** Representative image of polyQ128 aggregate (CFP, blue panels) accumulation within anterior (ALM) and posterior neurons (PLM) (YFP, green panels). White arrows in YFP panels indicate the location of the cell body of each neuron. **(D)** The effect of RNAi treatment on the number of distinct CFP-labeled extended polyglutatmine huntingtin protein aggregates in whole animal and anterior and posterior neurons of polyQ128 animals. **(E)** Sub-cellular localization of poyQ128 aggregates within anterior neurons of polyQ128 animals that contained aggregates. **(F)** Location of polyQ128 aggregates in posterior neurons of polyQ128 animals. *Denotes significance of $p < 0.01$ and #denotes significance of $p < 0.10$ relative to appropriate empty vector control following generalized linear model analysis with a log link function (Poisson regression) and Wald tests for significance of treatment effects. Each bar represents mean ± SE.

with, the proteotoxic stressor polyQ128, under RNAi conditions that mimic systemic, including neurons, low or high activation of the insulin signaling pathway. Our data suggest that insulin signaling plays complex roles in neuronal maintenance in both healthy (polyQ0) and degenerate (polyQ128) neurons, with distinct outcomes on individual neurons notable even among the similar group of 6 mechanosensory neurons. Our data on individual neurons highlight remarkable neuronal diversity of responses to cellular signaling. Comparison of how the insulin signaling pathway impacts natural and proteotoxic-associated decline reveals some interesting differences in these two processes.

We conclude that RNAi down-regulation of the *daf-2* insulin receptor plays a role in morphological aging of mechanosensory neurons in both normally aging (polyQ0) and proteotoxically stressed (polyQ128) animals. Anterior and posterior neurons can be differentially affected by altered neuronal insulin signaling (**Table 3**). Importantly, our data suggest that decreased insulin signaling in normally aging systems (polyQ0) actually enhances some process aberrations in middle aged adults, with a focused impact on formation of ectopic branches on axons and dendrites. That *daf-2* mechanosensory function is improved raises the possibility that the structural aberrations (branching) we observe actually reflect consequences of a defensive response that enhances or protects neuronal health during aging (**Figure 4**). *age-1* may contribute partially to this process in polyQ0 animals, but evidence for the involvement of other pathway components is not compelling in our study. We also find that *daf-2* RNAi knockdown has distinctive impact on the proteotoxically stressed polyQ128 mechanosensory neurons: morphological aberrations are lower; and aggregates are fewer in number and smaller in size, with a striking subcellular restriction of aggregates to the cell body, as compared to empty vector controls. Somewhat surprisingly, other insulin signaling pathway disruptions have relatively modest impact on cell function, aggregate morphology, and aggregate distribution in polyQ128 animals; we found that *age-1* RNAi, which extends overall lifespan, did not confer dramatic changes in mechanosensory neurons, and often correlated with *daf-16* RNAi in inducing modest effects. Our data raise the possibility that, under conditions of extreme proteotoxic stress, neurons utilize a non-canonical *daf-2* pathway to enhance neuroprotection.

EXTENDING UNDERSTANDING OF THE INFLUENCE OF INSULIN SIGNALING ON HEALTHY AGING MECHANOSENSORY NEURONS

daf-2 insulin receptor

Mechanosensory neuron morphology changes with age in wildtype animals (Pan et al., 2011; Tank et al., 2011; Toth et al., 2012). Overall, genetic mutants and systemic RNAi treatments have suggested that *daf-2* mutants maintain youthful, aberration-free phenotypes longer than wildtype animals. Toth et al. (2012) distinguished among specific abnormality classes to measure decreases in cell body outgrowths and wavy process phenotypes, with a trend toward delaying branching in posterior mechanosensory neurons. Tank et al. (2011) showed that 10 day old *daf-2(e1370)* mutants had decreased process branching. Using a neuron-targeted *daf-2* RNAi approach, we measured an increase in neuronal aberrations at day 5 of adulthood, the vast majority of which are novel branches (**Figures 4D,E**). Because we find increased aberrations in *daf-2* RNAi coincident with a period of enhanced function (**Figure 4A**), we raise the question

Table 3 | RNAi treatments targeting the insulin signaling pathway differentially effect polyQ128 specific mechanosensory neuronal aberrations.

Specific types of aberrations per	Cell type	RNAi treatment on polyQ128 animals									
		Empty vector ($N_{anterior}$ = 226; $N_{posterior}$ = 217)	(*daf-2* $N_{anterior}$ = 199; $N_{posterior}$ = 202)		*age-1* ($N_{anterior}$ = 185; $N_{posterior}$ = 185)		*daf-18* ($N_{anterior}$ = 92; $N_{posterior}$ = 94)		*akt-1* ($N_{anterior}$ = 93; $N_{posterior}$ = 92)		*daf-16* ($N_{anterior}$ = 143; $N_{posterior}$ = 140)	
		Mean ± *SE*	Mean ± *SE*	*p*-value	Mean ± *SE*	*p*-value	Mean ± *SE*	*p*-value	Mean ± *SE*	*p*-value	Mean ± *SE*	*p*-value
Number of cell body outgrowths	Anterior	1.11 ± 0.05	0.86 ± 0.04	*0.00	1.32 ± 0.06	*0.01	1.21 ± 0.08	*n.s.*	1.24 ± 0.08	*n.s.*	1.29 ± 0.06	*0.03
	Posterior	0.01 ± 0.01	0.01 ± 0.03	*n.s.*	0.03 ± 0.03	*n.s.*	0.01 ± 0.01	*n.s.*	0.00 ± 0.00	*n.s.*	0.01 ± 0.01	*n.s.*
Number of branches	Anterior	0.15 ± 0.03	0.09 ± 0.02	*0.05	0.16 ± 0.03	*n.s.*	0.23 ± 0.05	*n.s.*	0.10 ± 0.03	*n.s.*	0.13 ± 0.03	*n.s.*
	Posterior	0.07 ± 0.02	0.05 ± 0.02	*n.s.*	0.08 ± 0.02	*n.s.*	0.05 ± 0.02	*n.s.*	0.03 ± 0.02	*n.s.*	0.05 ± 0.02	*n.s.*
Presence of punctae	Anterior	0.14 ± 0.02	0.04 ± 0.02	*0.00	0.37 ± 0.02	*0.00	0.27 ± 0.03	*0.02	0.39 ± 0.03	*0.00	0.33 ± 0.02	*0.00
	Posterior	0.71 ± 0.03	0.90 ± 0.03	*0.00	0.59 ± 0.04	*0.02	0.65 ± 0.05	*n.s.*	0.55 ± 0.05	*0.01	0.58 ± 0.04	*0.01
Presence of abnormal cell body	Anterior	0.13 ± 0.01	0.03 ± 0.01	*0.00	0.19 ± 0.01	*n.s.*	0.10 ± 0.02	*n.s.*	0.15 ± 0.02	*n.s.*	0.11 ± 0.01	*n.s.*
	Posterior	0.00 ± 0.01	0.02 ± 0.01	*0.02	0.03 ± 0.01	*0.01	0.02 ± 0.02	*n.s.*	0.00 ± 0.02	*n.s.*	0.03 ± 0.01	*0.02

Table depicts mean number and significant difference for anterior (ALML or ALMR) and posterior (PLML or PLMR) mechanosensory neurons following RNAi treatment for each specific neuronal aberration observed (i.e., cell body outgrowths, process branching, presence of process punctae, and cell body abnormality). p-values indicate significance compared to appropriate empty vector control following generalized linear model analysis. Sample sizes for anterior ($N_{anterior}$) and posterior ($N_{posterior}$) neurons of each RNAi treatment are shown. Example outgrowths (A), branching (B), punctae (C), and abnormal cell body (D) are shown in ***Figure 4B****. *Denotes significance of* $p < 0.05$.

of whether mid-life branching might be a manifestation of a normal neuronal defense mechanism that actually improves sensory capacity, although this issue remains to be resolved with single animal functional imaging. This finding is also significant in that it suggests that all changes in morphology seen with age or treatments are not the same mechanistically and that some phenotypes, such as process branching, may be protective to neurons. Differences from other studies (Pan et al., 2011; Tank et al., 2011; Toth et al., 2012) could arise from differences in methods for reducing *daf-2* expression (with RNAi knockdown being distinct from modulation of specific amino acid resides in receptor reduction-of-function mutants) and different timing of scoring during adult life.

age-1 PI3 kinase and other insulin signaling molecules

We found that *age-1* RNAi modestly increased branching of mechanosensory neuron processes at day 5 of adult life, but did not alter touch sensitivity. The latter observation establishes that morphological phenotype and function are not always linked. *akt-1*, *daf-18*, and *daf-16* RNAi interventions did not alter the trajectory of age-associated morphological change in otherwise healthy neurons. The role of *age-1, akt-1*, and *daf-18* in morphological aging of the mechanosensory neurons has not been previously reported. Overall, *age-1* knockdown can have an effect on mechanosensory neuron morphological aging (**Figures 4D,E**) but impact on function is not large at mid-life. Other pathways that run in parallel may be important in mid-adult life (Tank et al., 2011).

daf-16

For natural aging of mechanosensory neurons, we find that *daf-16* RNAi exerts a small but statistically significant effect on day 5 adult posterior touch response, but not on aberrations. Tank et al. (2011) also came to the conclusion that aberrations for day 10 branching in a *daf-16* deletion mutant were similar to wildtype; whereas Pan et al. (2011) and Toth et al. (2012) noted a small increase in aberrations in *daf-16* mutants at days 9 and 10 (note the latter study, like ours, found no change at adult day 5). Still, time course studies do not support a profound impact of *daf-16* disruption on morphological aging of the mechanosensory neurons. *daf-16* appears to be needed for *daf-2(rf)*-mediated suppression of excess branching, though cell autonomy of this activity is disputed (Pan et al., 2011; Tank et al., 2011).

Overall, although compelling data support that insulin signaling a factor in natural aging of mechanosensory neurons, with reduced signaling correlating with reduced function, other pathways likely influence the process as well (Tank et al., 2011).

DISTINCTIVE OUTCOMES OF daf-2 RNAi IN THE PROTEOTOXICALLY STRESSED HUNTINGTON'S DISEASE MODEL STRAIN

daf-2

In middle-aged polyQ128 animals, we found that *daf-2* RNAi improved mechanosensory function, limited the number of morphological aberrations, and decreased overall aggregate number and size, compared to empty vector controls (**Figure 5**). Thus, reduced insulin receptor signaling through DAF-2 confers neuroprotection that is associated with diminished polyQ128 aggregate load in this model. Our data are consistent with studies in other disease models (Cohen et al., 2010) implicating *daf-2* in enhanced proteostasis during toxic protein challenge.

age-1 PI3 kinase and other insulin signaling molecules

We found that *age-1* RNAi modestly impairs touch sensitivity at a time point at which the mean number of aberrations and of aggregates are elevated relative to empty vector controls (**Figure 4**). Unexpectedly, *akt-1* and *daf-16* RNAi, which should have opposing impacts on the signal transduction pathway, induce similar outcomes in these proteotoxically challenged mechanosensory neurons. Because *age-1* RNAi [and sometimes *akt-1* (Hertweck et al., 2004)] extend lifespan (**Table 2**), dysfunction is not the anticipated outcome of such interventions. We emphasize two points here: first, our data raise the possibility that the most commonly outlined downstream pathway for DAF-2 signaling may not be the operative signaling pathway for the mechansensory neuron proteotoxicity pathway; and second, as it has previously been noted that other healthspan phenotypes differentially affected by *daf-2* vs. *age*-1 mutations. For example, in *age-1* mutants the biphasic profile for rate of increase in lipofuscin/age pigments during adulthood shows a temporal shift (delay in onset without change in time course), whereas for *daf-2* mutants the rate of lipofuscin accumulation remains low across adulthood (Gerstbrein et al., 2005). In other words, *daf-2* is more effective in preventing long term elevation in age pigment accumulation, while *age-1* delays onset of accumulation. This anomaly is a precedent for differential health outcomes following closely related insulin signaling pathway interventions.

In previous work, Morley et al. (2002) found that *C. elegans* expressing polyQ82 in body wall muscle show slower development of aggregates and motility defects with *age-1* RNAi and *age-1* mutants. While those authors showed the opposite effect of *age-1* RNAi on polyQ accumulation and toxicity compared to our study using a different cell type, they stressed the importance of threshold stresses in their interpretation. We note that our model carries an elevated polyglutamine load (polyQ128) as compared to polyQ82. In humans, longer polyglutamine expansion in huntingtin is well known to result in earlier onset of Huntington's disease and lower life expectancy.

daf-16

As expected, we observed *daf-16* RNAi confers diminished mechanosensory function together with increased anterior aberration and increased number and size of aggregates. Our results are consistent with previous studies showing that *daf-16* deficiency is associated with exacerbated polyQ128 proteotoxicity in young adult mechanosensory neurons (Parker et al., 2005, 2012; Lejeune et al., 2012) and with enhanced proteotoxicity in a polyQ82 model (Morley et al., 2002) and an Alzheimer's disease model (Cohen et al., 2009).

Overall, changes in anterior and posterior cell function, morphology, and protein aggregation load and localization (**Figures 4**, **5**) correlate with whole animal observations (**Figure 3**) in both polyQ0 and polyQ128 strains. However, with

polyQ128 *daf-16* RNAi we observed decreased anterior and posterior mechanosensory function (**Figure 5A**) and increased protein aggregate area and number in whole animal and both cell types (**Figure 5D** and Supplemental Figure 2), while only anterior mechanosensory neurons (ALML or ALMR) increased significantly in total neuronal aberrations (**Figure 5B**). This maintenance of neuronal morphology with worsened function and protein aggregation suggests other mechanisms can mediate these endpoints.

daf-2 RNAi UNIQUELY CHANGES DISTRIBUTION OF polYQ128 AGGREGATE LOAD

A striking observation of *daf-2* RNAi animals is a profound difference in the distribution of polyQ128 aggregates (**Figures 5E,F**). *daf-2* RNAi is the only intervention we tested that induces localization of CFP-labeled polyQ128 aggregates nearly exclusively to the neuron cell body. Most aggregates we concentrated in only a few dots, which resembled perinuclear lysosomes. Although this subcellular domain restriction remains to be definitively identified, our observations suggest that for both anterior and posterior mechanosensory neurons, *daf-2* may exert neuroprotection by sequestering aggregates to prevent them from interfering with other cellular functions. The lower aggregate size suggests that enhanced degradation may occur when DAF-2 signaling is low. Our data also suggest that DAF-2 signaling could influence axonal transport of aggregate proteins or their retention in the cell body. Since we did not observe the cell body restricted pattern of *age-1* RNAi, a non-canonical downstream signaling pathway might be responsible for the observed effect.

NEURONS AGING UNDER EXTREME AGGREGATE CHALLENGE EXHIBIT DIFFERENCES FROM NATURAL AGING

It is striking that the *daf-2* RNAi polyQ128 neuronal aberration level is below that of polyQ128 empty vector controls (**Figure 5B**), and similar to that of polyQ0 empty vector controls (**Figure 4C**), while polyQ0 *daf-2* RNAi increases aberrations. Together with stresses induced by polyQ128, low insulin signaling (*daf-2* and *age-1* RNAi treatment) is associated both with protection from and an increase in morphological restructuring. Without extreme proteotoxic challenge at mid-life (as in polyQ0), however, neuronal aberrations are more apparent when insulin signaling is low. One possibility to explain these differences between *daf-2* RNAi in the naturally aging (polyQ0) and proteotoxically stressed (polyQ128) models is that hormetic consequences induced by polyQ128 are involved in suppression of aberrations. Alternatively, the aberrations in polyQ0 may be a manifestation of cellular maintenance that cannot be executed in the face of extreme polyQ128 challenge. An interesting question is whether additional neuron classes in the polyQ128 animals are affected by the expression of polyQ aggregates in the mechanosensory neurons or whether these effects are cell autonomous.

