UNRAVELING NEUROPROTECTIVE AND NEURODEGENERATIVE SIGNALS IN NEURODEGENERATION

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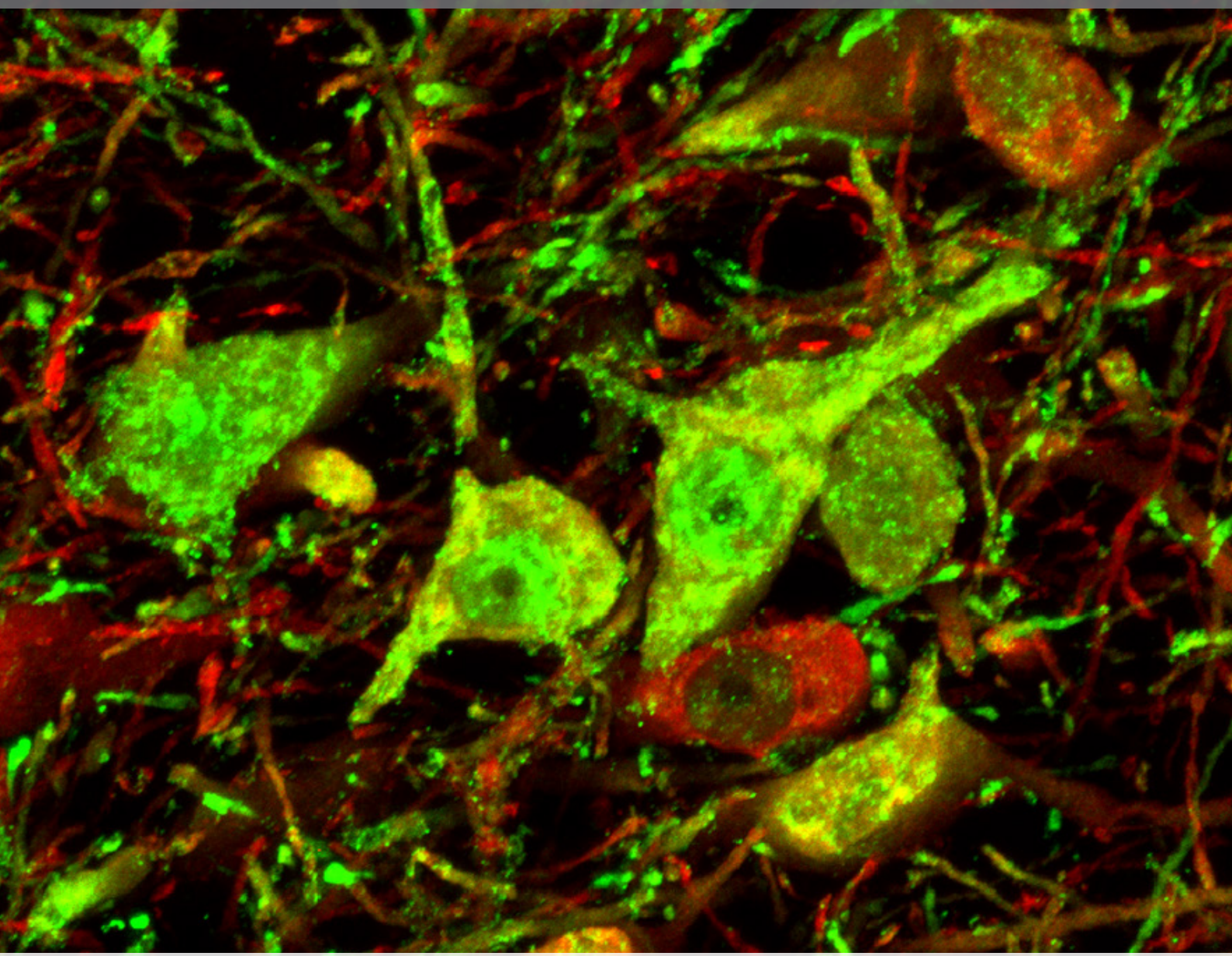

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UNRAVELING NEUROPROTECTIVE AND NEURODEGENERATIVE SIGNALS IN NEURODEGENERATION

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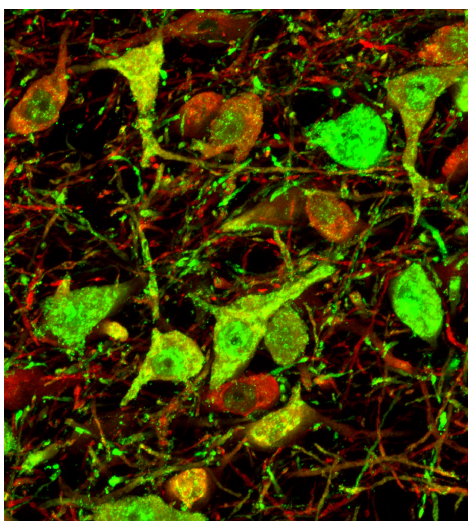

Alpha synuclein aggregation within nigrostriatal neurons. Adeno-associated virus expressing human alpha synuclein was used to transduce neurons of the substantia nigra pars compacta. Image shows alpha synuclein aggregates (green) within tyrosine hydroxylase immunoreactive (red) nigrostriatal dopamine neurons. Image courtesy of Matthew Benskey and Fredric Manfredsson.

Proteinopathy is a collective term used to classified neurodegenerative diseases associated with the progressive accumulation of toxic protein molecules in specific brain regions. Alzheimer's disease (AD) is a well-known proteinopathy characterize by the accumulation of A peptides and tau proteins. The accumulation of these toxic molecules in the brain starts many years before any clinical presentation, being the onset in the range of 65 to 72 years of age. Therefore, age is considered a risk factor due, in part, to the loss of molecular competence to clear the brain from these toxic protein molecules. This fact, supported by years of research, demonstrates that brain cells activate a neuroprotective mechanism upon detection of a pathobiological signal that (if the detrimental conditions persist) precedes the activation of the neurodegeneration pathway. The progressive brain region specific neuronal death in neurodegenerative diseases also indicates that the transition from neuroprotection to neurodegeneration is individually triggered in cells of the affected brain region. Thus, molecular understanding of the pathophysiology associated with proteinopathies needs to take in consideration this intricate transition process, especially when genomics and proteomics approaches are used.

Research directed to understand the pathogenesis and pathophysiology of neurodegenerative diseases uncovered the putative role of different molecular mechanisms associated with neurodegeneration. Among the molecular mechanisms identified are proteolysis, epigenetics,

microRNA, transcriptional regulation, innate and adaptive immune system, phagocytosis and autophagocytosis, exo/endocytosis, unfolded protein response, cytoskeleton defects, unregulated signaling molecules (i.e. kinases and phosphatases), trafficking molecules, cell cycle, neurogenesis/neurodevelopment, among others. Interestingly, all these molecular mechanisms have been identified through the analysis of tissue from animal models or human post-mortem pathologically confirmed cases, but their specific role in neurodegeneration is still unclear. Thus, it is plausible to consider that all these pathways play a role at a particular phase of the neurodegeneration process or, simply, are drive by the agonal state of the tissue examined. Hence, an important conundrum that researchers face today is the use of heterogeneous brain tissue samples in the quest to identify biomarkers associated with the pathogenesis or pathophysiology of neurodegenerative diseases.

At this junction of the neurodegeneration field, this research topic aim to critically assess the current literature on molecular mechanisms associated with neurodegeneration and the approaches used to dissect their putative pathophysiological role. The studies could include the interplay between neuroprotective and neurodegenerative signals in neurodegeneration, dissecting the molecular role of identified biomarkers, bioinformatics tools that facilitate data mining, dissecting pathways or molecular mechanisms, stages of protein aggregation (oligomers vs tangles; who did it?), aging brain and brain fitness (A natural selection process), adaptive protein response to environmental insults and cellular signals, expression profile associated with neurological disorders and health. Therefore, this Research Topic is expected to cover a wide range of subjects related to unravel the interplay between neuroprotective and neurodegenerative signals in neurodegeneration.