A second striking difference between natural aging and aging under extreme proteotoxic stress resides in *daf-16* RNAi treatment effects. In polyQ0 animals, *daf-16* RNAi impairs posterior touch sensation, but does not markedly alter neuronal morphology (only affecting anterior touch sensitivity). In contrast, in polyQ128 animals, *daf-16* RNAi results in both impaired touch response and increased neuronal aberrations. Also, *daf-16* RNAi in polyQ128, aggregate load increases and lifespan decreases proportionally more than in polyQ0. This suggests that polyQ128 expression in mechanosensory neurons induces stress signaling to other body tissues to disrupt the overall health of the animal.

A potential mechanism for the differences in effects of manipulating insulin signaling in polyQ0 and polyQ128 is increased basal insulin signaling in polyQ128 as a result of its protein load. However, we propose this is unlikely to be the mechanism underlying the observed differences. We see no difference in lifespan between empty vector control (L4440) treated polyQ0 and polyQ128 lifespan (**Figure 2A**). This is not surprising given that mechanosensory neurons do not seem to modulate lifespan (Chalfie et al., 1985). Further, microarray analysis of polyQ128 FACS-purified $P_{mec\text{-}3}$ cells (mechanosensory neurons) showed no apparent dysregulation of genes in the insulin signaling pathway compared to non-toxic huntingtin:polyglutamine-expressing controls (Tourette and Neri, personal communication). Thus, the differences in neuron morphology and function seen in empty vector control treated polyQ0 and polyQ128 animals are likely due to some mechanism other than insulin signaling.

We have seen that with age, the effects on touch response and mechanosensory morphology are negatively correlated in polyQ0 and polyQ128 models (Vayndorf et al., in preparation). We showed that this correlation between increased morphological aberrations and decreased function remains in all RNAi treated polyQ128 animals (**Figure 5**). However, when polyQ0 animals receive RNAi treatment targeting components of the insulin signaling pathway, touch response and accumulation of aberrant morphology are no longer negatively correlated (**Figure 4**). Interestingly, while only *daf-2* RNAi showed overall significant effects on mechanosensory morphology and function in polyQ0 animals, RNAi treatment of polyQ128 animals yielded a higher number of significant changes. Also, we observed empty vector control polyQ128 animals decreased touch response and increased changes in neuronal morphology when compared to polyQ0 animals. Perhaps the decreased baseline neuronal function and worsened baseline neuronal morphology of the polyQ128 animals makes them more susceptible to changes in expression of insulin signaling pathway proteins, whereas naturally aging animals have more ability to compensate with other signaling pathways when there are changes in insulin signaling protein expression.

Overall, our findings suggest that improving neuronal aging outcomes and neuronal dysfunction associated with elevated protein aggregate stress may not be as simple as decreasing classical insulin signaling. This is not surprising because there are 40 insulin ligands with different expression levels in various cell types all vying for binding sites at the *C. elegans* insulin receptor, DAF-2. Others have shown that this insulin network is complex and works together to respond to varying stresses (Ritter et al., 2013), including protein misfolding and dysfunction like in our polyQ128 model. In addition, in mammalian and cell culture models, huntingtin is a phosphorylation substrate for AKT/PKB (Humbert et al., 2002; Dong et al., 2012). AKT can be

modified and even cleaved into an inactive form in a rat model of Huntington's disease (Colin et al., 2005). Thus, in systems with endogenous huntingtin, unlike *C. elegans*, complex interactions between insulin signaling, expanded polyglutamine huntingtin aggregation, and neuronal morphology and function may be operative. Further genetic studies of the huntingtin polyQ128 model in *C. elegans* have the potential to elucidate mechanisms that influence morphological changes during neuronal aging.

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SUPPLEMENTARY MATERIAL

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Longevity pathways and memory aging

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The aging process has been associated with numerous pathologies at the cellular, tissue, and organ level. Decline or loss of brain functions, including learning and memory, is one of the most devastating and feared aspects of aging. Learning and memory are fundamental processes by which animals adjust to environmental changes, evaluate various sensory signals based on context and experience, and make decisions to generate adaptive behaviors. Age-related memory impairment is an important phenotype of brain aging. Understanding the molecular mechanisms underlying age-related memory impairment is crucial for the development of therapeutic strategies that may eventually lead to the development of drugs to combat memory loss. Studies in invertebrate animal models have taught us much about the physiology of aging and its effects on learning and memory. In this review we survey recent progress relevant to conserved molecular pathways implicated in both aging and memory formation and consolidation.

Keywords: Alzheimer's disease, autophagy, dietary restriction, insulin/IGF-1 signaling, learning, mitochondria, neurodegeneration,TOR signaling

INTRODUCTION

During the past century, age-related memory impairments have emerged as one of the top public health threats. Both psychiatric and neurodegenerative disorders comprising schizophrenia, depression, Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) are associated with age-related memory impairment. In humans, cognitive decline starts in midlife and deepens with advancing age suggesting that the greatest risk factor is age itself. Thus, ultimately, prevention of these pathologies necessitates thorough understanding of the molecular mechanisms underlying their links with the aging process (Bishop et al., 2010).

Our knowledge of the molecular regulatory mechanisms of aging impinging on cognitive capacity is steadily increasing in recent years. Interestingly, analyses of vertebrate and invertebrate model systems suggest that molecular and genetic pathways regulating cognitive aging are highly conserved in yeast, flies, worms, and mammals (Barco et al., 2006; Ardiel and Rankin, 2010; Bishop et al., 2010; Kauffman et al., 2010). Accumulating evidence from these models suggest a dynamic association between cognitive functions and aging. Similarly to several phenotypes and biomarkers of aging, which can vary substantially among individuals, cognitive decline displays significant severity fluctuation within a population. Consequently, it is important to identify key regulators of both cognitive impairment and longevity pathways. A plethora of molecular and cellular studies indicate a strong entanglement between lifespan regulation pathways and cognitive decline or neurodegeneration. In this review, we survey the molecular mechanisms and genes associated with longevity that have also been implicated in cognitive aging (summarized in **Table 1**). We further focus on recent work in invertebrate model organisms linking learning and memory impairment with age.

REDUCED INSULIN/IGF-1 SIGNALING PROMOTES LEARNING ABILITY DURING AGING

The insulin/IGF-1 (IIS) signal transduction pathway and its downstream effectors have been found to influence lifespan in a wide range of diverse organisms, suggesting a tightly conserved role of these mechanisms in aging. Reduction of IIS signaling promotes longevity in *Caenorhabditis elegans* and flies (Kenyon et al., 1993; Kenyon, 2010; Partridge, 2010). Whether this function is conserved in mice and humans remains unclear (Clancy et al., 2001; Tatar et al., 2001; Bluher et al., 2003; Suh et al., 2008; Bokov et al., 2011). Main components of insulin signaling in *C. elegans* are the insulin homolog INS-1, its receptor DAF-2, and the PIP3-kinase (phosphatidylinositol-triphosphate kinase) homolog AGE-1. Insulin signaling has been implicated in learning and memory, and in neuronal aging. Reduction of IIS attenuates protein aggregation and insolubility, and prevents amyloid-beta toxic effects. These processes are tightly associated with impaired nervous system function and age-related neurodegenerative diseases (Florez-McClure et al., 2007; David et al., 2010; Keowkase et al., 2010; Zhang et al., 2011; Tamura et al., 2013). In mouse models of AD, reduced IGF1 signaling protects from disease-associated neuronal loss and behavioral impairment, allthough IGF1R haploinsufficiency does not necessarily extent lifespan in mice (Cohen et al., 2009; Bokov et al., 2011).

In *C. elegans*, IIS has been shown to influence thermotaxis learning (Kodama et al., 2006) and salt chemotaxis learning (Tomioka et al., 2006). Moreover, long-lived IIS mutants show improved ability to associate temperature with food at both young and old age (Murakami, 2007). By contrast, some of these mutants are impaired, at young age, in their ability to associate NaCl with the absence of food (Vellai et al., 2006), or to intergrate sensory stimuli, such as Cu^{2+} and diacetyl

Table 1 | Conserved signaling pathway genes and cognitive aging in worms and flies.

Pathway	Gene	Cognitive aging effect	
		Caenorhabditis elegans	***Drosophila melanogaster***
Insulin signaling	*ins-1/INS*	Regulates gustatory associative learning, thermotaxis, and chemotaxis learning	NA[1]
	daf-2/IGFR	Inhibits growth cone initiation, axon growth and neuronal regrowth; loss-of-function improves thermotaxis associative learning and blocks chemotaxis/sensory integration learning	NA
	age-1/PI3K	Mutations improve thermotaxis learning with age but cause defects in chemotaxis associative learning	NA
	daf-18/PTEN	Loss-of-function decreases chemotaxis, odorant associative, and sensory integration learning	Inhibits axon regeneration
	daf-16/FoXO	Neuroprotective, promotes regeneration and neuronal migration; loss-of-function reduces associative and sensory integration learning	NA
Dietary restriction	*eat-2/DR*	Loss-of-function increases temperature–food association and impairs LTM[2]	DR[3] decreases STM[4] at mid-age, enhances MTM[5] at young-age
TOR signaling	*rheb*	NA	Overexpression induces morphology defects, and decreases odor-sucrose MTM
	rictor	NA	Deficiency blocks LTM
Autophagy	*cdk-5*	NA	Olfactory learning and memory defects
	apl-1/APPL	Olfactory and gustatory learning defects, habituation delay	NA
	unc-51/atg-1	NA	Influence axonal and dendritic development affecting olfactory learning
Mitochondria	*sod-1*	NA	Memory impairment associated with less synapses and mitochondrial dysfunction
	ucp-4, ucp-2	Promote neuronal toxicity in Huntington's disease models	Susceptible to Parkinson's and Huntington's diseases
	ced-9/Debcl	NA	Ameliorate cognition
	clk-1	Developmental and behavioral defects	Influence rhythmic behaviors

[1] *No information available,* [2] *Long-term memory,* [3] *Dietary restriction,* [4] *Short-term memory,* [5] *Mid-term memory.*

perception, towards decision-making (Jiu et al., 2010). Moreover, long-lived *age-1* mutant animals display delayed age-related decline of isothermal tracking and locomotion. Similarly, *age-1* and *daf-2* mutants associate temperature and starvation more efficiently compared to wild type controls, while young adults of these muants show increased temperature–food association. The enhanced association capacity of *daf-2* mutants is dependent on the neuronal Ca^{2+}-sensor NCS-1, which modulates isothermal tracking in the amphid interneurons, a key component of the thermosensory circuit (Murakami et al., 2005). AGE-1 also acts in the benzaldehyde-sensing amphid wing C (AWC) sensory neurons to direct benzaldehyde–starvation associative plasticity (Lin et al., 2010). While, mutations in the *daf-2* IIS receptor improve memory performance in *C. elegans* early in adulthood, maintaining learning ability with age, no extension in long-term memory (LTM) during aging is evident. Reduced insulin signaling does not alter neuronal plasticity but rather establishes an association more rapidly and prolongs the duration of this association early in adulthood (Kauffman et al., 2010).

Neuronal cells not only degenerate with age but the nervous system also loses the ability to regenerate after injury. Genetic experiments indicate that axon regeneration in aging *C. elegans* motor neurons is repressed by elevated IIS, which inhibits both growth cone initiation and axon growth (but not axon guidance) in aged animals. IIS impairs regeneration by blocking the function of DAF-16, a FOXO transcription factor and downstream effector of IIS. DAF-16/FOXO is necessary and sufficient to promote neuronal regeneration in a cell-autonomous manner. (Byrne et al., 2014). DAF-16 has also been shown to promote developmental neuronal migration and to affect aspects of neuronal cell morphology, such as neurite outgrowth (Christensen et al., 2011; Kennedy et al., 2013). DLK-1, a mitogen activated kinase kinase kinase (MAPKKK) that regulates presynaptic development is downregulated by IIS. DAF-16 upregulates expression of *dlk-1* in a neuron-specific manner, to promote neuronal regeneration

indipendently of lifespan (Byrne et al., 2014). In addition, DAF-16 and HSF-1, the *C. elegans* heat shock transcription factor ortholog, show neuroprotective characteristics since their activation can defer the morphological and functional defects that emerge on the synapses of touch receptor neurons with physiological aging (Toth et al., 2012).

The totality of these findings suggest that in addition to extending lifespan, reduced IIS signaling also promotes learning ability with age. However, this effect is not accompanied by maintenance or extension of long term-memory during aging. Instead, IIS signaling appears to play a more significant role in the retrieval rather than acquisition of memory.

DIETARY RESTRICTION AND LONG-TERM MEMORY

Dietary restriction (DR), a reduction in total food intake, has been shown to increase lifespan and reduce fecundity in a wide range of organisms such as yeast, nematodes, flies, and rodents (Masoro, 2005; Mair and Dillin, 2008; Piper and Bartke, 2008). Recent studies in primates indicate that DR prevents from aging-related pathologies like brain atrophy,but it is still under debate wether it extends lifespan (Colman et al., 2009; Mattison et al., 2012; Cava and Fontana, 2013; Colman et al., 2014). Little is known about the genes mediating these effects of DR. In *C. elegans*, knock-down of *mekk-3* a homolog of the mammalian mitogen-activated MEKK3-like kinase, recapitulates DR and extends lifespan. MEKK-3 deficiency leads to reprograming of fatty acid metabolism and lowering reactive oxygen species (ROS) generation, through the nuclear hormone receptor NHR-49 and DAF-22, an ortholog of human sterol carrier protein SCP2 (Chamoli et al., 2014).

The *C. elegans* feeding-defective mutant *eat-2* has been utilized as a model of DR. *eat-2* mutants ingest food poorly and, as a consequence, are long-lived. Lifespan extension by *eat-2* mutations is at least in part mediated through a *daf-16*-independent pathway (Avery, 1993; Raizen et al., 1995; Lakowski and Hekimi, 1998; Panowski et al., 2007). DR has also been suggested to attenuate age-related cognitive decline in rats (Adams et al., 2008). In *C. elegans*, young adult *eat-2* mutants show increased consistency of isothermal tracking (temperature–food association; Murakami et al., 2005). Contrary to *daf-2*, *eat-2* mutants exhibit significantly impaired LTM during young adulthood, but maintain memory capacity longer with age. Although young *eat-2* mutants display normal benzaldehyde chemotaxis, they require more training to form long-term memories. The duration of short-term memory in *eat-2* animals is similar to wild type, contrary to significant short term associative memory extension observed in *daf-2* mutants (Kauffman et al., 2010).

Dietary restriction also affects learning performance during aging in *Drosophila melanogaster*. The performance of young and old flies in an aversive learning test, where an odor is associated with a noxius mechanical shock, has been examined. These experiments showed that dietary-restricted flies, that live on average 14% longer than rich-diet fed flies, appear to have a better learning ability, even at old age. Young, dietary restricted flies show enhanced mid-term memory but their short-term memory is not affected. By contrast, short-term memory of mid-aged flies is poorer, compared with flies that grew on rich diet. Mid-term memory performance of mid-aged and old flies is not improved (Burger et al., 2010). These results are consistent with findings in *C. elegans*, in that only long term-memory is affected by DR during aging (Kauffman et al., 2010). While DR and reduced IIS signaling both increase longevity, the two pathways influence cognitive ability of young adults in an opposing manner.The differential effects of IIS and DR on learning and memory decline with age are likely due to their differential regulation of expression levels and activity of CRH-1, the cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) transcription factor homolog in *C. elegans* (Kauffman et al., 2010).

MITOCHONDRIAL FUNCTION AND COGNITIVE AGING

Mitochondria play pivotal role in adenosine triphosphate (ATP) production, calcium homeostasis, and apoptosis regulation, and are the main source of endogenous ROS. The functionality of these organelles influences aging through multiple pathways that may be directly or indirectly relevant to cognitive decline. The link between mitochondrial dysfunction, neurodegeneration, and cognition has been a subject of intensive study in many metazoans, ranging from *C. elegans* to humans (Bishop et al., 2010; Aksenov et al., 2013). A growing body of evidence suggests that neuronal structure and function are particularly vulnerable to mitochondrial function impairment (Stein and Murphy, 2012). However, the contribution of mitochondria to selective neurodegeneration in a variety of neurodegenerative pathologies associated with cognitive decline remains a matter of debate.

Aging studies in invertebrate model organisms provide a common ground for mitopathology and cognitive research. Several conserved groups of genes influencing mitochondrial metabolism, neural plasticity and synaptic function show expression changes during aging. In *C. elegans*, loss of α-tubulin acetyltransferase gene *mec-17* causes axon degeneration, thereby leading to neuronal dysfunction. Axons lacking MEC-17 contain less mitochondria, display transport defects, and loss of synaptic integrity (Neumann and Hilliard, 2014). Atat1, the mouse homolog of MEC-17 is associated with the formation of dentate gyrus, which is essential for learning and memory (Kim et al., 2013). Moreover, studies in *C. elegans ric-7* mutants, where axonal mitochondria trafficking is impaired, suggest that mitochondria are important for protection of axons against degeneration (Rawson et al., 2014). During physiological aging, nematode touch receptor neurons display morphological and functional abnormalities, such as neurite outgrowth defects and reduced number of synapses. Positioning of mitochondria in branches required for neurite outgrowth and the accumulation of vesicles in neuronal processes suggests that trafficking deficiency underlies these age-related abnormalities (Toth et al., 2012). These morphological changes of neurons have been associated with a decline in cognition, learning, and memory during aging (Vohra et al., 2010; Kimata et al., 2012; Kim et al., 2013; Wang et al., 2013).

The nematode genome encodes five superoxide dismutases (SODs) that function in cytoplasm, mitochondria, and extracellularly. Specifically, SOD-1 regulates detoxification of superoxide radicals in mitochondria and guards from accumulation of oxidative damage during aging (Harman, 1968; McCord and Fridovich,

1969; Yanase et al., 2009; Back et al., 2010). Nonetheless, lifespan extension in mutants overexpressing *sod-1* is not related with reduction of oxidative damage (Cabreiro et al., 2011). Transgenic *C. elegans* expressing the human G93A SOD1 variant, associated with familial amyotrophic lateral sclerosis (ALS), in motor neurons show motor defects and increased autophagy in an age-dependent manner (Aksenov et al., 2013; Li et al., 2013). SOD-1 overexpression has also been associated with mitochondrial swelling, and learning and memory impairment in flies, mice, and humans (Shin et al., 2004; Perluigi and Butterfield, 2012; Haddadi et al., 2014). For example, transgenic flies expressing a zinc-deficient SOD1 mutant display behavioral defects, including impairment of locomotion, associated with mitochondrial respiratory chain deficiency and matrix vacuolization, that is not accompanied by shortening of lifespan (Bahadorani et al., 2013). Moreover, SOD-1 activity and expression levels decline during normal aging of *Drosophila*. At the same time, knock-down of *sod-1* in the mushroom bodies deteriorates mid-term memory and LTM. These memory defects associate with reduced synapse formation and mitochondrial damage during *Drosophila* aging (Haddadi et al., 2014).

Converging evidence implicates members of the antiapoptotic BCL-2 family of proteins in neuronal injury and synapse deformation, through impairment of mitochondrial dynamics (Berman et al., 2009). CED-9, the *C. elegans* homolog of BCL-2, interacts with the mitofusin FZO-1 and the dymanin related protein EAT-3 to promote mitochondrial fusion under specific conditions. The *C. elegans eat-3* encodes a homolog of human OPA-1 which is associated with Dominant Optic Atrophy disorder (Breckenridge et al., 2009; Rolland et al., 2009). In *Drosophila*, the BCL-2 homologous proteins, Buffy and Debcl are involved in the permeabilization of mitochondria to cytochrome-*c* that is mediated by pro-death mitochondrial proteins including Reaper and Hid (Abdelwahid et al., 2011). Unlike in worms, Buffy inhibition results in normal flies, while knockdown of Debcl protects against polyglutamine (polyQ)-induced neurodegeneration through maintaining mitochondrial homeostasis. The Debcl ortholog in mice, Bax/Bak, was found to regulate neurogenesis in adult brain regions such as hippocampus and cerebellum and promote discrimination learning without affecting significantly spatial memory and learning (Senoo-Matsuda et al., 2005; Galindo et al., 2009; Sahay et al., 2011; Hardwick and Soane, 2013).

Neurons are particularly vulnerable to mitochondrial dysfunction. Interestingly, expression of the human mitochondrial uncoupling protein (UCP) *ucp2* in *Drosophila* dopaminergic neurons increases ATP production and locomotion activity, and results in neuroprotection against pathogenic stress associated with PD (Islam et al., 2012). Beyond neurons, enhanced expression of mitochondria UCPs in flies ameliorates HD phenotypes in glia cells by moderating ROS and ATP production (Besson et al., 2010). In *C. elegans*, depletion of UCP-4 exacerbates neuronal toxicity in animals expressing an expanded polyQ repeat protein in touch neurons, suggesting that similarly to flies, under normal conditions UCP-4 protects from neuronal injuries in worms (Parker et al., 2012). However, overexpression of *ucp-4* in worms does not extend lifespan (Sagi and Kim, 2012). Alterations in the expression of mitochondrial respiratory chain genes result in similar effects. For example, mutations in the *mev-1* and *gas-1* genes, encoding subunits of complex II and I of the respiratory chain, respectively, increase ROS production, shorten lifespan, and retard behavioral rates (Kayser et al., 2004). In another example, animals carrying mutations in mitochondria complex IV *sft-1* gene, show increased lifespan that is dependent on DAF-16 (Maxwell et al., 2013). Depletion of SURF1, the mouse ortholog of *sft-1*, also increases lifespan and improves cognitive function in mice (Lin et al., 2013a). Knockdown of *clk-1*, a gene required for ubiquinone biosynthesis reduces respiration rates and increases *C. elegans* lifespan, also delaying behavioral rates (Rea et al., 2007). Loss-of-function mutations in *clk-1* extend lifespan and slow development and behavioral rates (Takahashi et al., 2012). Similarly, knockdown of the mouse *clk-1* ortholog causes mild mitochondrial dysfunction and extends lifespan (Lapointe and Hekimi, 2008; Deepa et al., 2013). In *Drosophila*, reduced expression of complex I and IV genes specifically in adult neurons is sufficient to extend lifespan (Copeland et al., 2009). Furthermore, observations in *clk-1* mutants indicate that neurite outgrowth is inhibited in aged worms (Tank et al., 2011). The association between neuronal morphology and behavioral effects suggests that mitochondria dysfunction may, in part, underlie memory and learning decline during aging (Ardiel and Rankin, 2010; Kimata et al., 2012; Stein and Murphy, 2012). However, little is known about the molecular mechanisms that mediate the effects of alterations in mitochondrial metabolism on both cognitive capacity and longevity.

AUTOPHAGY AND PROTEIN HOMEOSTASIS IN LEARNING AND MEMORY

The autophagic pathway has also been implicated in aging and cognitive decline. Autophagic activity decreases during the course of aging and genes that control this process are strongly associated with lifespan regulation in flies and worms (Lionaki et al., 2013). In *Drosophila*, overexpression of autophagy-related genes in neurons enhances longevity, while their repression causes neuronal defects and shortening of lifespan (Simonsen et al., 2008). Similarly, increasing autophagy mediates lifespan extension in worms (Hansen et al., 2008). UNC-51, a nematode autophagy regulator also controls axonal and dendritic development and its homolog affects olfactory learning in flies (Mochizuki et al., 2011). Worms lacking UNC-51 display axonal membrane defects, indicating a role of autophagy in synaptic plasticity, which indirectly interferes with learning and memory (Sigmond et al., 2008; Ragagnin et al., 2013). In *Drosophila*, inhibition of the cyclin-dependent kinase 5 (cdk5) kinase ortholog decreases autophagy, shortens lifespan and causes structural defects in central brain regions associated with olfactory learning and memory (Trunova and Giniger, 2012). In both flies and worms, autophagy deficiency leads to abnormal accumulation of protein aggregates thus promoting pathological mechanisms associated with neurodegenerative disorders, such as HD and AD (Ling et al., 2009; Low et al., 2013). For example, accumulation of intracellular APL-1, a β-amyloid precursor protein, upon autophagy impairment, causes behavioral deficiencies, including olfactory and gustatory learning defects, and habituation delay in *C. elegans* (Ewald et al., 2012; Ewald and Li, 2012;

Chen et al., 2013). Accumulation of APL-1 also occurs during normal aging and can reach pathological levels contributing to the pathogenesis of AD (Nilsson et al., 2013).

Age-induced memory impairment studies in *Drosophila* suggest that cognitive aging is strongly associated with the autophagic pathway. Indeed, spermidine-induced autophagy reduces aggregation of ubiquitinated proteins and protects from age-related memory impairment, in the aged *Drosophila* brain (Gupta et al., 2013). Spermidine activates autophagy to also promote longevity in different metazoans ranging from *C. elegans* to mice (Eisenberg et al., 2009; Wang et al., 2012). Other studies suggest that spermidine may not act directly through autophagy to facilitate neuroprotection and memory during aging. Instead, spermidine administration may influence histone acetyltransferase activity to modulate autophagy (Simonsen and Tooze, 2009; Davis, 2013; Graff and Tsai, 2013). These findings indicate that although the protective effect of spermidine does require activation of the autophagy pathway, the involvement of additional regulatory pathways remains to be elucidated. In conclusion, the exact mechanism by which autophagy controls cognitive aging is multifaceted and remains poorly understood. Additional studies are required to elucidate the contribution of autophagy in both longevity and cognitive capacity maintenance during aging.

TOR SIGNALING AND LONG-TERM MEMORY

Reduced signaling through the target of rapamycin (TOR) kinase has been shown to extend lifespan in diverse organisms (Vellai et al., 2003; Jia et al., 2004; Kapahi et al., 2004; Kaeberlein et al., 2005; Powers et al., 2006; Kenyon, 2010). The evolutionarily conserved mTOR functions in two complexes, mTORC1 and mTORC2 (Hay and Sonenberg, 2004; Wullschleger et al., 2006; Guertin and Sabatini, 2007). Tight regulation of the upstream components of the TOR pathway is important for proper neural growth and function throughout development and adulthood in *C. elegans* (Goldsmith et al., 2010). Overexpression of the small GTPase RAS homolog enriched in brain (Rheb), an upstream activator of TOR, in *Drosophila* photoreceptor cells downregulates autophagy, causes axon guidance defects and induces cell death (Knox et al., 2007; Wang et al., 2009). Selective overexpression of Rheb in distinct subsets of central brain neurons results in enlarged cell bodies and projections. In addition, Rheb overexpression in the mushroom bodies decreases mid-term odor-sucrose memory (Brown et al., 2012).

In the PIP3/PTEN/Akt/TOR pathway phosphorylated Akt activates TOR to regulate cell cycle and protein synthesis. In flies, the PTEN/Akt pathway is implicated in axon regeneration (Song et al., 2012). Similarly, axon regeneration is evident after the loss of DAF-18/PTEN in young adult worms (Byrne et al., 2014). Reduced TORC2 activity causes LTM deterioration in fruit flies (Huang et al., 2013). Rapamycin, a protein synthesis inhibitor that acts through the TOR pathway (mainly mTORC1), blocks long-term facilitation (LTF) in *Aplysia californica* (Hu et al., 2006). Moreover, rapamycin completely disrupts pre-existing long-term synaptic plasticity in *Aplysia* (Hu et al., 2011). While rapamycin extends lifespan mainly by blocking the TOR pathway, it may exert its effects on cognition through a different mechanism (Neff et al., 2013).

The TOR pathway controls translation of 5′TOP mRNAs containing a 5′ terminal oligopyrimidine tract. 5′TOP mRNAs encode proteins of the translational machinery. Under physiological conditions, 5′TOP mRNAs are largely repressed. Serotonin, which activates the TOR pathway, aleviates this repression, in a rapamycin-sensitive manner (Garelick and Kennedy, 2011; Labban and Sossin, 2011). eEF2 (eukaryotic elongation factor 2) is implicated in LTF in *Aplysia*, but is differentially regulated by eEF2 kinase in the neurites and the soma of sensory neurons involved in LTF (Weatherill et al., 2011). TORC1 mediates regulation of phosphorylation of eEF2 through the eEF2K (Carroll et al., 2004). Both in *Aplysia*, and in rodents, eEF2K function is associated with increased memory processing, through enhancing expression of genes implicated in the regulation of syntaptic strength (Weatherill et al., 2011).

Similarly, long term administration of rapamycin eliminates neuronal demyelination and neurodegeneration observed during aging in senescence-accelerated OXYS rats, a strain characterized by overproduction of free radicals (Kolosova et al., 2013). In mouse models of AD, rapamycin administrated either prior or after the onset of AD symptoms, improves animal cognition, probably through the preservation of brain vascular integrity and function (Lin et al., 2013b). Moreover, chronic treatment with rapamycin enhances spatial learning and memory with age, as well as the ability to recall a memory, even when the administration takes place late in life (Halloran et al., 2012). However, short-term administration following the emergence of learning and memory defects with aging, is not accompanied by such positive effects. The improvement of cognitive ability with rapamycin is mediated through reduction of TOR signaling and of IL-1β levels in the hippocampus, the facilitation of NMDA signaling, and increased CREB phosphorylation (Majumder et al., 2012). Furthermore, increased phosphorylation of S6, a target of TOR, is observed in the prefrontal cortex, after the administration of rapamycin, in OXYS rats (Kolosova et al., 2013).

CONCLUSION

Understanding how neuronal aging and cognitive impairment are influenced by mechanisms that modulate lifespan is an ongoing challenge. Such well-studied mechanisms include the IIS signaling pathway, DR, mitochondrial dysfunction, autophagy, and the TOR signaling pathway. Accumulating evidence indicates that these pathways also impinge on age-related neuronal dysfunction and memory-impairment. Indeed, manipulation of these pathways in a variety of metazoans affects neuronal structure and function and consequently promotes age-related memory impairment. It is likely that the decline in different forms of memory is independently mediated by distinct aging mechanisms (**Figure 1**). Decreased IIS signaling promotes decision making and associative learning. However this is not a general rule and, instead, appears to be dependent on different types of association. Nonetheless, DAF-16 activation likely delays morphological changes that occur with aging and promotes neuronal regeneration. DR exerts negative effects on LTM but enhances association making and memory. DR effects on short-term and mid-term memory appear to be age dependent.