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EDITORIAL
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Editorial: Unraveling Neuroprotective and Neurodegenerative Signals in Neurodegeneration

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Keywords: neurodegeneration, neuroprotection, brain imaging, Alzheimer's disease, Parkinson's disease

The Editorial on the Research Topic

Unraveling Neuroprotective and Neurodegenerative Signals in Neurodegeneration

Our understanding of the etiology of human neurodegenerative disorders is limited by the necessity to infer an active sequence of events, often expressed over protracted periods of time, from a collection of still-life images that often represent the end of the sequence with no clear view of how we got there. Adding to the collective confusion, it often is not clear whether our measures of nervous system structure and function at a given point in time represent active contributors to degeneration, the tombstones reflecting consequences of degeneration, active attempts to protect the nervous system, products of failed protection, or unrelated events. As a result, we rely on observations and manipulations of fragments of the process in experimental models and human samples, pieced together to create versions of what is possible. In this collection of articles, the iterative process is on full display, with a focus on Alzheimer's disease, Parkinson's disease, and related disorders, and reports ranging from tools to mechanisms to treatment.

ALZHEIMER'S DISEASE AND RELATED DISORDERS

For decades, the relative contributions and timing of accumulation of β-amyloid peptides (senile plaques) and oligomerization of the microtubule associated protein tau (neurofibrillary tangles) has been an open question in the etiology of Alzheimer's disease (AD). In this collection, Khan and Bloom review evidence suggesting that tau plays a central pathological role in AD as the mediator of β-amyloid neuronal toxicity. The authors discuss reports indicating that accumulation of β-amyloid oligomers affects tau's biological function, promoting microtubule instability, axonal transport defects, aberrant activation of kinases and synaptotoxicity. The authors argue that tau plays a central role in a "pathogenic signaling nexus that underlies AD."

Sabbagh and Dickey discuss the known structural features and posttranslational modifications that contribute to tau function and toxicity in AD and other tauopathies. The authors review evidence indicating that posttranslational modifications and the function of chaperones influence tau structural dynamics, transitioning from its physiological conformation to pathological structures that lead to neurodegeneration.

The structural dynamics of tau is of great interest as a means to differentiate between normal and pathological tau. The contribution of Brelstaff et al. describes the use of pentameric formyl thiophene acetic acid (pFTAA), an amyloid specific fluorescent dye, to detect fibrillary tau in living neurons. The authors demonstrate that pFTAA can be used as a tool to assess the efficacy of compounds that block the accumulation of pathological tau and/or tau-mediated neurodegeneration in cultured neurons.

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While, pFTAA specificity for amyloid proteins is of significant value in cell models, it will not distinguish between pathological β-amyloid and tau proteins in AD brain, limiting applications to *in vivo* imaging. Shimojo et al. review the advances in PET and fluorescence imaging as tools for the diagnosis of AD. The authors discuss the advantages of these techniques for detecting and dissecting disease progression in living organisms. The authors also discuss the limitations and pitfalls of PET and fluorescence imaging, providing a technical framework to improve the available technology.

Despite progress in the understanding of structural dynamics and detection of β-amyloid and tau in the context of the disease state, it remains unclear if the observed pathological structures are toxic or neuroprotective. Both pathological hallmarks also have been detected in cognitively normal individuals, suggesting that compensatory plasticity could mask the undergoing pathological process and, avert clinical presentation. Here, Avila et al. propose an alternate neuronal axis to explain how cognitive capacity is maintained in asymptomatic individuals with plaques and tangles. The authors argue that activation of the "default mode network (DMN)" bypasses the role of the AD-damaged entorhinal cortex, preserving function.

However, the progression of pathological processes could lead to synaptotoxicity, affecting the compensatory role of the DMN and contributing to clinical presentation. In this context, the role of hyperactivation of NMDA receptors and increased glutamate in AD is reviewed by Lewerenz and Maher. The authors extensively discuss evidence supporting the role of glutamate toxicity in neurodegeneration.

Study of other pathophysiologies associated with AD, in addition to β-amyloid and tau, hold potential to lead to development of tools that contribute to the understanding, diagnosis and treatment of the disease. For example, the contribution by Vega reviews published work that links the novel amyloid EFhd2 protein to AD and other neurodegenerative diseases. EFhd2 is a calcium binding protein which exhibits altered expression in AD and is found to co-aggregate with tau. Weinberg et al. present evidence that specific microRNAs may provide neuroprotection from β-amyloid toxicity through regulation of translation of specific RNA transcripts. In this study, they focus on sirtuin 1 and its neuroprotective role in β-amyloid toxicity. Thus, the understanding of alternate molecular mechanisms could contribute to unravel the pathophysiology of AD and related disorders.

PARKINSON'S DISEASE AND RELATED DISORDERS

Like AD, Parkinson's disease (PD) is a multi-factorial syndrome that likely arrives at its clinical phenotype via a variety of combinations of risk factors and molecular pathways. One consistent feature of post-mortem PD brain is evidence of inflammation. Whether neuroinflammation contributes to PD neurodegeneration as cause, effect, or both, remains a topic of debate. Here, Walker et al. present findings from a quantitative multiplex protein analysis of 160 molecules, including those associated with inflammation, in substantia nigra (SN) and striatum of neuropathological confirmed cases of PD, incidental Lewy body disease (ILBD) and controls. The patterns of bidirectional changes in inflammation and growth factor proteins differ in SN and striatum, including novel changes in markers suggestive of reduced vascular integrity.

The accumulation of cytoplasmic protein aggregates containing alpha-synuclein (α-syn) in affected neurons is a neuropathological hallmark of PD. Delenclos et al. used a protein fragment complementation assay to monitor α-syn aggregates *in vivo*, providing a suitable model for therapeutic testing. However, whether α-syn aggregation elicits a toxic effect, neuroprotective mechanism, or both is debatable. While the association of α-syn with genetic forms of PD has been interpreted as disease-related toxic-gain-of-function, it is conceivable that sequestering the protein in aggregates results in toxic-loss-of-function. The contribution by Collier et al. presents new evidence from nonhuman primates consistent with the view that knock-down of endogenous α-syn in the nigrostriatal system faithfully reproduces the pattern of degeneration observed in PD.

Finally, the contribution of Lazzara and Kim reviews evidence addressing the neuroprotective potential of lithium for PD, AD and other neurodegenerative disorders. With the advent of drug repurposing as a strategy for a quicker path to clinical translation, many considered "dirty drugs," due to side-effect profile and non-specificity for a single target, as the compounds best suited to treatment of complex, multi-factorial diseases. For the example of lithium, effects favoring reduced oxidative stress, reduced inflammation, inhibition of apoptosis, increased authophagy and stimulation of neurotrophic factors all address interacting systems implicated in neurodegenerative disease. Perhaps, for complex syndromes, drugs with highly focused targets may not be the best approach to treatment. The alternate approach of "dirty drugs" for complex diseases may hold significant value.

To make progress in our understanding of the complex etiology of AD, PD, and related disorders, it is clear that an appreciation of the web of interactions among multiple molecular pathways including neuroinflammation, energy metabolism, proteostasis and others will be critical to unraveling the mixed contributions of neuroprotective and neurodegenerative signals, supporting therapeutic efforts to harness one and limit the other.

AUTHOR CONTRIBUTIONS

IV and TC reviewed the submissions and wrote the editorial.

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MINI REVIEW
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Tau: The Center of a Signaling Nexus in Alzheimer's Disease

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Tau is a microtubule-associated protein whose misfolding, hyper-phosphorylation, loss of normal function and toxic gain of function are linked to several neurodegenerative disorders, including Alzheimer's disease (AD). This review discusses the role of tau in amyloid-β (Aβ) induced toxicity in AD. The consequences of tau dysfunction, starting from the axon and concluding at somadendritic compartments, will be highlighted.

Keywords: Alzheimer's disease (AD), Amyloid-β oligomers (AβOs), neurofibrillary tangles (NFTs), tau phosphorylation, synaptic dysfunction

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder that causes an insidious decline in cognitive function. The greatest risk factor for AD is age and the chances of developing the disease increases two-fold every 5 years after age 65. As the population continues to live longer, the toll that AD inflicts on healthcare costs for affected individuals will continue to rise. Thus, there is an urgent need to improve our understanding and therapeutic treatment of AD.

One of the barriers preventing the expeditious treatment of AD is our inability to detect at-risk individuals early enough for effective intervention. A diagnosis of AD can only be confirmed at autopsy following the detection of extracellular plaques containing Aβ peptides and intracellular neurofibrillary tangles composed of the neuron-enriched, microtubule-associated protein (MAP), tau. Since mutations in the amyloid precursor protein (APP) gene are linked to AD onset, and plaques often precede the formation of tau tangles, the amyloid cascade hypothesis, which states that Aβ initiates hyper-phosphorylation and aggregation of tau, and overall AD pathogenesis, was developed (Hardy and Higgins, 1992).