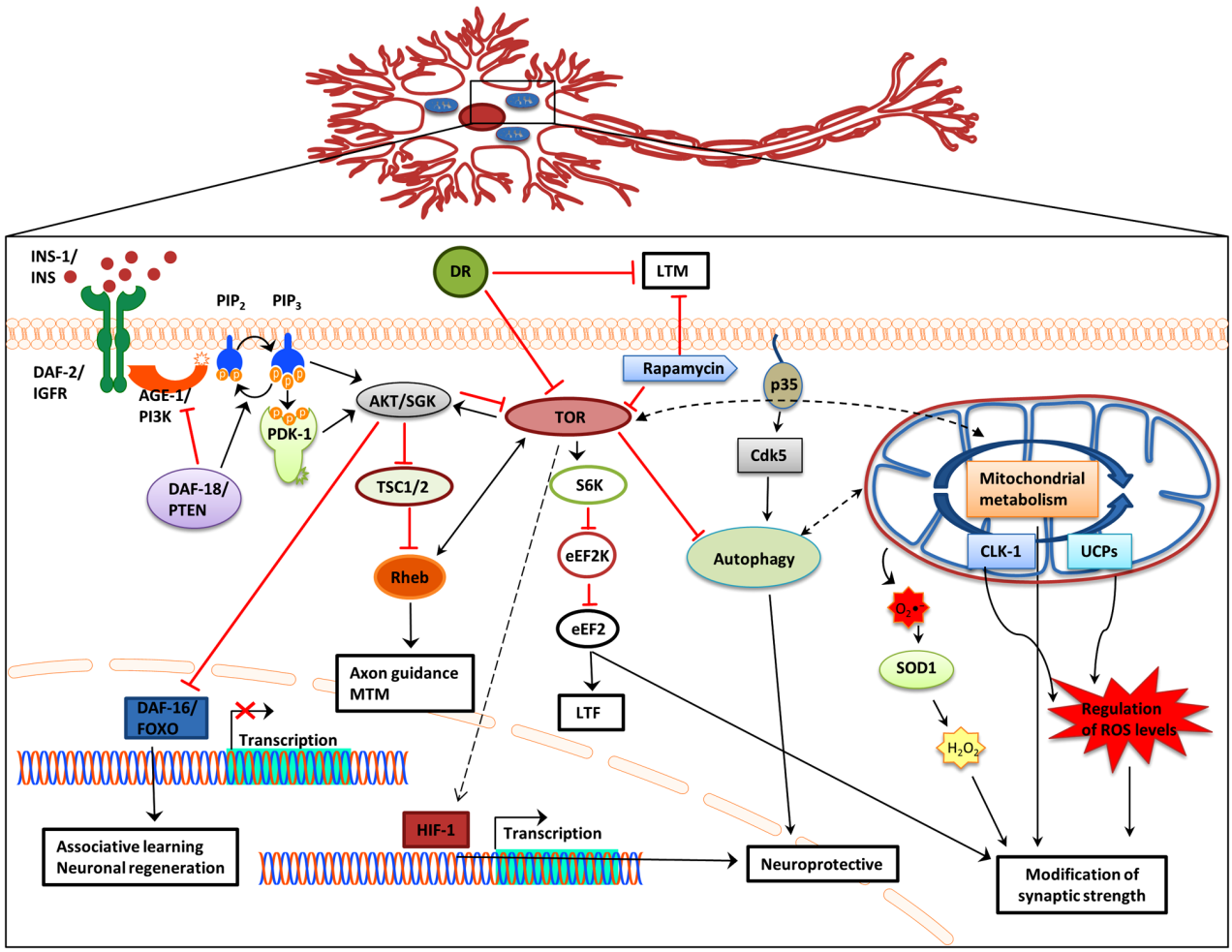

FIGURE 1 | Effects of IIS, DR, and TOR signaling, autophagy, and mitochondrial function on memory formation. In addition to extending lifespan, attenuation of IIS signaling, and subsequent de-repression of DAF-16 also reinforces associative learning and promotes axon regeneration and neuronal migration. Dietary restriction significantly impairs long-term memory, while it does not affect short-term memory. Blocking TOR signaling causes long-term-facilitation defects, while Rheb overexpression decreases mid-term memory and causes axon guidance defects. Regulation of autophagy through Cdk-5 affects lifespan and has also been associated with central nervous system structure defects, including olfactory learning and memory. Several mitochondrial genes promote neuroprotection. Impairment of mitochondrial function causes over production of ROS and concomitant synaptic deficiency. AGE-1/PI3K,phosphoinositide 3-kinase; AKT, protein kinase B; Cdk5, cyclin-dependent kinase 5; CLK-1, clock 1; DAF-2/IGFR, insulin-like growth factor receptor; DAF-16/FOXO, forkhead box O; DAF-18/PTEN, phosphatase and ensin homolog; DR, dietary restriction; eEF2, eukaryotic elongation factor 2; eEF2K, eukaryotic elongation factor 2 kinase; HIF-1, hypoxia-induced factor 1; INS, insulin; LTF, long-term facilitation; LTM, long-term memory; MTM, mid-term memory; PDK-1, 3-phosphoinositide-dependent kinase 1; PIP_2, phosphatidylinositol (4,5)-bisphosphate; PIP_3, phosphatidylinositol (3,4,5)-trisphosphate; Rheb, Ras homolog enriched in brain; S6K, ribosomal protein S6 kinase; SGK, serum- and glucocorticoid-inducible kinase; SOD1, superoxide dismutase 1; TSC1/2, tuberous sclerosis 1/2; TOR, target of rapamycin; UCPs, uncoupling proteins; Black arrow, direct stimulation; black dashed arrow, indirect stimulation; black dashed double head arrow, interplay; red arrow, inhibition.

The TOR pathway influences cognition by controlling protein biosynthesis, cell cycle, and metabolism (Garelick and Kennedy, 2011; Santos et al., 2011). Studies in invertebrates suggest that increased TOR signaling downregulates autophagy and causes axon guidance defects, while also promoting memory processing and synapse integrity. Conversely, in addition to increasing lifespan, downregulation of TOR signaling, mainly through rapamycin treatment, blocks LTM and LTF but also causes axon regeneration. In rodents rapamycin administration offsets the negative impact of aging on spatial learning and memory, increases memory recall ability, and enhances the vascular integrity of the brain. Moreover, morphological signs of AD and aging, such as neuronal demyelination and neurodegeneration are ameliorated. Activation of mTORC1 promotes mRNA translation, which likely enhances synapse formation. These distinct, but not necessarily conflicting results of manipulating TOR signaling could reflect a dose or compartment dependent regulation of cognition through TOR signaling.

In both worms and flies, activation of autophagy appears to promote lifespan and cognitive function. In mammals, little it is known about the role of autophagy during aging. However, recent studies in mice suggest that overexpression of autophagy-related

gene Atg5 also increases lifespan (Pyo et al., 2013). Manipulation of mitochondria function during aging causes similar effects, either causing axon degeneration, or promoting axon integrity and stability (Keller et al., 2011; Fang et al., 2012). These ostensibly contradictory observations may suggest a neuron-specific function of mitochondria in aging. Despite recent progress and findings, several open questions need to be addressed. The involvement of epigenetic mechanisms and environmental conditions on nervous system aging is largely unknown. In addition, whether aging differentially affects subpopulations of neurons or different brain areas and to what extent remains unclear. While significant progress has been achieved towards deciphering the link between pathways that modulate both lifespan and aspects of neuronal function in invertebrate models, the relevance of these findings to neuronal aging and pathophysiology in higher organisms including humans has not been evaluated yet. Addressing these key issues will contribute towards developing informed strategies and therapeutic approaches aiming to battle age-related decline of nervous system performance and numerous neurodegenerative conditions associated with aging.

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Worming forward: amyotrophic lateral sclerosis toxicity mechanisms and genetic interactions in *Caenorhabditis elegans*

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Neurodegenerative diseases share pathogenic mechanisms at the cellular level including protein misfolding, excitotoxicity and altered RNA homeostasis among others. Recent advances have shown that the genetic causes underlying these pathologies overlap, hinting at the existence of a genetic network for neurodegeneration. This is perhaps best illustrated by the recent discoveries of causative mutations for amyotrophic lateral sclerosis (ALS) and frontotemporal degeneration (FTD). Once thought to be distinct entities, it is now recognized that these diseases exist along a genetic spectrum. With this wealth of discoveries comes the need to develop new genetic models of ALS and FTD to investigate not only pathogenic mechanisms linked to causative mutations, but to uncover potential genetic interactions that may point to new therapeutic targets. Given the conservation of many disease genes across evolution, *Caenorhabditis elegans* is an ideal system to investigate genetic interactions amongst these genes. Here we review the use of *C. elegans* to model ALS and investigate a putative genetic network for ALS/FTD that may extend to other neurological disorders.

Keywords: *C. elegans*, ALS (Amyotrophic lateral sclerosis), TDP-43, FUS, C9orf72, SOD1, genetic networks, motor neuron disease

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder affecting 1–2/100,000 individuals. Most cases of ALS are sporadic, but 10% of cases are familial (Turner et al., 2013b). Mutations in the gene *superoxide dismutase 1 (SOD1)* were identified in 1993 (Rosen et al., 1993) as the first cause of familial ALS. Thanks to the recent advances in genetics, more than 20 genes are now linked to ALS (Chen et al., 2013) (**Table 1**). Genes recently shown to be mutated in ALS include the DNA/RNA binding proteins *TAR DNA binding protein 43 (TARDBP)* and *Fused-in-sarcoma (FUS)* (Kabashi et al., 2008; Sreedharan et al., 2008; Vance et al., 2009). More recently, mutations in *C9ORF72* have turned out to be a major cause of familial and sporadic ALS (DeJesus-Hernandez et al., 2011; Renton et al., 2011).

ALS is characterized by the selective loss of motor neurons in the motor cortex, the brainstem and the spinal cord, the loss of myelin in the spinal cord, and the presence of neuroinflammation (Robberecht and Philips, 2013). Onset of the disease usually begins in the lower limb and spreads toward the upper motor neurons leading to muscle weakness, fasciculation, and wasting. Death occurs 3–5 years after the beginning of the symptoms (Kiernan et al., 2011) and is caused by respiratory failure due to denervation of the respiratory muscles.

50% of ALS patients show cognitive impairment, of which 15% met the criteria of frontotemporal dementia (FTD) (Ringholz et al., 2005). FTD is a group of non-Alzheimer dementias characterized by atrophy of the frontal and/or temporal lobes causing mid-life behavioral changes or language impairment (Warren et al., 2013). Over the past few years, the identification of *TDP-43*, *C9ORF72* and *UBQLN2* as genes causing ALS and FTD has suggested a similarity for both diseases (Morris et al., 2012). Similar pathogenic mechanisms have been suggested for ALS and FTD (Van Langenhove et al., 2012; Ling et al., 2013) but so far it is unclear how patients with the same genetic mutations can have either ALS, FTD or both.

The genes involved in ALS have diverse functions and we still do not know how they interact to cause motor neuron degeneration. Most of the research over the past 20 years has focused on the toxicity caused by mutant SOD1. Among the proposed mechanisms of toxicity are mitochondrial dysfunction, axonal dysfunction, excitotoxicity and neuroinflammation (Turner et al., 2013b). However, TDP-43, FUS, and C9ORF72 proteins seem to point toward RNA toxicity (Ling et al., 2013). Most importantly, only one drug, riluzole, is used to slow disease progression and has only modest effects (Kiernan et al., 2011). Diagnosis is difficult and requires an experienced neurologist to differentiate between ALS and other neurological diseases (Turner et al., 2013a). It is thought that the clinical manifestations of ALS are downstream events that occur much later after the initial insult to the nervous system (Turner et al., 2013a). Therefore, the identification of biomarkers is essential for the rapid, early diagnosis of ALS, and the identification of new drugs limiting the degeneration of motor neuron is an essential unmet need for ALS patients.

Table 1 | ALS genes and their *C. elegans* orthologs.

Human gene	Function	*C. elegans* gene
MOST COMMON ALS GENES		
SOD1	Superoxide metabolism	*sod-1*
TARDBP	RNA metabolism	*tdp-1*
FUS	RNA metabolism	*fust-1*
OPTN	Vesicular transport	–
VCP	Vesicular transport	*cdc-48.1/2*
UBQLN2	Proteasome	*ubqnl-1*
C9ORF72	Unknown, DENN protein	*alfa-1*
SQSTM1	Autophagy	*sqst-2*
PFN1	Cytoskeleton dynamics	*pfn-1*
OTHER GENES INVOLVED IN ALS		
DCTN1	Cytoskeleton dynamics	*dnc-1*
ALS2	Endocytosis	–
CHMP2B	Vesicular transport	–
FIG4	Vesicular transport	*C34B7.2*
HNRNPA2B1	RNA metabolism	*hrp-1*
ELP3		–
SETX	RNA processing	–
HNRNPA1	RNA processing	*hrp-1*
ATXN2		*atx-2*
ANG	Blood vessels formation	–
SPG11	DNA damage	–
VAPB	Vesicular transport	*vpr-1*
NEFH	Cytoskeleton dynamics	*H39E23.3*
ARHGEF28	RNA metabolism	*rhgf-1*

To understand better the impact of the genetic mutations on the function of the different proteins involved in ALS, *in vivo* models have proved to be essential. Ever since the first SOD1 mouse was developed in 1994 (Gurney et al., 1994), several groups have tried to investigate ALS pathogenesis by expressing different ALS related mutations in mice, an approach that has recently been extended to other genes including TDP-43 and FUS for example. While the over expression of wild type SOD1 causes a mild denervation of neurons (Epstein et al., 1987), the over expression of $SOD1^{G93A}$ causes a loss of motor neurons, neuroinflammation, and reduces life span (Gurney et al., 1994; Guo et al., 2009). One model expressing mutant $TDP\text{-}43Q^{331K \text{ or } M337V}$ in the mouse central nervous system has shown selectivity for large caliber motor neuron neurodegeneration (Arnold et al., 2013), while others over expressing mutant $TDP\text{-}43^{G348C,\ A315T}$ and $FUS^{R512C,\ 14\Delta}$ have limited neuronal loss (Swarup et al., 2011; Verbeeck et al., 2012). Some rodent models display relevant ALS pathology, but given the time and expense to develop models for many of the recently discovered ALS genes, not to mention the difficulty of manipulating several genes at once, some laboratories have turned to simpler organisms to model ALS toxicity.

One model showing increasing popularity is the nematode *Caenorhabditis elegans*. This 1 mm long worm has a painstakingly characterized, invariant cell lineage that includes 302 neurons. The nervous system, its interconnections and its synapses are well studied which makes it an ideal model to study mechanisms of neuronal toxicity. The *C. elegans* genome was the first to be fully sequenced in 1998 and includes more than 19,000 genes on 6 chromosomes (*C. elegans* Sequencing Consortium, 1998). Since then, deletion mutants have been produced for many *C. elegans* genes and approximately 80% of *C. elegans* genes have a human homolog (Lai et al., 2000) (Shaye and Greenwald, 2011). *C. elegans* behavior is well studied and many experimental assays are available, including for worm locomotion. Worms initiate movement by bending their body to advance forward in a sinusoidal pattern, a process that is orchestrated by GABAergic and cholinergic neurons. Cholinergic neurons initiate the contraction along the dorsal or ventral body wall muscles while the GABAergic neurons send an inhibitory signal on the opposite side (Jorgensen, 2005).

C. elegans has been an important tool for the characterization of many neurodegenerative disorders (Li and Le, 2013). Many protein-misfolding disorders have been modeled in worms including Alzheimer's disease, Parkinson's disease, Huntington's disease and different spinocerebellar ataxias. Also, the toxicity of non-coding mutations in *C. elegans* resemble the toxicity in mammalian tissues (Wang et al., 2011b). Since many cellular stress and survival pathways are conserved in worms, our group and others have used *C. elegans* to model ALS. This review aims to summarize the work done modeling ALS in *C. elegans* and highlights the future possibilities and applications.

USE OF *C. elegans* TO MODEL SOD1 TOXICITY

SOD1 is an enzyme that catalyzes the conversion of O_2- into O_2 and H_2O_2. More than 160 mutations causative for ALS have been found in *SOD1* since 1993 (Al-Chalabi et al., 2012). Phenotypic heterogeneity is observed among *SOD1* mutation carriers where $SOD1^{A4V}$ seems to cause an aggressive form of ALS while $SOD1^{D90A}$ causes a milder, long duration ALS (Renton et al., 2014). It is hypothesized that *SOD1* mutations cause toxicity through a gain of function, even though a loss of enzyme activity have been observed in patients and some models (Saccon et al., 2013). Many pathogenic mechanisms have been hypothesized but no consensus has been reached, although it is thought that the misfolding of mutant SOD1, and sometimes wild type SOD1, may be an important first step of the pathogenesis observed in patients (Pickles and Vande Velde, 2012). Based on pathological evidence, it is now acknowledged that ALS caused by mutations in SOD1 is a distinctive form of ALS (Mackenzie et al., 2007).

Several groups have used *C. elegans* to model SOD1 toxicity (**Table 2**) starting with Oeda and colleagues who showed that the ubiquitous expression of human mutant SOD1 impairs the worm's response to oxidative stress and causes protein aggregates (Oeda et al., 2001). It was later shown that expression of mutant SOD1 throughout the worm's entire nervous system resulted in locomotion defects and impaired neuronal transmission (Wang et al., 2009). Interestingly, the formation of aggregates seemed to be restricted to certain mechanosensory neurons despite the pan neuronal expression of SOD1. Other models are non-neuronal in nature and have relied on the expression of SOD1 proteins in the body wall muscles where it was observed that distinct SOD1 mutations have varying propensities to aggregate (Gidalevitz et al., 2009). More recently a *C. elegans* model was generated

Table 2 | Summary of transgenic SOD1 models.