Although, it has been popular to focus primarily on Aβ in AD, tau plays an equally important role in AD pathogenesis. For instance, individuals with substantial plaque loads but no evident tau pathology can lead healthy lives with no symptoms of cognitive decline (Sperling et al., 2009). Additionally, research has long supported the notion that tau is the major component of neurofibrillary tangles that positively and robustly correlate with AD severity Grundke-Iqbal et al., 1986; Nukina and Ihara, 1986; Kondo et al., 1988; Kosik et al., 1988; Braak and Braak, 1991; Götz et al., 2001; Lewis et al., 2001). Tau depletion also protects against Aβ-associated neuron death (Leroy et al., 2012; Nussbaum et al., 2012). Thus, it is generally accepted that tau dysfunction, manifested as hyper-phosphorylation and aggregation, are major proximal causes of neuron loss in AD (Bloom, 2014). This review emphasizes the role of tau as a central player in a pathogenic signaling nexus that underlies AD.

BACKGROUND ON TAU

Tau was originally identified as a predominant MAP present in mammalian brain (Weingarten et al., 1975). In the CNS, alternative splicing leads to the formation of six isoforms (Goedert et al., 1989a). Variation among the six isoforms lies in the number of exons expressed at the N-terminus

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(0, 1, or 2) and microtubule binding repeat domains near the C-terminal end of the protein (3 or 4). During early stages of mammalian development, the 3 repeat domain tau isoforms predominate and it is heavily phosphorylated (Goedert et al., 1989b). A proposed function for elevated tau phosphorlyation during development is that it contributes to synaptic plasticity (Frandemiche et al., 2014). As the brain ages, however, phosphorylation of tau decreases and the presence of 4 repeat to 3 repeat tau reaches an approximate 1:1 ratio (Goedert et al., 1989a,b; Himmler et al., 1989; Kosik et al., 1989; Goedert and Jakes, 1990; Hong et al., 1998).

TAU FUNCTION

The first antibody generated against tau helped determine that its expression is predominantly axonal (Binder et al., 1985). Subsequent experiments using the same antibody identified tau, and what would be later shown as hyper-phosphorylated tau, as the primary component of neurofibrillary tangles (Grundke-Iqbal et al., 1986; Wood et al., 1986; Kondo et al., 1988; Kosik et al., 1988). The putative functions of tau include stimulation of tubulin polymerization, stabilization of microtubules (Witman et al., 1976), and a "speed bump" property whereby tau constrains the fast transport of organelles along microtubules (Stamer et al., 2002; Dixit et al., 2008). Tau is most concentrated in the distal portions of axons, where it helps regulate microtubule dynamics.

Methods designed to knockdown or knockout (KO) tau have identified additional roles for the protein in the CNS. Early work on tau depletion using antisense nucleotides suggested that tau is required for proper axon development and neuronal polarity (Caceres and Kosik, 1990). However, follow-up studies *in vivo* using tau KO mice were less convincing (Harada et al., 1994), and subsequent work in several tau KO strains reported no change in reproduction, physical appearance, or behavior (Dawson et al., 2001; Tucker et al., 2001). Nevertheless, during mouse postnatal development reduction of tau alters the migration and morphology of neurons, and also intracellular mitochondrial transport (Sapir et al., 2012). In *Drosophila*, global tau KO is developmentally lethal and targeted KO in neurons or the eye results in progressive neurodegeneration (Bolkan and Kretzschmar, 2014). However the lethality and neurotoxicity of tau KO in *Drosophila* might reflect a general paucity of microtubule-associated proteins in flies.

Much of the original work using tau KO mice extends to 6 months of age, leaving the long-term effects of tau depletion uncertain. Recent work has attempted to investigate the effects of prolonged tau ablation, with very inconsistent results. Work from one group demonstrated that aged tau KO mice (older than 6 months) develop iron accumulation, motor deficits, Parkinsonism with dementia, significant brain atrophy, and impaired Y-maze performance (Lei et al., 2012, 2014). Similarly, others have reported motor deficits in aged tau KO mice (Ma et al., 2014; Lopes et al., 2016), or decreased brain weight and mild hyperactivity in aged tau KO homozygotes (Li et al., 2014). Evidence of impaired learning, as determined by Barnes maze performance, was also recently reported (Regan et al., 2015).

However, other studies reported no difference in iron accumulation (Li et al., 2014), parkinsonian abnormalities in dopamine levels (Li et al., 2014), dopamine-related motor deficits (Morris et al., 2013; Ahmed et al., 2014; Li et al., 2014), or impaired Y-maze performance in aged tau KO mice (Li et al., 2014). Furthermore, results demonstrated that Morris water maze performance is either improved or unaffected (Morris et al., 2013; Ahmed et al., 2014) which presents conflict to other data (Ma et al., 2014). Likewise, the literature reports inconsistent fear conditioning results in aged tau KOs. One study showed no difference in contextual fear conditioning (Li et al., 2014) while another reported impaired cue and contextual fear conditioning (Ahmed et al., 2014). The literature also reports contrasting data on long-term depression (LTD), with severe impairments in long-term potentiation and no effect on LTD shown by one group (Ahmed et al., 2014), and deficits in LTD demonstrated by others (Kimura et al., 2013; Regan et al., 2015).

These discrepancies might reflect strain-dependent phenotypic differences among the various tau KO mouse lines. Hence, as more studies are completed, and as methods become more standardized, we will be better able to resolve what consequences, or lack thereof, arise with prolonged tau ablation. Understanding the effects of prolonged tau ablation is not only important to elucidate tau function, but is also necessary to ascertain the best therapeutic strategy to employ when treating tau-related neurodegenerative disorders.

TAU IMPAIRMENT AT THE AXON IN AD

Among tau post-translational modifications its phosphorylation is the best characterized. There are 80 serine or threonine and 5 tyrosine sites at which tau can theoretically be phosphorylated. Once tau becomes aberrantly phosphorylated its functional capacity to stabilize microtubules is reduced, contributing to axon deficits in AD. Furthermore, axonal swellings, or varicosities, that are frequently observed during early-stage AD, are hypothesized to reflect tau-associated defects in transporting cargo-containing vesicles (Krstic and Knuesel, 2012). Expression of tau phosphomimics lends supporting data to this theory by demonstrating that sustained tau (psuedo)phosphorylation impairs its axonal transport and degradation (Rodríguez-Martín et al., 2013). Other tau post-translational modifications, such as *cis* or *trans* isomerization, also affect the ability of tau to maintain microtubule assembly at axons (Nakamura et al., 2012).

In support of the amyloid cascade hypothesis, Aβ oligomers (AβOs) are reported to impair the ability for tau to stabilize microtubules. Our lab previously reported that pre-fibrillar Aβ stimulates tau-dependent disassembly of microtubules (King et al., 2006). It was likewise reported that Aβ treatement promoted tau hyperphosphorlyation, microtubule-related deficits, and organelle dysfunction (Silva et al., 2011). Similarly, tau is implicated to trafficking deficits in cell surface receptors, particularly those that bind glutamate, following AβO treatment (Li et al., 2009; Hoover et al., 2010). Thus, under atypical conditions Aβ and tau interact to cause significant microtubule and transport dysfunction.

SOMADENDRITIC TAU

A conspicuous property of tau in AD is its ectopic mislocalization to somatodendritic compartments (Götz et al., 1995; Hoover et al., 2010). It is hypothesized that an Aβ and tau interaction causes the synaptotoxicity commonly observed in AD. For instance, double transgenic mice that overexpress the human forms of APP (containing the Swedish and London mutations, for example) and WT tau acquire significant dendritic spine loss with age (Chabrier et al., 2012, 2014). Mechanistically, Aβ exposure promotes complex formation between the non-receptor tyrosine kinase, fyn, and PSD95, in a tau-dependent manner, to mediate aberrant activation of the NMDA receptor in dendritic spines (Ittner et al., 2010). Some synapse dysfunction following Aβ exposure also requires formation of a complex containing fyn and the cellular prion protein, and fyn-dependent phosphorylation of tau (Larson et al., 2012). Tau phosphorylation by other kinases, such as AMP-activated kinase (AMPK) is further necessary for AβO synaptotoxicity (Mairet-Coello et al., 2013). In addition to the requirement of tau for the AβO-initiated activation of the NMDA receptor, recent work has shown that AβOs elicit phospho-tau infiltration to dendrites and AMPA receptor dysfunction (Miller et al., 2014).

Importantly, cell-cycle re-entry (CCR) by post-mitotic neurons preludes much of the massive neuron death that occurs in AD (Arendt, 2012) and is also associated with impaired synaptic plasticity (Arendt and Brückner, 2007). AβO treatment of primary neurons results in activation of the kinases CaMKII, PKA, and fyn, which induce ectopic CCR by a mechanism that relies on site-specific tau phosphorylation catalyzed by those kinases (Seward et al., 2013). Neuronal CCR is also present in hAPPJ20 AD model mice at 6 months, but absent in comparable tau KO littermates (Seward et al., 2013).

NUCLEAR TAU

Although, it is predominately expressed in the axon, tau can also be found in other cellular compartments, including the nucleus, under normal physiological conditions. Its nuclear role is unresolved, but one theory proposes a protective role against DNA damage for nuclear tau, depending on its phosphorylation state (Sultan et al., 2011). Interestingly, tau was recently shown to induce chromatin relaxation, which subsequently leads to DNA damage and global changes in transcription (Frost et al., 2014).

CONCLUSIONS

The current understanding of tau identifies its hyper-phosphorylation and subsequent mislocalization as seminal steps for AD pathogenesis. Once it becomes appropriately phosphorylated, tau loses its affinity for microtubules and becomes potently cytotoxic (Alonso et al., 2010). Over the course of AD, hyper-phosphorylated tau ectopically enters the somadendritic compartment, where in conjunction with AβOs, it promotes excitotoxicity at synapses. Additionally, tau phosphorylation modulates DNA integrity under cellular stress, and global changes in protein transcripts. Inhibiting aberrant tau phosphorylation may prove useful in the treatment of AD. However, targeting tau phosphorylation will require a greater understanding on how site-specific tau phosphorylation alters its function.

AUTHOR CONTRIBUTIONS

SK wrote an advanced draft of this mini-review, which was then edited by GB.

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The Metamorphic Nature of the Tau Protein: Dynamic Flexibility Comes at a Cost

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Accumulation of the microtubule associated protein tau occurs in several neurodegenerative diseases including Alzheimer's disease (AD). The tau protein is intrinsically disordered, giving it unique structural properties that can be dynamically altered by post-translational modifications such as phosphorylation and cleavage. Over the last decade, technological advances in nuclear magnetic resonance (NMR) spectroscopy and structural modeling have permitted more in-depth insights into the nature of tau. These studies have helped elucidate how metamorphism of tau makes it ideally suited for dynamic microtubule regulation, but how it also facilitates tau self-assembly, oligomerization, and neurotoxicity. This review will focus on how the distinct structure of tau governs its function, accumulation, and toxicity as well as how other cellular factors such as molecular chaperones control these processes.

Keywords: tau, microtubules, structure, neurodegenerative diseases, NMR

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INTRODUCTION

The microtubule-associated protein tau accumulates in a number of neurodegenerative diseases termed tauopathies, including Alzheimer's disease (AD), progressive supranuclear palsy (PSP), frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), and several others. Many of these diseases are caused by missense mutations in the *MAPT* gene coding for tau, while several others are caused by environmental factors: chronic traumatic encephalopathy (CTE) is a sequelae of traumatic brain injury (TBI), postencephalitic parkinsonism (PEP) results from infection, while the cause of age-related AD is unknown. The sheer diversity of the factors that ultimately lead to tauopathy and neurodegeneration is quite remarkable and suggestive of a broad neurological reaction in response to a variety of insults. Because the disordered structure of tau lends itself to heavy post-translational modifications, signaling events caused by these environmental factors can have a multitude of effects on tau structural dynamics.

Tau aggregates into ß-sheet fibrils in tauopathies, leading to the formation of neurofibrillary tangles (NFTs) and subsequent cell death. Although precisely what triggers tau assembly into these ß-sheet structures in the brain is unclear, it is known that a number of post-translational modifications can regulate this process, including phosphorylation, acetylation, cleavage, ubiquitination, and misfolding. This mini-review will reveal what has been recently discovered about tau structure and how distinct cellular mechanisms such as molecular chaperones can control tau folding to promote or block its toxic assembly [for more comprehensive reviews on tau structure and pathology, we direct readers to Kolarova et al. (2012) and Wang and Mandelkow (2016)].

OVERVIEW OF TAU STRUCTURE

Tau is an intrinsically disordered protein with a strong propensity for self-aggregation into ß-sheet structures which compose the core of NFTs. Several factors can enhance the propensity of tau to aggregate, including mutations in the *MAPT* gene and post-translational modifications such as phosphorylation and acetylation (Goedert et al., 1988; Hutton et al., 1998; Spillantini et al., 1998; Von Bergen et al., 2001; Augustinack et al., 2002; Cohen et al., 2011; Mandelkow and Mandelkow, 2012; Cook et al., 2014; Min et al., 2015). Another post-translational modification to tau, ubiquitination, has been shown to be required for tau ß-sheet assembly *in vivo*; but when ubiquitination was blocked, soluble tau intermediates, typically termed oligomers, developed in the brain that were highly toxic (Dickey et al., 2006b). This idea of soluble tau oligomers being the major toxic species in the brain has gained recent support because of key studies that have emerged over the past decade (Santacruz et al., 2005; Oddo et al., 2006; Spires et al., 2006; O'leary et al., 2010). Using tools that have been developed to specifically investigate oligomeric tau species, it has been demonstrated that these structures were responsible for much of the neurotoxicity due to tau accumulation (Lasagna-Reeves et al., 2011; Blair et al., 2013). Moreover, several studies have shown that reducing soluble tau mitigates neuronal loss and functional deficits in tau transgenic mice, despite the lack of change in ß-sheet aggregates (Santacruz et al., 2005; Oddo et al., 2006; Spires et al., 2006; O'leary et al., 2010). Thus, it is clear that elucidating the processes governing tau oligomerization and aggregation is critical for not only understanding tau pathogenesis but also for developing tau-based therapeutics.

The structure of tau has been extensively analyzed in an effort to gain insight into the mechanisms of its aggregation and toxicity. Initial approaches using fluorescence resonance energy transfer (FRET) theorized a "paper clip" model of tau wherein the N and C termini transiently fold onto the microtubule-binding repeat domains as well as each other (Jeganathan et al., 2006). The tau protein contains either 3 or 4 microtubule-binding domain repeats (3R or 4R) which have been shown to be essential for both the ability of tau to bind to microtubules and its assembly into paired helical filaments (PHFs). Specifically, stretches of amino acid residues within these repeat domains, which include the hexapeptide motifs VQIIYK and VQIVYK, are capable of serving as seeds for aggregation (Von Bergen et al., 2001; Mukrasch et al., 2005). In fact, these motifs are uniquely critical for the intermolecular contact between tau molecules which gives rise to initial oligomer formation and eventual self-aggregation (Peterson et al., 2008). Moreover, the hexapeptide motifs have intramolecular contacts with proline-rich regions on tau, suggesting proline-directed phosphorylation may alter tau structure, affecting its aggregation propensity (Mukrasch et al., 2009). More recent work has corroborated this idea, demonstrating that intramolecular interactions in the repeat domains promote aggregation, whereas the more unstructured N terminus folds longer stretches which prevent aggregation (Wegmann et al., 2011). Disulfide cross-linking of tau can also play a substantial role in its aggregation propensity: 3R tau contains only one cysteine, oxidation of which permits cross-linking, oligomerization, and aggregation. Conversely, 4R tau contains two cysteines which readily form intramolecular contacts and can suppress cross-linking and aggregation (Schweers et al., 1995; Barghorn and Mandelkow, 2002). The consequences of these intra- and intermolecular tau interactions *in vivo* likely depend on a multitude of factors such as phosphorylation state, proteostatic burden, and binding to microtubules.

TAU-MICROTUBULE INTERACTIONS INFLUENCE TAU STRUCTURE, ASSEMBLY, AND TOXICITY

It is likely not a coincidence that the regions within tau necessary for binding to microtubules are also important for ß-sheet assembly and aggregation. The first discovered function of tau was its ability to promote microtubule assembly (Weingarten et al., 1975) and subsequent studies have only reinforced these findings, showing that tau stabilizes microtubules with a high affinity through interactions within its microtubule-binding repeats (Goode et al., 1997; Sillen et al., 2007). The mechanism by which tau stabilizes microtubules was only very recently established using nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry. Zweckstetter and colleagues revealed that tau binds to microtubules at the interface between tubulin heterodimers, using small groups of residues that have previously been shown to be critical for tau ß-sheet assembly and aggregation (Kadavath et al., 2015). The authors highlight the implication that competition may arise between the physiological interaction of tau with microtubules and tau misfolding and aggregation. Phosphorylation of tau can prevent its binding to microtubules, which could potentially give rise to a feed-forward loop under pathological conditions. In this scenario, hyperphosphorylation of tau, characterized by abnormal tau conformations, and self-assembly into PHFs, causes gain of toxicity as well as loss of function if it is unable to stabilize microtubules. Thus, loss of axonal stability coupled with tau self-assembly and aggregation contribute to neurodegeneration in tauopathies.