Study	Promoter	Gene	Motor phenotype	Aggregation	Neuro-degeneration	Synaptic dysfunction
Oeda et al., 2001	*hsp-16.2:* all tissues except the germline	$SOD1^{A4V}$	n.d	n.d.	n.d	n.d
		$SOD1^{G37R}$	n.d	n.d	n.d	n.d
		$SOD1^{G93R}$	n.d	n.d	n.d	n.d
	myo-3: muscle cells	$SOD1^{A4V}$	n.d	yes	n.a	n.a
Wang et al., 2009	*snb-1:* entire nervous system	$SOD1^{WT}$	No	No	n.d	Normal
		$SOD1^{G83R}$	Yes	Yes	n.d	Impaired
Gidalevitz et al., 2009	*unc-54:* muscle cells	$SOD1^{WT}$	No	No	n.a.	n.a
		$SOD1^{G85R}$	Yes	Yes	n.a.	n.a.
		$SOD1^{G93A}$	Yes	Yes	n.a.	n.a.
		$SOD1^{127X}$	Yes	Yes	n.a.	n.a.
Li et al., 2013a	*unc-25:* GABAergic motor neurons	$SOD1^{WT}$	Yes	Yes	Yes	n.d.
		$SOD1^{G93A}$	Yes	Yes	Yes	n.d.

n.d., not determined; n.a., not applicable.

based on the expression of SOD1 in the worm's motor neurons showing neurodegeneration in the absence of caspases (Li et al., 2013a), an intriguing finding since the motor neuron loss observed in mouse models is associated with caspase activation (Pasinelli et al., 2000). Whether this reflects a difference between invertebrate and vertebrate systems, or reflects a novel mechanism of neurodegeneration remains to be determined.

The *C. elegans sod-1* gene has a similar function to human *SOD1*. *sod-1* loss of function mutants have increased O_2- levels, shorter lifespan and are sensitive to some environmental stresses (Yanase et al., 2009). Inversely, overexpression of the worm *sod-1* increases lifespan and increases the level of H_20_2, the by-product of the catalase reaction of SOD1. However, the increased lifespan seems to be independent of SOD-1 catalase activity, but may be due to altered endoplasmic reticulum (ER) stress signaling (Cabreiro et al., 2011). Interestingly, Van Raamsdonk et al. have generated a *sod* null worm, where all five *C. elegans sod* genes have been mutated and these worms have a normal lifespan and response to oxidative damage but are sensitive to many acute environmental stresses (Van Raamsdonk and Hekimi, 2012).

In summary, many aspects of SOD1 function and toxicity are conserved in worms, but some questions remain. It is known that mutant SOD1 is found in association with the mitochondria in SOD1 mouse model and ALS patients (Pickles and Vande Velde, 2012). To our knowledge, no group has yet investigated the effects of human mutant SOD1 in worm mitochondria. However, it was recently shown that a cleavage product of *vpr-1,* the ortholog of VAPB also involved in ALS, affects mitochondrial organization in muscle cells (Han et al., 2013). A similar analysis of the different SOD1 transgenic models would be interesting and could help identify pathways and drugs that act specifically on this important aspect of ALS pathogenesis.

USE OF *C. elegans* TO MODEL TDP-43 TOXICITY

TDP-43 is a protein encoded by the *TARDBP* gene on chromosome 1. The protein contains two RNA binding domains, a glycine rich domain and nuclear export and import signals. TDP-43 is similar to the members of the ribonucleoprotein family. TDP-43 was identified in 2006 as the main constituents of sporadic and familial ALS/FTD aggregates (Neumann et al., 2006). In patients, the ubiquitinated aggregates are present in the most affected regions of the brain and spinal cord. These aggregates contain a hyperphosphorylated form of TDP-43 and the C terminus cleaved fragment (Neumann et al., 2006). In 2008, mutations in the *TARDBP* gene were linked to familial and sporadic ALS/FTD cases (Kabashi et al., 2008; Sreedharan et al., 2008; Sreedharan and Brown, 2013). So far, more than 40 mutations in *TARDBP* have been linked to ALS/FTD and most of them are found in the C terminus region of the protein, a region involved in protein-protein interactions (Al-Chalabi et al., 2012).

Under normal cellular conditions, TDP-43 protein shuttles from the nucleus to the cytoplasm. The normal function of TDP-43 is still unclear but the protein participates in transcription, miRNA processing, mRNA splicing, RNA transport and stress granule formation (Ling et al., 2013). The pathogenic effect of the mutant proteins is not well understood and it is still unclear if the toxicity is a gain of function, a loss of function, or both (Ling et al., 2013; Vanden Broeck et al., 2014). An important aspect of TDP-43 toxicity was discovered when characterizing TDP-43 wild type mice. Mice with elevated expression of wild type TDP-43 also have characteristics of TDP-43 mutant proteins (Xu et al., 2010). Therefore, expression level is important and should be considered when generating different transgenic models.

To clarify the toxicity caused by the expression of mutant TDP-43, several groups have developed *C. elegans* models (**Table 3**). In 2010, Ash and colleagues developed the first TDP-43 overexpression model in *C. elegans*. The pan neuronal expression of human TDP-43 and *C. elegans* TDP-1 resulted in worms with uncoordinated, slow movements and defasciculation of the GABAergic motor neurons (Ash et al., 2010). The results regarding the expression of human TDP-43 were confirmed by Liachko and colleagues who also observed motility defects and degeneration phenotypes from the expression of mutant TDP-43 proteins throughout the worms nervous system (Liachko et al., 2010).

Table 3 | Summary of transgenic TDP-43 models.

Study	Promoter	Gene	Motor phenotype	Aggregation	Neuro-degeneration	Synaptic dysfunction
Ash et al., 2010	*snb-1:* entire nervous system	TDP-1	Yes	n.d.	n.d.	n.d.
		TDP-43^{WT}	Yes	n.d.	GABAergic	n.d.
		TDP-43$^{\Delta RRM1}$	No	n.d.	n.d.	n.d.
		TDP-43$^{\Delta RRM2}$	No	n.d.	n.d.	n.d.
		TDP-43$^{\Delta C\ terminus}$	No	n.d.	n.d.	n.d.
		TDP-43$^{no\ caspase}$	Yes	n.d.	n.d.	n.d.
		TDP-43$^{no\ NLS}$	No	n.d.	n.d.	n.d.
Liachko et al., 2010	*snb-1:* entire nervous system	TDP-43^{WT}	Yes	Yes	No	n.d.
		TDP-43^{G290A}	Yes	Yes	GABAergic and dopaminergic	n.d.
		TDP-43^{A315T}	Yes	Yes	GABAergic and dopaminergic	n.d.
		TDP-43^{M337V}	Yes	Yes	GABAergic and dopaminergic	n.d.
Zhang et al., 2012b	*snb-1:* entire nervous system	TDP-43^{WT}	Yes	Yes	No	Impaired
		TDP-43^{G331K}	Yes	n.d.	No	Impaired
		TDP-43^{M337V}	Yes	n.d.	No	Impaired
		TDP-43$^{C\ terminus}$	Yes	Yes	No	Impaired
Vaccaro et al., 2012c	*unc-47*: GABAergic neurons	TDP-43^{WT}	No	No	No	No
		TDP-43^{A315T}	Yes	Yes	GABAergic	Impaired

n.d., not determined; NLS, nuclear localization signal; ΔRRM, deletion of RNA recognition motif; ΔC terminus, deletion of C terminus; no caspase, mutations in TDP-43 that block caspase cleavage.

These phenotypes also highly correlated with protein phosphorylation levels where hyperphosphorylation increased the toxicity of mutant TDP-43 proteins similarly to what is observed in ALS patients (Liachko et al., 2010). The TDP-43 C terminus fragment shows another similarity with patients. Zhang and colleagues showed that the pan neuronal expression of human TDP-43 C′ fragment caused a phenotype similar to the expression of wild type or mutant TDP-43 (Zhang et al., 2011). Even though no neuronal loss was observed in the latter model, all strains displayed synaptic transmission abnormalities. In worms, GABAergic neurons seem to be particularly sensitive to the expression of TDP-43 (Liachko et al., 2010). To evaluate if the effect of TDP-43 expression in these neurons could cause locomotor defects, our group developed models in which human wild type or mutant TDP-43 were expressed specifically in GABAergic motor neurons as directed by an *unc-47* promoter (McIntire et al., 1997). Interestingly, the overexpression of mutant TDP-43, but not wild type TDP-43, caused an adult-onset, progressive paralysis phenotype accompanied by GABAergic neurodegeneration and synaptic transmission impairment (Vaccaro et al., 2012b). Finally, some of these models showed aggravation of the phenotypes during aging recapitulating an important feature of ALS and neurodegeneration (Liachko et al., 2010; Vaccaro et al., 2012b).

It is still unclear if the toxicity of mutant TDP-43 proteins in ALS patients arises from a gain of function, a loss of function or if both mechanisms are employed. The transgenic *C. elegans* models of TDP-43 are based on the overexpression of TDP-43 in worms and likely represent a gain of function rather than a loss of function. However, the *C. elegans* genome has an ortholog of TDP-43 called TDP-1. TDP-1 is a primarily nuclear protein expressed in most tissues including body wall muscles, pharynx and neurons (Vaccaro et al., 2012c; Zhang et al., 2012b). TDP-1 contains two RNA binding motifs, a nuclear localization signal and an export signal but lacks the glycine rich domain found in human TDP-43. TDP-1 seems to be functionally conserved because the expression of human TDP-43 can rescue the toxicity of a loss of function of a *tdp-1* mutant (Zhang et al., 2012b).

Mutant *tdp-1* animals show numerous phenotypes including slow development, and locomotion defects (Liachko et al., 2010; Zhang et al., 2012b). TDP-1 was also shown to be involved in lifespan and the cellular stress response. Somewhat paradoxically, worms lacking *tdp-1* have a longer lifespan but are more sensitive to oxidative and osmotic stresses (Vaccaro et al., 2012c; Zhang et al., 2012b). The expression of *tdp-1* can be induced by oxidative stress, either chemically or from activation of the ER stress response, and it is thought that chronic induction of *tdp-1* by stress is ultimately cytotoxic and reduces the worms lifespan (Vaccaro et al., 2012c). Furthermore, several studies have shown that wild type TDP-1 protein may contribute to the neurodegeneration elicited by mutant protein in *C. elegans*. Neurodegeneration was suppressed by deleting *tdp-1* from worms in several ALS models (Vaccaro et al., 2012c; Zhang et al., 2012b) as well as in a *C. elegans* model of Huntington's disease (Tauffenberger et al., 2013a) suggesting there may be genetic interactions amongst genes linked to neurodegeneration. Interestingly, a transcriptome analysis of *tdp-1(ok803)* showed that one of the biological process that was highly affected in the mutant worms was the ER unfolded protein response (Zhang et al., 2012b). ER stress and proteostasis have been a recurrent theme in ALS research (Matus et al., 2013; Musarò, 2013) which

is of interest since sporadic and familial cases of ALS are known to have an abnormal ER stress response (Ilieva et al., 2007; Atkin et al., 2008; Hetz et al., 2009; Ito et al., 2009).

USE OF *C. elegans* TO MODEL FUS TOXICITY

After the identification of TDP-43, several groups examined related RNA-binding proteins for their potential contributions to ALS. In 2009, a protein with a similar function, FUS, was identified as causative of ALS (Kwiatkowski et al., 2009; Vance et al., 2009). Similar to TDP-43, FUS contains a RNA binding domain and a glycine rich domain but also has a two arginine glycine rich regions and one large glutamine, glycine, serine, tyrosine domain in N terminus. Because of their high degree of structural similarity, it was hypothesized that FUS and TDP-43 share common functions. It is known that FUS can bind DNA and RNA and is involved in many of the same RNA processing activities of TDP-43 (Ling et al., 2013). FUS transgenic models are relatively recent additions to the research field and much remains to be learned about the function of FUS and the implication of the mutant protein in neurodegeneration.

Two transgenic models have been developed in *C. elegans* for FUS (**Table 4**). Murakami et al. (2012) expressed several *FUS* mutations and two truncated FUS proteins throughout the worm's nervous system. Interestingly, only the mutations that caused aggregation resulted in motor phenotypes in worms. The motor phenotype could not be rescued by the expression of wild type *FUS* suggesting a gain of function mechanism. Our group confirmed a similar toxicity mechanism in models expressing *FUS* in the worm motor neurons. Expression of *FUS* wild type did not cause aggregation but expression of mutant *FUS* caused aggregation accompanied by paralysis, neuronal synaptic impairment and neurodegeneration (Vaccaro et al., 2012b).

FUS is well conserved and the *C. elegans* ortholog is named *fust-1*. In contrast to *tdp-1*, a *fust-1* deletion mutant could not alleviate the toxicity induced by the expression of C′ TDP-43 fragment (Zhang et al., 2012b), suggesting a different role in proteotoxicity. In *Drosophila*, *Cabeza* (*Caz*), the *Drosophila* ortholog of *FUS*, is expressed in motor neurons and a decreased expression of *Caz* causes a motor phenotype and motor neuron degeneration (Wang et al., 2011a; Sasayama et al., 2012). These results suggest a link between the expression and function of FUS, and the specificity of ALS neurodegeneration and we await further investigations of *fust-1* in *C. elegans*.

USE OF *C. elegans* TO MODEL C9ORF72 TOXICITY

A region of chromosome 9 had been linked to ALS for several years (Morita et al., 2006; Vance et al., 2006; van Es et al., 2009; Shatunov et al., 2010) but the gene was only identified in 2011 (DeJesus-Hernandez et al., 2011; Renton et al., 2011) and has since been shown to be a major cause of sporadic and familial ALS (Turner et al., 2013b). The basis of the mutation is a GGGGCC repeat expansion within the first intron of *C9ORF72*. Many questions remain to be answered about the role of *C9ORF72* in the pathogenesis of ALS. It is still not clear whether the GGGGCC repeat expansion results in a loss of function, a gain of function or both, or if the size of the repeat has differential effects on these potential mechanisms. Recent reports have observed decreased expression of *C9ORF72* when the GGGGCC repeat reaches pathogenic length (DeJesus-Hernandez et al., 2011; Ciura et al., 2013; Xi et al., 2013). Whether decreased expression contributes to ALS pathogenesis is unknown since very little is known about the biological role of C9ORF72 other than its sequence similarity to the GDP/GTP exchange factor "Differentially Expressed in Normal and Neoplasia" (DENN) (Zhang et al., 2012a; Levine et al., 2013). DENN proteins are involved in the regulation of Rab-GTPases and endocytosis. Recently, C9ORF72 was shown to be implicated in endosomal trafficking (Farg et al., 2014), confirming its role as a DENN protein. In *C. elegans*, work has been previously done regarding some Rab proteins using deletion mutants and GFP reporters (Sato et al., 2008) to investigate endocytosis (Fares and Grant, 2002). *C. elegans* would be an ideal model to confirm the involvement of *C9ORF72* in this pathway. The *C. elegans* homolog of *C9ORF72* is named *alfa-1* (**AL**S/**F**TD **a**ssociated gene homolog). Our group characterized the loss of function mutant *alfa-1(ok3062)* where we observed that decreased expression of *alfa-1* causes a motility defect, neurodegeneration specifically of the motor neurons and sensitivity to osmotic stress (Therrien et al., 2013). Further characterization still remains to be done but it is interesting that loss of *alfa-1* is linked

Table 4 | Summary of transgenic FUS models.

Study	Promoter	Gene	Motor phenotype	Aggregation	Neuro-degeneration	Synaptic dysfunction
Murakami et al., 2012	*rgef-1*: entire nervous system	FUS^{WT}	No	No	n.d.	n.d.
		FUS^{R514G}	No	No	n.d.	n.d.
		FUS^{R521G}	No	No	n.d.	n.d.
		FUS^{R522G}	Yes	Yes	n.d.	n.d.
		FUS^{P525L}	Yes	Yes	n.d.	n.d.
		$FUS^{501trunc}$	Yes	Yes	n.d.	n.d.
		$FUS^{513trunc}$	Yes	Yes	n.d.	n.d.
Vaccaro et al., 2012c	*unc-47*: GABAergic neurons	FUS^{WT}	No	No	No	Normal
		$FUS^{S57\Delta}$	Yes	Yes	GABAergic neurons	Impaired

n.d., not determined; trunc, truncation.

to neuronal integrity specifically for GABAergic motor neurons in worm.