POST-TRANSLATIONAL MODIFICATIONS INFLUENCE TAU STRUCTURE

As discussed above, tau structure and conformation can be altered by several primary post-translational factors, but the best characterized of these are phosphorylation and proteolytic cleavage. Tau phosphorylation has a dramatic effect on the structure and function of tau, potentially obscuring the microtubule binding sites within the repeat domains. However, not all phospho-epitopes behave the same. For example, when tau is phosphorylated at the AT8 (S202/T205) and AT180 (T231) sites, it loses the ability to drive microtubule assembly, but can still associate with preformed microtubules (Amniai et al., 2009). In contrast, MARK-dependent phosphorylation of tau at S262 induces conformational changes around this phosphorylation site, altering tau structure and attenuating

microtubule binding (Fischer et al., 2009). A recent investigation of tau phosphorylated at T231 revealed that this residue can selectively form a salt bridge with R230, which competes with the bridge necessary for tau-microtubule interaction (Schwalbe et al., 2015). Tau phosphorylation not only affects local conformations but has also been shown to weaken the transient long-range interactions common to intrinsically disordered proteins (Bibow et al., 2011; Sibille et al., 2012), potentially making tau more susceptible to aggregation. These studies and others have made it clear that phosphorylation of tau not only influences the aggregation potential of the protein, but can also have more immediate consequences on microtubule binding and folding.

Tau can also be modified by proteolytic cleavage events, altering its structure, functional capacity, and self-association. Tau can be cleaved by caspase-3, most notably at D421, or by the calcium-dependent protease calpain, which leads to a 17 kDa tau fragment (Hanger and Wray, 2010). Cleavage by either of these proteases produces truncated tau forms which are more neurotoxic than full-length tau (Chung et al., 2001; Park and Ferreira, 2005). Cleaved tau has been found in the brains of individuals with AD and other tauopathies, indicating it may play a role in disease pathogenesis (García-Sierra et al., 2008). Although most cleaved tau species that have been identified are C-terminal truncations, a recent report showed that an N-terminal truncation at Q124, identified from human brains via proteomics, actually enhanced microtubule stability (Derisbourg et al., 2015).

Evidence suggests that tau phosphorylation may also influence cleavage of tau; phosphorylation at S422 is commonly observed in tauopathy brains and has been shown to inhibit caspase cleavage of tau (Rissman et al., 2004; Guillozet-Bongaarts et al., 2006). Furthermore, tau phosphorylation and cleavage differentially affect the ability of tau to interact with microtubules (Drewes et al., 1997; Ding et al., 2006), suggesting tau may adopt an alternative structure that promotes aggregation following cleavage. It was also recently discovered that tau can be cleaved by asparagine endopeptidase, a lysosomal cysteine protease, which (1) abrogates the ability of tau to stabilize microtubules, (2) induces aggregation, and (3) enhances neurodegeneration (Zhang et al., 2014). Collectively, these studies suggest that post-translational modifications such as phosphorylation and cleavage can affect the ability of tau to interact with microtubules and make it more susceptible to self-assembly and aggregation. Further work may begin to develop a clearer picture of how the interplay between these modifications influences the propensity of tau to form ß-sheet structures and eventual irreversible aggregates. Other factors such as molecular chaperones also play an important role in determining tau fate both in concert with and independent of post-translational modifications.

CHAPERONES CONTROL TAU STRUCTURE AND ASSEMBLY

Molecular chaperones such as Hsp70 and Hsp90 have been long recognized as vital mediators of protein folding and structure (Young et al., 2004). These proteins help maintain proteostasis by attempting to refold misfolded proteins or shuttling them to the proteasome if refolding is unsuccessful. A seminal paper by Hu and colleagues showed that Hsp70 and Hsp90 can each directly interact with tau, promoting tau-microtubule binding while decreasing tau phosphorylation and aggregation (Dou et al., 2003). Inhibition of Hsp70 or Hsp90 with siRNA or small molecules reduces intracellular tau levels in cells and the brain (Dickey et al., 2005, 2006a, 2007; Jinwal et al., 2009). The heat shock response typically produced by this inhibition increases levels of Hsp72, an inducible Hsp70 isoform, leading to enhanced tau turnover (Dickey et al., 2006a). In fact, it is known that Hsp72 and Hsc70, the predominant, constitutively active isoform of the Hsp70 family, have opposing effects on tau clearance, with Hsc70 preserving tau in the cell and Hsp72 promoting tau degradation (Jinwal et al., 2013).

Tau phosphorylation seems to be an important determinant of chaperone activity. The carboxyl terminus of the Hsc70-interacting protein (CHIP) is a major co-chaperone of Hsp90 and Hsc70. Tau phosphorylation is required for the Hsc70-CHIP complex to shuttle tau to the proteasome for ubiquitination, an event that mitigates cell death induced by tau hyperphosphorylation (Shimura et al., 2004). Similarly, the Hsp90-CHIP complex selectively degrades phosphorylated tau species (Dickey et al., 2007). However, these processes appear to be phospho-epitope specific; although tau phosphorylated at S202/T205 and S396/S404 could be degraded by Hsp induction, tau phosphorylated at S262/S356 was not affected (Dickey et al., 2006a). Interestingly, deletion of CHIP promotes accumulation of phospho-tau without promoting its aggregation (Dickey et al., 2006b). Thus, distinct chaperones clearly recognize structural features of tau to govern the degradation and assembly of tau. This point is illustrated by data demonstrating that Hsp70 and Hsp90 actually compete for binding to shared residues on tau, leading to differential effects on tau clearance; Hsp70 stabilizes tau levels while Hsp90 facilitates tau removal (Thompson et al., 2012). Hsp70 and Hsp90 also have opposing effects on tau assembly and structure *in vitro*. While Hsp70 prevents tau aggregation (Patterson et al., 2011), Hsp90 stimulates it (Blair et al., 2013). Because the dynamic chaperone landscape changes with age and especially AD (Yoo et al., 2001; Jinwal et al., 2010; Blair et al., 2013), it is likely that dysfunction in the ability of Hsp70 or Hsp90 to regulate tau contributes to its misfolding and aggregation.

Although Hsp90 is one of the more well-characterized chaperones, it was only recently discovered how it interfaces with a substrate (Karagoz et al., 2014). In fact, tau was the model substrate used to determine this. Through this work, it was found that Hsp90 interacts with a long stretch of the tau microtubule binding repeats, essentially stretching tau out along the N-terminal and middle domains of Hsp90 (Karagoz et al., 2014). Although tau is intrinsically disordered, it adopts conformations that facilitate its pathogenicity, a property that is referred to as "meta-stability" and Hsp90 may constrain this flexibility to some extent. But in doing so, Hsp90 can facilitate tau ß-sheet assembly. Of course Hsp90 does not work alone within the cell. The dynamic network of other major chaperones and co-chaperones can influence several aspects of tau biology.

For example, the Hsp90 co-chaperones FKBP51 and FKBP52 have opposing effects on tau assembly, oligomerization, and aggregation (Blair et al., 2013; Giustiniani et al., 2015). Thus, a number of cellular factors coordinate to regulate tau structure and proteostasis; an imbalance in these factors, as occurs with aging, could shift regulation of tau to a more pathogenic state.

CONCLUSIONS

To describe proteins like tau as intrinsically disordered may be somewhat misleading. Perhaps proteins with unstructured domains should rather be considered dynamically flexible, providing a way for the cellular proteome to adapt to distinct environments and needs. These metamorphic proteins come with a cost however, in that some structures can promote proteotoxicity. The factors that promote these toxic structures are critical for our understanding of tauopathies and could each be viable therapeutic targets. Moreover, if these transient structures can be identified, it could usher in a new era of structure-based drug discovery for tauopathies and other diseases associated with metamorphic proteins. With the advent of improved methods of 3D electron microscopy and high resolution mass spectrometry, examining these large, aggregate structures is now feasible and will certainly yield key insights for therapeutic development in these disorders.

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JS and CD each contributed to the writing of the manuscript.

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frontiers in Neuroscience

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The fluorescent pentameric oligothiophene pFTAA identifies filamentous tau in live neurons cultured from adult P301S tau mice

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Identification of fluorescent dyes that label the filamentous protein aggregates characteristic of neurodegenerative disease, such as β-amyloid and tau in Alzheimer's disease, in a live cell culture system has previously been a major hurdle. Here we show that pentameric formyl thiophene acetic acid (pFTAA) fulfills this function in living neurons cultured from adult P301S tau transgenic mice. Injection of pFTAA into 5-month-old P301S tau mice detected cortical and DRG neurons immunoreactive for AT100, an antibody that identifies solely filamentous tau, or MC1, an antibody that identifies a conformational change in tau that is commensurate with neurofibrillary tangle formation in Alzheimer's disease brains. In fixed cultures of dorsal root ganglion (DRG) neurons, pFTAA binding, which also identified AT100 or MC1+ve neurons, followed a single, saturable binding curve with a half saturation constant of 0.14 μM, the first reported measurement of a binding affinity of a beta-sheet reactive dye to primary neurons harboring filamentous tau. Treatment with formic acid, which solubilizes filamentous tau, extracted pFTAA, and prevented the re-binding of pFTAA and MC1 without perturbing expression of soluble tau, detected using an anti-human tau (HT7) antibody. In live cultures, pFTAA only identified DRG neurons that, after fixation, were AT100/MC1+ve, confirming that these forms of tau pre-exist in live neurons. The utility of pFTAA to discriminate between living neurons containing filamentous tau from other neurons is demonstrated by showing that more pFTAA+ve neurons die than pFTAA-ve neurons over 25 days. Since pFTAA identifies fibrillar tau and other misfolded proteins in living neurons in culture and in animal models of several neurodegenerative diseases, as well as in human brains, it will have considerable application in sorting out disease mechanisms and in identifying disease-modifying drugs that will ultimately help establish the mechanisms of neurodegeneration in human neurodegenerative diseases.

Keywords: hyperphosphorylated tau, neurofibrillary tangles, transgenic P301S tau mouse, dorsal root ganglion neurons, Alzheimer's disease, frontotemporal dementia (FTD), fluorescent vital fibrillar tau dye

Introduction

Tau is a soluble microtubule-associated protein with dynamic phospho-epitopes that is expressed throughout the CNS and PNS (Cleveland et al., 1977; Binder et al., 1985). Its primary role is the stabilization of microtubules thereby facilitating axonal transport and maintaining proper neuronal morphology (Mandelkow and Mandelkow, 2012). However, insoluble hyperphosphorylated filamentous tau forms the neurofibrillary tangles (NFTs) and Pick bodies that are diagnostic hallmarks of numerous neurodegenerative diseases including Alzheimer's disease (AD), Pick's disease, frontotemporal dementia with Parkinsonism linked to chromosome 17, progressive supranuclear palsy and cortical basal degeneration (Spillantini and Goedert, 2013). Indeed, the *MAPT* (tau) gene haplotype H1/H1 is also associated with memory dysfunction in patients with Parkinson's disease (Winder-Rhodes et al., 2015) and acts as an independent genetic risk factor in pathologically proven PD (Charlesworth et al., 2012). Moreover, abnormal NFTs of tau were also found in the brains of Huntington's disease patients (Fernandez-Nogales et al., 2014; Vuono et al., 2015). The driving force behind tau aggregation is not known but familial mutations in the MAPT gene such as P301S can increase aggregation propensity by reducing tau binding to microtubules, and possibly by introducing a new phosphorylation site (Hong et al., 1998). Individuals affected by this mutation have an early to midlife age of onset and an aggressive disease progression (Bugiani et al., 1999; Sperfeld et al., 1999; Yasuda et al., 2000; Lossos et al., 2003).

Although both beta-amyloid and tau form abnormal filaments in AD, there is increasing evidence suggesting that tau is necessary for mediating the ultimate neurodegeneration (Pooler et al., 2013; Bloom, 2014). However, despite extensive research into the mechanisms of degeneration, it is still unclear how tau mediates its toxic effects neither in pure tauopathies, nor in the context of beta amyloid plaques in AD. There is a continued debate as to whether tau+ve NFTs induce cell death (Tomlinson et al., 1970; Gomez-Isla et al., 1997; Mocanu et al., 2008; Fatouros et al., 2012) or whether protein aggregates are benign (Kuchibhotla et al., 2014) or even protective (Morsch et al., 1999; Spires et al., 2006; Fox et al., 2011). Perhaps a "one hit" model, which predicts that abnormally misfolded tau is both necessary and sufficient for induction of toxicity, as was suggested to account for the progression of several neurodegenerative diseases (Clarke et al., 2000, modified by Clarke and Lumsden, 2005), needs to be replaced by a "two hit" model, where another event, which is not toxic *per se*, is required for tau to cause cell death. The second hit could come from a myriad of sources, which might also explain why it has been so difficult to demonstrate a clear single mechanism of cell death in AD, unlike the more defined cases of regulated cell death such as apoptosis during development (Dekkers and Barde, 2013; Kole et al., 2013), which fit a modified one hit model (Edwards and Tolkovsky, 1994), or parthanatos (Fatokun et al., 2014).

To study whether cell autonomous mechanisms lead to tau pathology and neurodegeneration, we turned to the transgenic P301S tau mouse model, which develops progressive hallmarks of tau pathology that culminate in neuronal cell death over 5 months. We showed previously that a subset of Dorsal Root Ganglion (DRG) neurons from transgenic human P301S tau mice develop progressive hallmarks of tau pathology *in vivo* and *in vitro* with the same time course as that of CNS neurons (Allen et al., 2002; Mellone et al., 2013). These neurons are unique in that they can be cultured from adult mice for months, enabling tau-dependent processes to be defined as tau evolves from a soluble to a filamentous form with a characteristic acquisition of different hyperphosphorylation and conformational patterns (Mellone et al., 2013). Nevertheless, despite the presence of sarkosyl-insoluble filamentous tau in DRG extracts and evidence for conformational changes associated with filamentous tau in cultured DRG neurons (Mellone et al., 2013), supported, for example, by staining with the conformation-specific antibody MC1 (Jicha et al., 1997, 1999), there has been no direct evidence that tau adopts these conformations in living DRG neurons. This is most likely due to the low intensity of specific signals elicited by beta-sheet-reactive dyes such as thioflavins (Allen et al., 2002) or FSB (Velasco et al., 2008) in the context of the large volumes of the proprioceptive and mechanoceptive neurons that express P301S tau in the transgenic model. The ability to visualize filamentous tau aggregates in specific cells within a heterogeneous culture would allow investigations of the cell autonomous processes leading to toxicity or protection.

Recently a new family of luminescent conjugated polythiophene dyes were described that detect various beta-sheet containing proteins. Some members of this family are capable of discriminating between different conformational strains of PrP^{Sc}, the prion disease associated aggregate form of normal prion protein PrP^{C}, that would be indistinguishable by immunohistochemistry alone (Sigurdson et al., 2007), while others can detect beta amyloid and tau filaments in post-mortem AD brains (Aslund et al., 2009). In particular, the pentameric formyl thiophene acetic acid (pFTAA) is a highly promising compound, as it can be used to distinguish different conformations of beta-sheet containing proteins due to specific conformation-dependent shifts in emission spectra depending on the nature of the protein (Klingstedt et al., 2013). Intravenous injection of pFTAA into APP23 mice was reported to produce no discernable toxic effects on body weight, whole blood cell counts, or histology of peripheral organs (Wegenast-Braun et al., 2012). In AD brains, pFTAA co-labeled neurons immunostained for AT8, an antibody that correlates with tau pathology (Aslund et al., 2009), but given that AT8 is not specific to insoluble filamentous tau aggregates, more complete characterization with markers of filamentous tau was lacking. The clarity of staining of neurons in a brain stem section from 6-month-old P301S tau transgenic mice (Klingstedt et al., 2013), a stage at which there is considerable accumulation of filamentous tau in cortical and motor neurons in the CNS and spinal cord, and the lack of apparent toxicity, prompted us to investigate whether we could use pFTAA to identify living DRG neurons that express filamentous tau. Here we show that pFTAA binds filamentous tau aggregates in DRG neurons of P301S tau mice by co-labeling with antibodies that detect aggregated/filamentous tau. Moreover, we show that both live and fixed cultured DRG neurons that are immunoreactive for these antibodies co-label

with pFTAA. Importantly, we show that we can follow the same fields of living pFTAA+ve neurons in long-term cultures and quantify their death, an observation that will allow us to underpin the mechanism of toxicity and identify drugs that may alleviate it.