GGGGCC repeat expansions are found in the first intron of *C9ORF72* and the presence of such long non-coding repeats is suggestive of a toxic gain of function mechanism driving neurodegeneration as seen in many of the trinucleotide repeat expansion diseases. In patients, the repeat was shown to induce abnormal translation (non-ATG translation of the repeat, also called RAN translation) leading to the production of different dipeptides(Ash et al., 2013; Mori et al., 2013b). Also of interest were the presence of RNA foci containing the expanded GGGGCC repeat in patients (DeJesus-Hernandez et al., 2011). It is unknown whether a toxic gain of function is caused by the presence of toxic RNA or the presence of toxic protein, or both. So far, no groups have generated transgenic worms to model this aspect of the toxicity however the expression of the non-coding GGGGCC repeat in *Drosophila* causes neurodegeneration (Xu et al., 2013). *C. elegans* may be useful to model non-coding repeats based on previous efforts studying the expression of non-coding CUG repeats that were toxic to worms (Chen et al., 2007) and recapitulated aspects of RNA foci toxicity (Wang et al., 2011b).

STRESS RESPONSE AND AGE-DEPENDENT NEURODEGENERATION IN *C. elegans*

With the identification of TDP-43 in most ALS aggregates and later the identification of mutations affecting *TARDBP* and *FUS* genes, RNA metabolism has become an important area of investigation in ALS research. Under normal conditions, both proteins are mainly observed in the nucleus but the mutant proteins are also found in the cytoplasm. FUS and TDP-43 contain a low-complexity prion-like domain and a RNA binding domain suggesting a role in RNA metabolism (Li et al., 2013b). High throughput RNA-sequencing experiments have been used to identify targets of TDP-43 and FUS in normal or disease states. In worms, the transcriptome of the *tdp-1(ok803)* mutant has been studied (Zhang et al., 2012b) and showed the involvement of TDP-1 in various aspects of development.

Under cellular stress, wild type and mutant TDP-43 and FUS proteins form RNA granules (Bosco et al., 2010; Dormann et al., 2010; Liu-Yesucevitz et al., 2010; Gal et al., 2011; McDonald et al., 2011). These granules are usually formed in order to protect RNA from degradation under stress conditions. In worms, a variety of different RNA granules exist: P granules, P bodies and stress granules. P granules are the most characterized RNA granules in worms and are highly involved in cellular development (Updike and Strome, 2010). However, human proteins found in P bodies and stress granules, such as TIA1, the decapping enzymes and polyA binding proteins, have *C. elegans* ortholog and their role seem conserved regarding RNA granules (Jud et al., 2008; Sun et al., 2011). An active area of research concerns whether mutant TDP-43 and/or FUS proteins interfere with stress granule homeostasis. In a transgenic model of FUS, wild type and mutant FUS were shown to colocalize to stress granules after a heat shock but only the recruitment of mutant FUS to the stress granules caused persistent motility defects in the worms (Murakami et al., 2012). Most work done in *C. elegans* to study stress granules have used thermal stress as an inducer of the granule (Sun et al., 2011). In cells, formation of granules containing FUS is also initiated by other environmental stresses such as osmotic stress (Baron et al., 2013) and oxidative stress (Vance et al., 2013), thus the effect of these other stresses would be interesting to evaluate. Since most of the components of the granules are conserved in worms, *C. elegans* could be a powerful system to investigate stress biology in the context of aging, an aspect not easily studied in cellular systems.

Since TDP-43 and FUS are components of stress granules, this has led to the hypothesis that both proteins may be involved in the cellular stress response. The genetic pathways governing cellular stress signaling have been studied to great success in *C. elegans*. The different stress response pathways are highly characterized in worms with the insulin/IGF-1 pathway being a major, conserved signaling axis (Lau and Chalasani, 2014). In worms, the insulin/IGF-1 pathway has a sole insulin/IGF-1 receptor, DAF-2, that acts through the kinases AGE-1, PDK and AKT to phosphorylate the FOXO transcription factor DAF-16, and regulate stress resistance and longevity (Lapierre and Hansen, 2012). The most common stresses applied to worms in laboratory settings include exposure to thermal, oxidative, osmotic or hypoxic stresses (Rodriguez et al., 2013). While each is a damaging stress, they can elicit distinct genetic signaling pathways with diverse outcomes. An open question in the field of late-onset neurodegeneration is whether environmental components exist to account for the range in disease onset and progression for what are many highly penetrant, monogenic, dominantly acting disorders. A stress intrinsic to ALS and many neurodegenerative diseases is proteotoxicity. Here mutant proteins misfold leading to a diverse range of proteotoxic consequences. Thus, cells maintain an extensive network of mechanisms, including the insulin/IGF-1 pathway, to maintain protein homeostasis in the face of environmentally derived damage or genetically encoded misfolded proteins.

Work from *C. elegans* directly linked *tdp-1* to the insulin/IGF-1 pathway and proteotoxicity. In *C. elegans tdp-1* is required for the stress resistance of *daf-2* mutants and the stress-induced expression of *tdp-1* was dependent on *daf-16*. These data suggest a role for TDP-1/TDP-43 in the insulin/IGF-1 pathway and it remains to see if insulin/IGF-1 signaling is altered by disease-associated TDP-43 mutations.

Interestingly, in Vaccaro et al., mutant TDP-43 and mutant FUS proteins were only expressed in the 26 GABAergic motor neurons but activated the ER unfolded protein response chaperone HSP-4 in intestinal tissue (Vaccaro et al., 2012c). This observation suggests that proteotoxic insults can induce stress-signaling pathways in other tissues. It is not known if this is due to a diffusible signaling molecule, or if the mutant proteins make their way from the nervous system to adjacent tissue. TDP-43, FUS, HNRNPA1, HNRNAP2B and TAF15 all contain a prion-like domain (Couthouis et al., 2011; Polymenidou and Cleveland, 2011; Kim et al., 2013) and misfolded SOD1 protein may be able to self propagate (Grad and Cashman, 2014). Thus, these proteins could share properties with toxic prion protein (PrPsc) that misfolds, become infectious, and spreads from cell to cell (Kabir and Safar, 2014). The development of ALS symptoms, starting usually in the lower limb and spreading upward, also suggests a propagation mechanism. Little is known about

the propagation potential of the ALS associated misfolded proteins in *C. elegans* transgenic models. Mutant TDP-43 and FUS proteins in the worm's motor neurons were shown to induce the expression of HSP-4 in the intestine, but the proteins were not visualized outside of the neurons where they were expressed (Vaccaro et al., 2012c). A prion model was however characterized expressing Sup35NM, a yeast prion protein, in the body wall muscle of the worm. The most toxic form of the protein was shown between muscle cells, in the intestine and the coelomocytes, and the toxic fibrils were able to induce protein misfolding (Nussbaum-Krammer et al., 2013). Also, proteostasis, ER stress resistance and longevity, all major ALS research topics, have been recently shown to have important cell-non-autonomous components (Taylor and Dillin, 2013; van Oosten-Hawle et al., 2013). Since *C. elegans* is transparent, direct visualization of tagged proteins during development and aging is possible. The development of additional tools should help establish if a propagation mechanism exists for mutant TDP-43, FUS and SOD1 proteins.

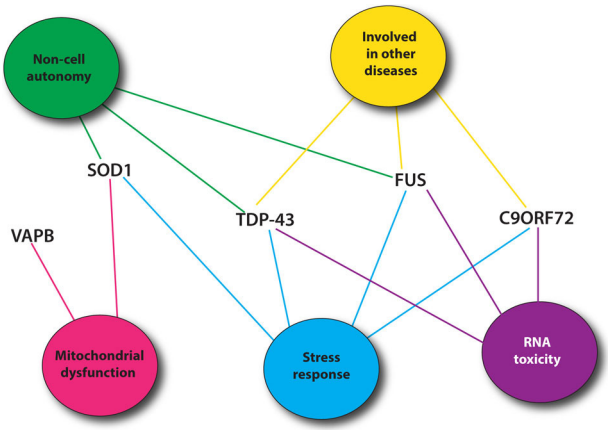

FIGURE 1 | Most common ALS genes and pathogenic pathways. Shown are mutant proteins associated with ALS and putative, shared toxicity mechanisms including RNA toxicity, the cellular stress response, mitochondrial impairment and cell autonomy/non-autonomy. Many of these pathways can be easily investigated using *C. elegans*.

IDENTIFICATION OF GENETIC INTERACTIONS

Recent genetic advances have identified many new causative genes for familial cases of ALS (**Table 1**). Moreover, genome-wide association studies (GWAS) have also been done in sporadic ALS cohorts to identify potential genes (Renton et al., 2014). With the increasing number of genes linked to ALS along with the diverse functions of these genes, it is essential to identify common pathological pathways relevant to ALS. Genetic interactions amongst genes can refer to functional relationships amongst a group of genes (Boucher and Jenna, 2013). However, genetic interactions are not always easy to interpret and do not necessarily point toward genes that function in the same pathway but rather identify functional similarity between genes that could be in the same pathway or in compensatory pathways (Boucher and Jenna, 2013). Therefore, identification of genetic interactions between ALS genes could point toward potential therapeutic avenues for ALS patients (**Figure 1**).

Among the proteins identified, TDP-43, SOD1, FUS, OPTN, UBQLN2, and NEFH proteins are found in familial and sporadic ALS inclusions (Al-Chalabi et al., 2012). In zebrafish and *Drosophila*, FUS, and TDP-43 were shown to interact genetically together but independently of SOD1 (Kabashi et al., 2011; Lanson et al., 2011; Wang et al., 2011a). The rapid development of phenotypes and the availability of multiple mutants or RNAi clones make *C. elegans* an expedient model to study genetic interactions. In worms, TDP-1 was shown to participate in the neurotoxicity observed in motor neuron caused by human TDP-43 and human FUS (Vaccaro et al., 2012c). However, FUS and TDP-43 seem to interact differently with PGRN, a gene involved in FTD, and C9ORF72/ALFA-1 (Tauffenberger et al., 2013a; Therrien et al., 2013). Those results provide an interesting start to the identification of common pathological pathways in ALS. Finally, the characterization of the loss of function mutant of *pgrn-1*, the *C. elegans* ortholog of progranulin, showed that PGRN-1 is involved in apoptotic cell clearance (Kao et al., 2011). Understanding how *pgrn-1* interacts with the different genes involved in ALS/FTD could help to better understand the variation observed along ALS/FTD continuum (Mackenzie et al., 2011).

At a broader level, screening for potential genetic modifiers using RNAi has brought a new understanding of the pathogenesis. For example, SOD1 aggregation was linked to motor dysfunction in worms, but upon decreasing the expression of chaperone proteins, the worms exhibited larger aggregates and increased locomotion deficits (Wang et al., 2009). A non-biased screening approach has recently demonstrated that targeting proteins that affect the phosphorylation levels of TDP-43 greatly affects its neuronal toxicity, setting the stage for novel therapeutic approaches (Liachko et al., 2013). Thus far, only a handful of genetic screens have been described for *C. elegans* ALS models but future studies may help uncover pathogenic mechanisms and therapeutic strategies.

TDP-43 aggregates were shown to be the main protein found in non-SOD1 ALS cases (Neumann et al., 2006). However, the presence of TDP-43 aggregates is not exclusive to ALS. TDP-43 aggregates are observed in other neurodegenerative diseases such as Huntington's disease, Parkinson's disease, Alzheimer's disease, and FTD (Mackenzie et al., 2010). FUS is also found in the aggregates of polyglutamine disorders (Woulfe et al., 2010) and mutations in *FUS* were linked to essential tremor (Merner et al., 2012). Recently, FIG4 and VCP were also identified in different neurodegenerative aggregates (Mori et al., 2013a; Kon et al., 2014). Uncovering specific genetic interactions that involve these proteins could also help our understanding of their recruitment to the aggregates of so many neurodegenerative disorders. Using other models, groups have shown that intermediate polyglutamine repeat of *ATXN2* gene and EPHA4 are potent modulators of ALS toxicity (Elden et al., 2010; Van Hoecke et al., 2012). Therefore, a genetic interaction map may extend the role of these genes beyond ALS and perhaps into other neurodegenerative disorders.

USING *C. elegans* MODEL FOR ALS DRUG DISCOVERY

The small size of *C. elegans*, its rapid life cycle, its ease of cultivation and ability to obtain large numbers of animals makes it an attractive model for drug discovery. Furthermore, worms can be grown on solid media or in liquid culture, the latter being relatively easy to adapt for drug screening purposes often through use of multiwell plates and/or automated screening methods (O'Reilly et al., 2013). The transparency of *C. elegans* makes it an ideal model for neurodegeneration applications since protein aggregation and neuronal morphology can be easily assayed as a complement to behavioral phenotypes.

Boyd and colleagues have shown that drugs identified from cell based systems often have relevance in *C. elegans* (Boyd et al., 2013). A screen to identify compounds that decrease TDP-43 aggregation was performed in cell lines and many of the molecules identified were able to suppress the impaired motility phenotype of worms expressing mutant human TDP-43 (Boyd et al., 2013).

Our group has also developed a high-throughput drug-screening assay. We observed that the paralysis phenotype that typically manifests over 5–12 days on solid media can be observed after just hours when the worms are placed in liquid culture (Vaccaro et al., 2012b; Therrien et al., 2013). Using this technique, more than 4000 FDA approved compounds were screened in our laboratory. From this screen, we identified a number of molecules including methylene blue and others acting on the ER stress response that decrease the toxicity of TDP-43 (Vaccaro et al., 2012a, 2013). Interestingly, these drugs were also confirmed in zebrafish ALS models confirming that these compounds can be effective across species. These compounds are therefore promising leads for testing in mammalian models.

Even though in the disease state, aging and neurodegeneration seem to go hand in hand, we have shown that the drugs that act on neurodegeneration can be separated from those that broadly affect lifespan (Tauffenberger et al., 2013b) suggesting that lifespan extension is not a strong predictor of neuroprotection.

OTHER MOTOR NEURON DISEASES

ALS is part of the neurological group of disorders called motor neuron diseases. This group also includes spinal muscular atrophy (SMA), primary lateral sclerosis (PLS), hereditary spastic paraplegia (HSP) and many others affecting the upper and/or lower motor neurons. The causative genes of these diseases are involved in many cellular functions, however they all share a common toxic pathways since they mainly affect motor neurons. Finding similarities and differences among those diseases could highly increase our understanding of motor neuron toxicity. *C. elegans* has been used to study two of these, SMA and HSP.

HSP is a group of disorders affecting mainly the lower motor neurons. More than 40 loci have been linked to HSP and the genes identify are involved in axon pathfinding and myelination, mitochondrial maintenance and membrane trafficking (Blackstone, 2012). Recently, a large network including many of these genes have been identify and this network is highly similar to Parkinson's, ALS and Alzheimer's diseases (Novarino et al., 2014). Using *C. elegans*, the function and toxicity of two HSP genes have been investigated. First, *spas-1*, the *C. elegans* ortholog of *spastin*, also called *SPG4*, was shown to be involved in the development of microtubules. SPAS-1 is expressed in the cytoskeleton and is involved specifically in the disassembly of microtubules (Matsushita-Ishiodori et al., 2007). Then, the pan neuronal expression of *NIPA-1* associated mutations led to motor deficits and shortened the lifespan of transgenic worms probably through the activation of caspases and increased ER toxicity (Zhao et al., 2008). With the rapid discovery of new HSP genes, more models are surely to come and will help unravel similarities between these diseases.