Materials and Methods

Animals

Homozygous mice transgenic for P301S tau (Allen et al., 2002), or wild-type human 2N4R tau (Alz17) (Probst et al., 2000; Clavaguera et al., 2009), and C57BL/6S (C57BL/6 OlaHsd; Harlan) control mice were maintained as described previously (Mellone et al., 2013). All procedures were performed in accordance with the UK Home Office Regulations for the Care and Use of Laboratory Animals and the UK Animals (Scientific Procedures) Act 1986 and were approved by the Cambridge University local ethical committee. To label neurons *in vivo*, 150 nmole of pFTAA in 150 μl saline were injected intravenously via the tail vein. After 48 h, the mouse was perfused transcardially with PBS followed by 4% paraformaldehyde in PBS. Brains and DRGs were removed and treated as described below.

Cultures

DRG neurons were cultured on 13 mm coverslips or glass-bottom dishes (MatTek) coated with poly-D-lysine and laminin and maintained as described previously (Mellone et al., 2013). For live labeling experiments, neurons were cultured for 7 days in growth medium containing fluorodeoxyuridine (to remove non-neuronal cells and neurons that die due to injury during preparation), then washed and labeled in medium containing 3 μM pFTAA and 0.1 μg/ml propidium iodide (PI) for 30 min at room temperature. Medium containing PI was replaced every 3 days but no new pFTAA was added to ensure that no new pathological tau assemblies forming over time as the culture progressed were counted.

Immunocytochemistry

The brain and DRGs removed from P301S tau mice after perfusion were incubated overnight in 4% PFA, washed and stored in 30% sucrose in PBS (brain) or PBS containing 0.1% sodium azide (DRG). Brain was cut into 30 μm sections on a sliding microtome (Leica SM2000 R). Prior to antibody staining, brain sections or DRG were either immersed in acetone for 10 min (Nilsson et al., 2012), and then rehydrated in water followed by PBS without detergent, or incubated in PBS containing 0.3% triton X-100. There was no difference in the staining intensity or pattern between the two procedures. To label whole DRG *ex vivo*, DRG were isolated from 5-month-old P301S tau mice or C57BL/6 OlaHsd (Harlan) mice and incubated with 15 μM pFTAA in DMEM medium containing 20 ng/ml NGF and 1% fetal bovine serum at 4°C for 15 h, then fixed in 4% PFA for 20 min at room temperature. Following studies of the time course and dose-dependence to establish the best conditions for pFTAA labeling of tau, it was determined that an incubation of 30 min with 3 μM pFTAA was sufficient to label filamentous tau+ve DRG neurons to the same extent as higher concentrations and longer times. Before labeling, ganglia were permeabilised by incubating with acetone or PBS with 0.3% triton X-100 as described for the brain sections. Ganglia were bisected with a scalpel blade to facilitate antibody penetration. Antibodies were either added in PBS (if acetone was used) or PBS with 0.3% triton X-100.

Dissociated DRG neurons were fixed on ice with 95% ethanol kept at −20°C, then rehydrated through 70% and 50% alcohol and water before storage at 4°C in PBS with 0.1% azide. Ice-cold methanol (100%) was equally effective at fixation. pFTAA diluted in PBS was added to fixed neurons or in growth medium when added to living neurons. Neurons were washed in PBS before the addition of primary antibodies. Incubation in primary antibody solution was at 4°C over night for cultured neurons, or 2 days at 4°C for brain sections or whole DRG to facilitate antibody penetration into the tissue. After washes in PBS, secondary antibody was applied for 1 h (cultures) or 1 day (brain sections, whole DRG) in the same buffers used for primary antibodies. Brain sections or cultures on coverslips were mounted in FluorSave™ (Calbiochem).

Antibodies

Anti-phospho-tau AT100 (epitope pT212/pS214, MN1060) and anti-human tau HT7 (epitope 159–163, MN1000) mouse monoclonal antibodies were from Thermo Scientific and used at 1:1000 or 1:500 dilution, respectively. Hybridoma culture supernatant containing MC1 was a kind gift from Dr. P Davies, Albert Einstein College of Medicine, New York and used at 1:500-1:1000 dilution. Anti-β-III tubulin antibody was from Covance (PRB-435P-100) and used at 1:1000 dilution. AlexaFluor®568/647/350 conjugated secondary antibodies (Invitrogen) appropriate for the species were used at 1:1000 dilution. pFTAA was excited using the L5 filter (Leica). No significant fluorescent emission of pFTAA bound to tau was detected upon excitation at 540/50 nm, the wavelength used to excite AlexaFluor®568.

Imaging and Data Analysis

Imaging was performed using a Leica DMI 4000 B microscope and Leica-LAF software. Images were exported in TIFF format and figures compiled using Adobe Photoshop CS3. Quantification of concordance rates between pFTAA+ve neurons and co-stains was achieved by imaging 30 random fields at 20x magnification across 3 biological replicates, yielding 133–224 pFTAA+ve cells. Subsequent analysis used ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2014). Sampling for calculation of the half saturation constant was achieved by imaging 15 randomly chosen pFTAA+ve cells per concentration. Relative fluorescence intensity of each pFTAA+ve neuron per concentration was quantified using the ImageJ Measure tool. Microscope settings were: filter cube L5, intensity 5, exposure 170 ms, gain 1. The half saturation constant and the coefficient of determination (R^2) values were determined using the non-linear least squares curve-fitting program http://faculty.gvsu.edu/carlsont/232lab/nonlin2.html. To determine the rate of cell death, the number of pFTAA+ve cells that became PI positive

and/or were subsequently lost from the culture, were expressed as a percent of total pFTAA+ve neurons imaged at $t = 0$ in 3 biological replicates. Significance of changes were calculated by one sample *t*-test. The number of pFTAA-ve neurons was identified by phase contrast morphology in the same fields as those used to image pFTAA+ve neurons, and were counted at onset of the experiment and after 25 days *in vitro*. Significance of difference between the percentage of pFTAA+ve and pFTAA-ve neurons remaining in the dish was determined by a paired *t*-test.

Results

We first examined whether DRG neurons in P301S tau transgenic mice—like CNS neurons (Klingstedt et al., 2013)—were labeled after injection of pFTAA *in vivo*, and whether pFTAA-labeled neurons co-label with antibodies that detect insoluble tau aggregates and fibrils. **Figure 1A** shows an overview of a brain section taken from the level of the anterior commissure; pFTAA labeled numerous neurons in the upper and deeper layers of the motor cortex, and most of the neurons in the piriform cortex, but none in the striatum. This labeling occurred exclusively in P301S tau+ve neurons, detected with the human-specific tau antibody HT7 (**Figures 1B–D**), and coincided with positive immunolabeling with the anti-phospho (pT212/pS214) tau antibody AT100 (**Figures 1B,C**), which only recognizes sarkosyl-insoluble, but not soluble, tau in the CNS of P301S tau mice (Delobel et al., 2008). Due to comparatively poorer antibody penetration compared to pFTAA, some structures that first appeared HT7- or AT100-negative, were found to be positively stained after increasing the camera exposure time. Colocalization was also obtained after immunolabeling with the anti-phospho (pSer202/pThr205) tau antibody AT8, which binds to filamentous tau but also binds to soluble hyperphosphorylated tau (Delobel et al., 2008) (data not shown). A subset of neurons in the DRG were also labeled with pFTAA and, as in the brain, this labeling coincided with immunolabeling with HT7 or the MC1 antibody (**Figure 1D**), which detects a conformational change in tau that is associated with neurofibrillary tangles in human AD brains (Weaver et al., 2000) and is only evident in DRG neurons from P301S tau mice when they are also AT100+ve (Mellone et al., 2013). In biochemical studies, MC1 was shown to bind with the highest affinity to a compacted "paper clip" form of misfolded tau when the protein is pseudo-phosphorylated at the three epitopes recognized by the antibody PHF-1 [which stains most of the HT7+ve DRG neurons from P301S tau mice (Mellone et al., 2013)], AT8 and AT100 antibodies (Jeganathan et al., 2012). It should be noted that only 7–20% of DRG neurons are P301S tau (HT7)+ve in ganglia from 5-month-old P301S tau mice (Mellone et al., 2013).