SMA is a rare autosomal recessive disorder and a leading genetic cause of infant death. All genetic causes of SMA lead to a decreased expression of the proteins survival of motor neuron (SMN) 1 and 2 (Arnold and Burghes, 2013). It mainly affects the lower motor neurons, but recent evidences suggest that it can be a systemic disease affecting the vascular, cardiac and hepatic functions as well as affecting bone formation (Hamilton and Gillingwater, 2013). *C. elegans* possesses one ortholog of the *SMN* gene, *smn-1*. In 1999, Miguel-Aliaga and colleagues showed that decreased expression of *smn-1* in worms resulted in severe locomotion defects and sterility (Miguel-Aliaga et al., 1999). Then SMN-1 was shown to interact with SMI-1, a known interactor of SMN in humans (Burt et al., 2006). Briese and colleagues characterized the first *smn-1* deletion mutant observing that the mutants displayed early developmental arrest, which could be rescued by reintroducing expression of *smn-1* in the nervous system, while expression in muscle cells was ineffective (Briese et al., 2009). Little is known about any downstream targets of SMN and no drugs are available. Thus, several groups have used *C. elegans* to identify modifiers of the *smn-1* phenotypes. In a cross-species study, it was shown that proteins involved in endocytosis and mRNA regulation could modify the toxicity (Dimitriadi et al., 2010). Also, knowing that the ubiquitin-proteasome pathway degrades SMN, decreased expression of *Mibl*, an E3 ligase, was shown to ameliorate *smn-1* phenotypes (Kwon et al., 2013). Since the *smn-1* deletion allele o*k355* is an early larval lethal phenotype, to aid the development of drug screening Sleigh and colleagues identified a less severe mutant allele that more closely resembles the severity of SMA (Sleigh et al., 2011). Using this mutant, they identified several small molecules that alleviate *smn-1* phenotypes of the worms, therefore, being highly promising compounds for SMA drug development (Sleigh et al., 2011).

PERSPECTIVES

With the discovery of many new ALS genes comes the need to better understand their functions, expression patterns and their modes of toxicity. *C. elegans* has proven to be an informative model to study neurodegeneration mechanisms arising from multiple ALS related proteins. We envision that the introduction of new transgenic and genetic models will help unravel important questions about the normal and pathogenic roles of these proteins.

Most models explained here recapitulate some if not many, important features of ALS, however, phenotypic variations are seen amongst the different models, for a number of reasons. First, the models do not all use the same mutations, thus the resulting mutant proteins may not all be equally toxic, or display the

same interactions with other proteins. Also, the level of expression is important to consider as for example, there is considerable evidence that TDP-43 levels are tightly regulated (Budini and Buratti, 2011), and elevated expression is toxic in nearly every system studied (Ash et al., 2010; Xu et al., 2010; Estes et al., 2011). The most common method to generate transgenic worms is by microinjection to create stable lines followed by radiation to integrate the transgene in the genome. This procedure typically produces transgenics with multiple copies of the gene inserted in the genome, thus some of the toxicity observed may be due to overexpression. Aware of this issue, a new generation of ALS transgenic worms should be constructed based on single copy integration (Frøkjaer-Jensen et al., 2008) or with the CRISPR-Cas9 method (Friedland et al., 2013) instead to ensure that transgenic lines have a similar level of expression from the same genomic location. Finally, the phenotypic variance may also be due to the promoter used. Some models have used pan neuronal expression constructs, while others have targeted transgenic expression to specific neuronal populations. In humans, most of these proteins are expressed ubiquitously but only specific neuronal populations are sensitive to degeneration. Thus, worm models based on motor neuron transgenics could be ideal model to uncover conserved mechanisms of motor neuron degeneration. To confirm the specificity of each phenotype, mutant and wild type proteins should be carefully compared and similar changes should be confirmed in higher eukaryotes. For example, mutant TDP-43 and FUS proteins induce an ER stress response in worms which is not observed when the wild type proteins are expressed (Vaccaro et al., 2012b). Also, the ER stress response was shown to be activated in other ALS models and in patients (see section above).

These models are setting the stage for novel toxicity hypothesis. The immune system seems to play an important role in the neurodegeneration observed in ALS. Protein aggregation could activate the immune response and neuroinflammation actively contributes to disease progression (McCombe and Henderson, 2011). *C. elegans* relies on an evolutionary conserved, innate immune response (Engelmann and Pujol, 2010) that coordinates its activity with the insulin/IGF-1 pathway (Singh and Aballay, 2009) suggesting these may be pathways worth investigating. Also, in the past year, a convergence of data has suggested a role for glial cells in ALS neurodegeneration (Parisi et al., 2013; Valori et al., 2013; Chiu et al., 2014). The worm has 56 glial cells and some are found at the neuromuscular junction (Oikonomou and Shaham, 2011). Characterization of the cross talk between the neurons and the glial cells would also be an interesting area of investigation.

An important topic related to ALS and to other neurodegenerative disorders is aging. The risk of ALS increases with age, peaking between 70 and 80 years old (Gordon, 2013). Aging pathways are well characterized in worms and among others, include the insulin/IGF-1, the target of rapamycin (TOR) and germline signaling pathways. There is a strong overlap between protetotoxicity and aging where autophagy and lipid metabolism are major targets (Lapierre and Hansen, 2012). Evaluating the toxic impact of mutant proteins during aging is not feasible in many models, but is easily accomplished using *C. elegans*. The development of models with age-related toxicity is essential and could help understand the link between the proteotoxicity and aging.

When using *C. elegans* or other animal models, most studies have focused on the toxicity of known ALS genes. It is important to note that almost 90% of ALS cases are sporadic ALS with no link to known genetic abnormalities. Therefore, we still do not know how most patients develop ALS. However, it is important to know that sporadic and familial cases of ALS are clinically indistinguishable (Al-Chalabi and Hardiman, 2013). Given that ALS patients can live between 6 months and 6 years after diagnosis, it has been hypothesized that environmental factors may influence disease onset and progression (Al-Chalabi and Hardiman, 2013). Many environmental factors have been examined in relation to ALS but there is no consensus for their contribution to the disease (Al-Chalabi and Hardiman, 2013). *C. elegans* could be useful to study some of the environmental risks hypothesized. In fact, several groups have identified compounds that could cause specific degeneration of motor neurons (Du and Wang, 2009; Negga et al., 2012; Estevez et al., 2014) opening the door to identifying environmental modifiers of degeneration in ALS models.

However, how relevant are any of these findings to humans? Will any of the drugs identified in *C. elegans* translate to mammalian models let alone ALS patients? So far, many drugs identified using rodent models focusing mainly on protein aggregation and cell death mechanisms have failed in subsequent clinical trials. Using *C. elegans* to identify drugs acting on early neuronal dysfunction mechanisms could be an effective way to prevent ensuing cellular decline and death. From a liquid culture screen, our group has identified a compound with this property (unpublished results). The compound is effective in vertebrate ALS animal models and is now being tested in ALS patients. Therefore, large screens using *C. elegans* targeting specific early aspects of neurodegeneration seem promising and show relevance in higher organisms.

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A semi-automated motion-tracking analysis of locomotion speed in the *C. elegans* transgenics overexpressing beta-amyloid in neurons

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Multi-Worm Tracker (MWT) is a real-time computer vision system that can simultaneously quantify motional patterns of multiple worms. MWT provides several behavioral parameters, including analysis of accurate real-time locomotion speed in the nematode, *Caenorhabditis elegans*. Here, we determined locomotion speed of the Alzheimer's disease (AD) transgenic strain that over-expresses human beta-amyloid1-42 (Aβ) in the neurons. The MWT analysis showed that the AD strain logged a slower average speed than the wild type (WT) worms. The results may be consistent with the observation that the AD patients with dementia tend to show deficits in physical activities, including frequent falls. The AD strain showed reduced ability of the eggs to hatch and slowed hatching of the eggs. Thus, over-expression of Aβ in neurons causes negative effects on locomotion and hatchability. This study sheds light on new examples of detrimental effects that Aβ deposits can exhibit using *C. elegans* as a model system. The information gathered from this study indicates that the motion tracking analysis is a cost-effective, efficient way to assess the deficits of Aβ over-expression in the *C. elegans* system.

Keywords: Alzheimer's disease, beta amyloid, age-related memory impairment (AMI), frailty, behavioral aging, motion tracking, automated system, Alzheimer's disease in children

INTRODUCTION

Alzheimer's disease (AD) is a type of amyloidosis and is the major neurodegenerative disorder that causes dementia. Amyloidosis is characterized by fibril deposits that contain at least 30 fibril proteins in humans and 10 fibril proteins in animals, according to the 2012 the Nomenclature Committee of the International Society of Amyloidosis (Sipe et al., 2012). Of them, accumulation of Aβ has been observed in the AD and congophilic cerebral angiopathy (CAA) (Selkoe, 2001; Revesz et al., 2003). AD leads to cognitive deficits due to abnormal deposits of β-amyloid (Aβ) peptides in the brain, while CAA is characterized by vascular deposits in the central nervous system, in which hemorrhage is a major clinical feature (Samarasekera et al., 2012). CAA is often observed in AD patients, in which case it has been diagnosed as AD (Wright, 2013).

In AD, deposits of the Aβ peptides arise from the proteolytic processing of amyloid precursor proteins (APP), commonly observed in patients with AD (Nicholson et al., 2012). Aβ deposits and tau tangles are well known hallmarks for AD, which may trigger inflammation worsening the disease (Nicholson et al., 2012; Jack and Holtzman, 2013). Aβ toxicity may be linked to tau hyperphysphorylation observed in tau tangles (Lloret et al., 2011; Ermak and Davies, 2013). Although it is believed that Aβ is involved in initiation of AD in the presence of tau pathologies and that tau is correlated with severity of AD (reviewed in LaFerla, 2010; Castillo-Carranza et al., 2014), molecular mechanisms of AD remain unclear. Details of clinical pathogenesis and biomarkers have been reviewed elsewhere (Jack and Holtzman, 2013).

This study aimed to assess the effects of Aβ toxicity on a behavioral parameter, average speed of movement. A transgenic strain overexpressing the signal peptide::$A\beta^{1-42}$ in the neurons (Dosanjh et al., 2010; Lublin and Link, 2013) was used for this study. Over-expression of Aβ in the neurons may affect the serotonin system (Dosanjh et al., 2010). Previously, locomotion has been assessed in a manual assay that measures the rate of body bend, which is an indirect measure with relatively high variability (Dosanjh et al., 2010; Lublin and Link, 2013); the manual assay may have been missed early signs of motility defect. In addition, another neural Aβ strain is tagged by a behavioral marker (i.e., roller phenotype) (McColl et al., 2012), which is difficult to assess locomotion speed. We used behavioral tracking software, Multi Worm Tracker (MWT) (Swierczek et al., 2010), to record and collect data on the speed. MWT is a program designed to quantify the behavior of multiple worms on a petri plate with minimal human effort. The main advantage is that it allows for a more accurate and detailed assessment of locomotion speed compared to the traditional method that measures body bends. Thus, our hypothesis is that the strain overexpressing Aβ in the neurons show defects in locomotion speed. We reason that motion tracking analysis, including MWT analysis, should contribute to understanding the harmful effects of Aβ toxicity in *C. elegans*.

MATERIALS AND METHODS

STRAINS, MEDIA, AND STAINING

Wild-type strain, N2, was used (referred to as WT strain). As a control, we also used the *smg-1*^{ts} strain [*smg-1*^{ts}*(cc546)*], which generated similar results as N2 in the assays (i.e., locomotion and hatchability) used in this study (data not shown). The strain CL2355 [*smg-1*^{ts}*(cc546)*; *snb-1*::Aβ_{1-42}::long 3′-UTR] that utilizes the *C. elegans* promotor of the synaptobrevin (*snb-1*) gene to cause a pan-neuronal overexpression of the signal peptide::Aβ^{1-42} (Dosanjh et al., 2010; Lublin and Link, 2013) was used (referred to as AD strain). For simplicity, the transgenic strain CL2355 is referred to as the AD strain for the duration of the paper. All strains were maintained at 15°C in a nematode growth media (NGM) spotted with *Escherichia coli*, OP50, as a food source (Murakami et al., 2005). The following procedure adopted from Stiernagle (2006) was used to prepare NGM plates. Before autoclaving, 17 g agar, 2.5 g peptone, 3 g NaCl, and 975 mL distilled H_2O was added to a flask and covered with aluminum foil. Then the flask was autoclaved for 50 min. After autoclaving the mixture, 1 mL of 1 M $CaCl_2$, 1 mL of 5 mg/mL cholesterol in ethanol, 1 mL of 1 M $MgSO_4$, and 25 mL of 1 M KPO_4 (pH 6.0) was added to the flask. Then petri plates were filled 2/3 with agar. Plates were left at room temperature for 2–3 days to allow excess moisture to evaporate. For immunofluorescence staining, we used the procedure described in Link (1995). Transgenic worms were fixed, permeabilized, and stained with the anti-Aβ monoclonal antibody 4G8 and anti-TOR-2 polyclonal sera as a counterstain. DNA was visualized using 4′,6-diamidino-2-phenylindole (DAPI).

GROWTH CONDITIONS

Strains were grown on NGM agar plates at 15°C. To assess hatchability, eggs were layed on NGM plates at 15°C and incubated at the temperature indicated in the text (15 or 25°C). Unhatched eggs were counted after 24 h or the time specified in the text. Hatched eggs (larvae) were also counted to confirm the result. To prepare adult worms, eggs were layed and grown into adults for 4 days. Adults at the age of day 5 (i.e., 1 day after they start to lay eggs) was defined as "younger adults." Adults at the age of day 7 was defined as "middle-aged adults." Adults at the age of day 13–14 was defined as "older adults."

MULTI WORM TRACKER (MWT) ANALYSIS

Videos of the worms were recorded under a stereomicroscope using ToupView, a video capturing software (Amscope.com, Irvine, CA). The parameters of 3 min for duration and 6 s for bin size was set when capturing the video, which created a video file that was 35 s in length. To analyze the speed of the worms, the video file was uploaded into MWT, a behavioral tracking software (Swierczek et al., 2010). In a typical assay, about five worms in a microscopic field was computed and average speed of the worms were calculated. A measure for speed (in pixels/s) was made at a series of ages indicated in the text. We used the conversion rate in the system: 1 pixel/s = 0.035 cm/s at the images of 72 PPI (pixels per inch). Death was determined by observing no movement of the worms. Statistical analysis has been performed by ANOVA using NCSS 2007 statistics software (NCSS, LLC, Kaysville, Utah, USA).

RESULTS

We sought to assay the locomotion of the transgenic *C. elegans* strain CL2355 (Dosanjh et al., 2010), which expresses a human Aβ_{42} minigene under the control of the pan-neuronal synaptobrevin (*snb-1*) promoter. Expression of Aβ using this promoter leads to intraneuronal deposition of Aβ, particularly detectable in the nerve ring area (**Figure 1**).

Video images of the worms on NGM agar were captured for 3 min and the speed of the worms was determined using MWT. We compared the results of the WT (wild type) and AD strains (over-expressing amyloid beta) (Materials and Methods). As described in the Method, adults that had grown for 5 days were defined as "younger adults." Adults that had grown for 7 days were defined as "middle-aged adults." Adults that had grown for 13 days were defined as "older adults."

THE AD TRANSGENIC STRAIN SHOWING SLOWER MOVEMENT

Table 1 summarizes the results. For the younger adults (day 5), WT had a mean speed of 0.18 ± 0.15 cm/s whereas the AD had a mean speed of 0.05 ± 0.06 cm/s ($p > 0.0001$; **Figure 2A**;

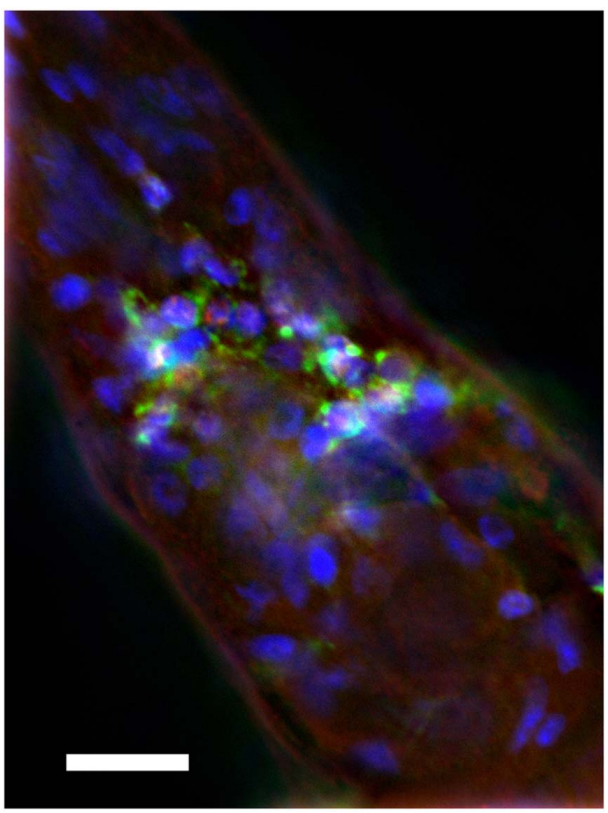

FIGURE 1 | Intraneuronal deposition of Aβ in transgenic *C. elegans*. Anterior region of transgenic *C. elegans* is shown. The strain was immune-stained by anti-Aβ monoclonal antibody 4G8 (green), anti-TOR-2 polyclonal sera (red), and DAPI (blue). Note cytoplasmic accumulation of Aβ in nerve ring neurons. Size bar = 10 μM.

Table 1 | Locomotion speed (mean ± standard deviation) as assessed by MWT analysis for various stages in the life cycle (*$p < 0.0001$).

Life stage (day #)	Type of strain	Average speed (cm/s)
Younger adult (day 5)	WT	0.18 ± 0.15
	AD	0.05 ± 0.06*
(day 6)	WT	0.52 ± 0.25
	AD	0.21 ± 0.17*
Middle-aged (day 7)	WT	0.43 ± 0.19
	AD	0.05 ± 0.05*
(day 10)	WT	0.10 ± 0.11
	AD	0.08 ± 0.12
Older adult (day 13)	WT	0.11 ± 0.07
	AD	0.12 ± 0.07

Table 1). Slow locomotion speed in the AD strain compared to WT was observed from day 5 (younger adults) to day 7 (middle age) ($p > 0.0001$; **Figures 2A,B**; **Table 1**). Interestingly, there was a peak of locomotion speed at the age of day 5 (**Figure 2E**), which was consistent with previous study as assessed in a classical assay that measured body bends, an indicator of locomotion speed (Murakami et al., 2008).

Slow locomotion speed in the AD strain was also evident in the still images (**Figure 3**) and **Supplementary Video** (Supplementary Figure). In the sill images taken every 7 s (**Figure 2**), the WT worms were located in different positions (**Figure 3A**), while the AD worms were nearly stagnant from 0 to 35 s (**Figure 3B**). Thus, a difference in the worm movements was clearly visible.

Older worms at day 10 and 13 showed similar results for the WT and AD strains. Day 10 WT worms had an average speed of 0.10 ± 0.11 cm/s whereas Day 10 AD worms moved at an average speed of 0.08 ± 0.12 cm/s for four worms (**Figure 2C**; **Table 1**). Finally, in the older adult at the age of Day 13, WT worms logged an average speed of 0.11 ± 0.07 cm/s and Day 13 AD worms showed similar locomotion speed (0.12 ± 0.07 cm/s) (**Figure 2D**; **Table 1**). Overall, worms with overexpressed Aβ resulted in slower movement up to middle-aged (**Figure 2**).

Aβ TOXICITY IN THE EMBRYONIC STAGE

Hatching of the eggs from the WT (N2) and AD (CL2355) strains were compared. We measured total number of unhatched eggs 24 h at restrictive temperature (25°C) after egg lay. We also counted hatched larvae to confirm the results. In the WT strain, there were 16.3% (37/227) eggs that remained unhatched and 83.7% (190/227) hatched (**Figure 4A**). For the AD strain, 89.2% (182/204) eggs remained unhatched and 10.8% (22/204) hatched. The rate of hatching in the control strain (*smg-1^{ts}*), was similar to WT (data now shown), excluding the possibility that the background mutation lowered the rate of hatching. Thus, the AD strain showed an approximately 7.8 folds lower hatching after 24 h of egg lay. We also investigated the time course of hatchability at permissive temperature, 15°C. Most of the eggs in the WT strain hatched 1 day after egg lay (Day 2; **Figure 4B**). In contrast, the AD strain hatched much slower than the WT strain, taking 2 days after egg lay (Day 3; **Figure 4B**). Thus, the AD strain shows reduced hatching and a delayed timing of egg hatching. We also observed a low rate of fecundity (Blood size: WT, 248 ± 22, $n = 10$; AD, 50 ± 12, $n = 20$; $p < 0.001$).

DISCUSSION

In this study, we used MWT analysis to measure locomotion speed. Compared to a manual assay to measure the number of body bends, MWT analysis provides a more accurate measure of multiple worms for a longer time (MWT for 3 min; Manual assay for 1 min). Young wild-type worm (locomotion speed: 0.18 ± 0.15 cm/s) was within the range of previously reported results (lower range of 0.13 cm/s, Ramot et al., 2008; upper range of 0.22 cm/s, Ryu and Samuel, 2002). They have also reported locomotion speed, ranging from 0.14 to 0.33 cm/s at the temperatures ranging from 17 to 27°C. The groups used other worm tracking systems, which suggests that MWT analysis results are consistent with those from the other worm tracking systems. It is worth noting that our MWT system is one of the most cost effective systems. The total cost was estimated to be up to US$200 for a routine USB-camera (as of April, 2014; our cost was zero since we recycled the camera, excluding the cost of software, computer, and microscope), which is much less than the cost estimated for MWT elsewhere (US$7,000) (Husson et al., 2012).

The AD strain showed an approximately 7.8-fold decrease in hatching compared to the WT strain. Likewise, the AD strain showed reduced hatching even at the permissive temperature, where Aβ is expressed, but at a lower level. The reduced level of hatching seen in the AD strain could be attributed to Aβ overexpression, suggesting the negative effect of Aβ on embryogenesis due to its toxicity. It is worth noting that some CL2355 worms are completely sterile (no eggs laid). This is a surprising observation if the defects in the CL2355 strain are restricted to neurons, as fertility in *C. elegans* is not strongly neuronally regulated. The fertility/sterility phenotypes of CL2355 were observed in the precursor extrachromosomal line, suggesting that these defects are not due to gene disruption caused by chromosomal integration of the transgene. We also observed that worms show larval arrest and death, suggesting defects in development caused by Aβ (data not shown).

The findings also suggest that Aβ accumulation has detrimental effects on locomotion speed in the adult life cycle, except for late-life in which all worms move poorly. In younger adults and middle-aged adults (corresponding to Day 5 and 7, respectively), the AD worms exhibited a slower speed than the WT worms (**Figure 1**). The middle-aged AD worms showed the greatest amount of difference from that of the WT worms; the approximate 8.3-fold difference in speed indicates that the presence of Aβ is correlated with the slower movement in the worms. This may be consistent with frailty seen in the Alzheimer's patients (Koch et al., 2013; Kulmala et al., 2014) and gait problems in the mouse models of AD (APP and APP/PS1 mice) (Lalonde et al., 2012; Wang et al., 2012a), though some other mice models [APP23, J20, APP + PDAPP, PS1 [Tg2576 + PS1 (M146L)], TgCRND8, TG2576, and 3 × Tg-AD mice] show increased locomotion due to

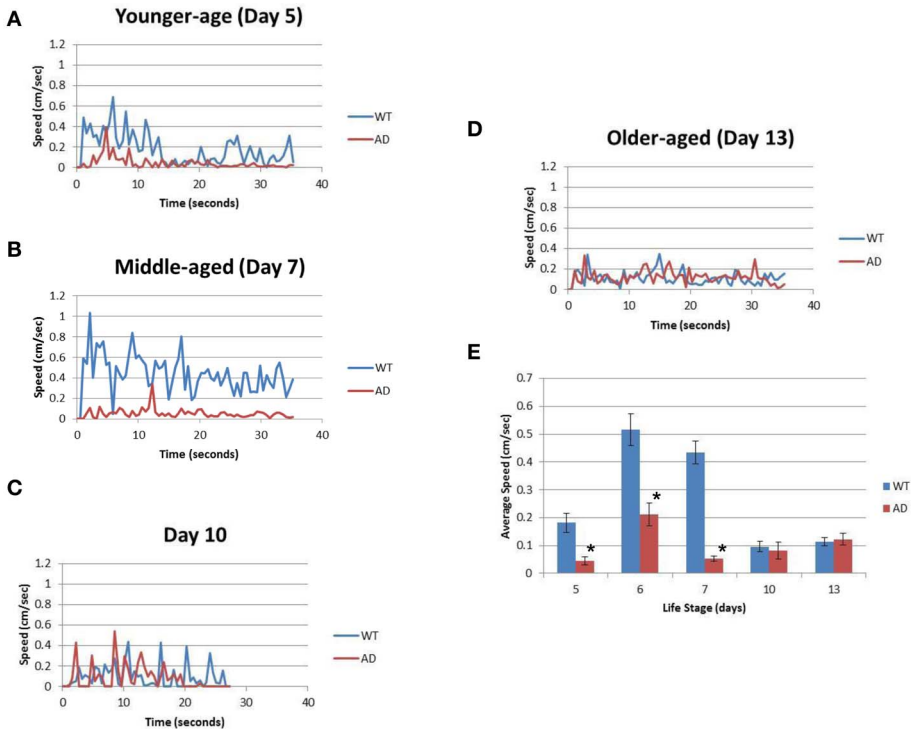

FIGURE 2 | The AD strain exhibits slower speeds than WT in the life cycle. (A) The graph represents the locomotion speed of adult worms measured at the age of day 5 (younger age). **(B)** The graph represents the speed for worms measured at the age of day 7 (middle age). **(C)** The graph represents the speed for middle-aged worms measured at the age of day 10. **(D)** The graph represents the speed for older adult worms measured at the age of day 13 (older age). **(E)** Comparison of the average speeds for WT (blue) and AD (red) worms at various ages over the course of their life span. Error bars indicate standard error of the mean. $*p > 0.0001$. WT worms (blue); and AD worms (red). See also **Table 1**.

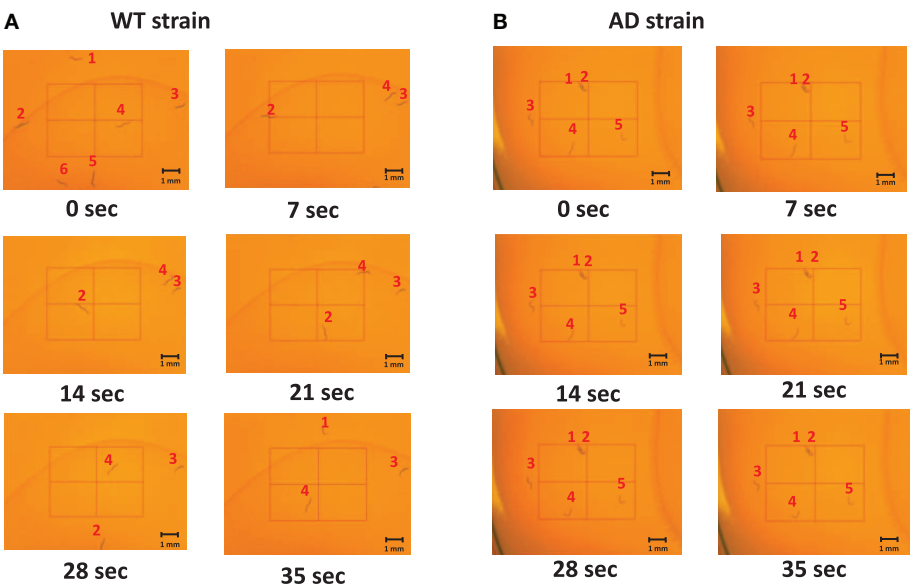

FIGURE 3 | Images of middle-aged (day 7) worms on the NGM plates at various time points. The images captured were taken every 7 s from 0 to 35 s. The worms were labeled 1–6 for the WT and 1–5 for the AD strain so that the difference in movement could be seen between and amongst frames. **(A)** An example of the WT worms. They moved throughout the plate as time progressed. **(B)** An example of the AD worms. They hardly moved as time elapsed.

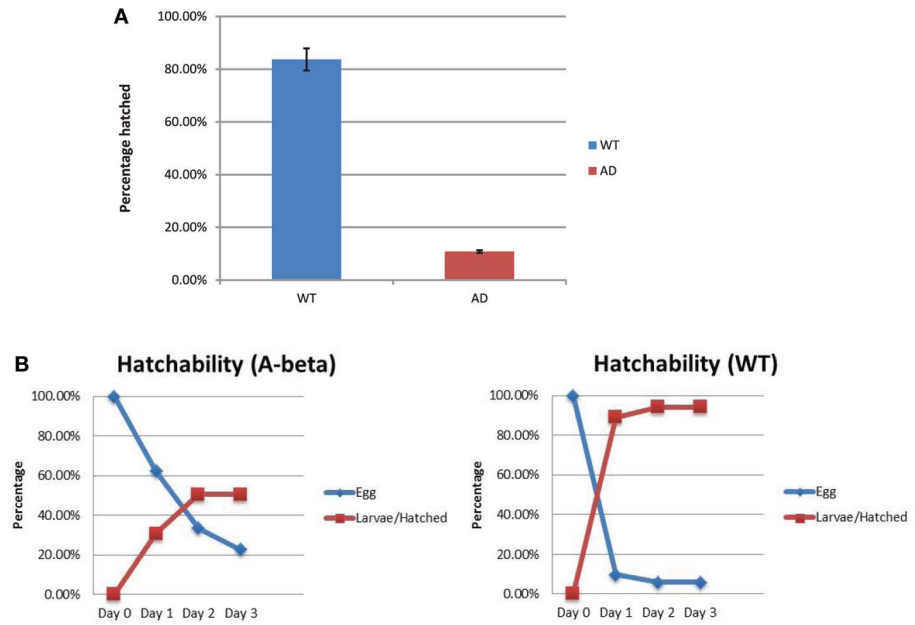

FIGURE 4 | Rate of egg hatching (hatchability) in the WT and AD strains. (A) Percentage of eggs unhatched after 24 h. There were more AD eggs (red) that remained unhatched compared to the WT eggs (blue). **(B)** Time course of the percentage of eggs that hatched from day 0 (the day of egg lay) to day 3 for both strains. Error bars indicate standard error of the mean.

aggression and other behavioral problems (Webster et al., 2014). Thus, locomotion is not always defective in AD patients and in AD models but rather altered. It is critical to assess locomotion in each AD system. In addition to our finding, locomotion defects have been observed in the fruit fly model of Aβ toxicity, while paralysis has been observed in the other strains over-expressing Aβ in the muscles and in the neurons (McColl et al., 2012; Wang et al., 2012b; Lublin and Link, 2013; Prüßing et al., 2013; Carrillo-Mora et al., 2014). Technical difficulties in the previous studies have been discussed above.

This study provides evidence that Aβ toxicity affects the embryonic stage as well as the adult phases. Since the AD transgenic strain used in this study has Aβ overexpression, the data can be used to assess the effects of Aβ toxicity on embryonic and behavioral parameters. Assessing the hatchability and speed of the worms allows for the analysis of the strains and the impact Aβ toxicity has on embryonic health and movement. The research will be beneficial as AD is one of the most common causes of dementia, and the number of Americans aged 65 and older affected by AD is predicted to triple by 2050 (Hebert et al., 2013). Currently, there are drugs there are drugs that can treat memory impairment in AD patients, but there are no cure for the disease itself. It is worth noting that a chaperone, HSP-16, is strongly associated with the Aβ deposits in the neuronal expression lines (reviewed in Lublin and Link, 2013). The future direction of this study aims to explore the relationship between AD drugs and potential Aβ clearance. The hope is that future studies will demonstrate the effectiveness of current FDA-approved AD treatment interventions to help alleviate the Aβ buildup seen in AD patients and enhance future treatments by potentially slowing or stopping the disease.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fgene.2014.00202/abstract

Supplementary Video | Video images of day 7 worms shown in Figure 2. (Video 1). Video shows the WT strain (File name: Machino et al. WT strain Day 7.mwv). **(Video 2)** Video shows the AD strain (File name: Machino et al. AD strain Day 7.mwv). As described in Materials and Methods, 3 min-long videos were shortened into 35 s in length. Due to the conversion from AVI to MP4 format to reduce the size of the files, the video have lower quality compared to the originals.

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