To investigate whether pFTAA also labels specific DRG neurons in whole ganglia *ex vivo*, whole DRG were excised from 5-month-old P301S tau mice or C57BL/6S wild type (wt) mice, labeled with pFTAA, then fixed and immunostained. **Figure 2A** shows that pFTAA did not label any DRG neurons in ganglia from wt mice but did label the same subset of neurons in DRG from P301S tau mice that were also immunolabeled with HT7. Because the serine 396/404 phospho epitope recognized by PHF-1 is implicated in high affinity binding of MC1 to tau fibrils, we also stained the DRG for PHF-1. **Figure 2A** (bottom row) shows that there was extensive co-localization of pFTAA and PHF-1+ve neurons.

To establish whether pFTAA labeled cultured DRG neurons, and determine whether this labeling was specific to P301S tau+ve cells expressing advanced forms of tau pathology, DRG neurons were cultured from 5-month-old wt C57BL/6S mice, or Alz17 mice, which express the 2N4R isoform of human tau under the same Thy1.2 promoter encoding a neuron-restricted element as that used to express P301S tau (Probst et al., 2000; Clavaguera et al., 2009), and 2- or 5-month-old P301S tau mice. After 2 days, cultures were fixed, and stained with pFTAA. **Figure 2B** shows that no staining of pFTAA was detected in wt DRG neurons, nor in HT7+ve neurons from Alz17 mice, which do not express filamentous tau aggregates at this age, nor in HT7+ve neurons from 2-month-old P301S tau mice, a stage when they express P301S tau protein but do not form insoluble tau fibrils (Delobel et al., 2008). By contrast, pFTAA stained $44.1 \pm 0.9\%$ of HT7+ve, $81.0 \pm 11.4\%$ of AT100+ve, and $87.3 \pm 4.3\%$ of MC1+ve neurons (mean $\pm$ SD, 3 independent cultures) cultured from 5-month-old P301S tau mice (**Figure 2C**). The lower percentage of pFTAA staining of HT7+ve neurons compared to AT100 or MC1+ve neurons reflects the fact that only ~40% of HT7+ve neurons taken from this stage have developed the advanced stages of tau aggregation that is detected by the AT100 and MC1 antibodies (Mellone et al., 2013).

To further verify that pFTAA was binding to insoluble filamentous tau, cultured DRG neurons were imaged before and after formic acid treatment, which solubilizes filamentous tau in brain sections from Alzheimer's disease patients (Bing et al., 2006) and in brain sections from P301S tau mice (Velasco et al., 2008). DRG neurons were fixed, stained with pFTAA, and immunolabeled with MC1 and HT7 antibodies. Cells were imaged, then treated with 90% formic acid for 15 min after which the same cells were re-stained with pFTAA, re-probed with the indicated antibodies, and re-imaged. **Figure 3** shows that no pFTAA or MC1 staining was detected after incubation with formic acid, whereas HT7 immunostaining was still strongly visible. Hence, the pFTAA binding site is present only in insoluble tau with a pathological conformation.

The high signal to noise ratio of the pFTAA staining pattern prompted us to run a dose response curve to determine the relative affinity of pFTAA to P301S tau in the neurons. For this purpose, DRG neurons from 5-month-old P301S tau mice were cultured for 2 days, fixed, and stained with varying concentrations (0.0015–15 μM) of pFTAA. **Figure 4A** shows representative images of neurons stained with the different concentrations of pFTAA, while the average relative fluorescence intensity measurements from 15 random neurons per concentration are plotted in **Figure 4B**. To determine the half saturation constant (EC50), the data were fitted to an equation that assumes a single, saturable binding site (Fl_{max}x [pFTAA])/(EC50 + [pFTAA]) by non-linear curve fitting, from which an EC50 value of 0.142 μM was calculated with a correlation coefficient of 0.983 over the entire fit. Such a high

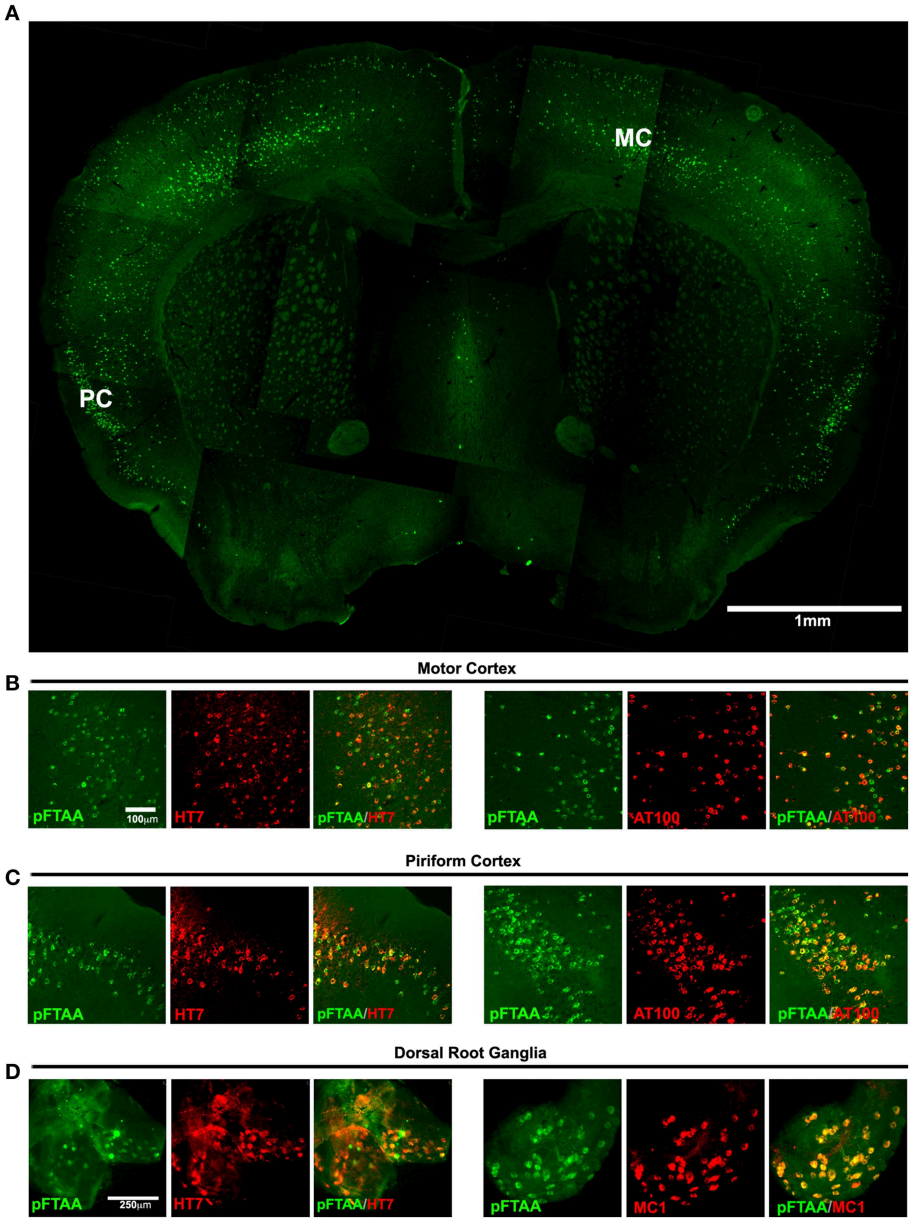

FIGURE 1 | Labeling of tau inclusions in the brain and DRG of 5-month-old P301S transgenic mouse by pFTAA. A P301S tau transgenic mouse injected with 150 nmole pFTAA via the tail vein was perfused after 48 h. **(A)** A composite image of a 30 μm coronal brain section at the level of the anterior commissure (5x objective) showing the general staining pattern of pFTAA+ve neurons; staining is found almost exclusively in the cortex. MC, motor M1 cortex, PC, piriform cortex. **(B)** motor M1 cortex and **(C)** piriform cortex stained with pFTAA (green) and immunolabeled with anti-human tau antibody HT7, or anti-fibrillar phospho-tau-specific antibody AT100 (red). **(D)** whole mount of a DRG stained with pFTAA *in vivo* (green) and immunolabeled with anti-human tau antibody HT7, or the conformational anti-tau antibody MC1 (red). Note that due to poorer penetration of the antibodies compared to pFTAA, some pFTAA+ve neurons that appear HT7-ve at the exposure shown are co-stained by the antibodies upon increased exposure. Scale bar, 1 mm **(A)** 100 μm **(B,C)**, 250 μm **(D)**.

affinity is unique to pFTAA as it was impossible to resolve any specific binding using FSB or thioflavin T/S at similar concentrations.

To examine whether live DRG neurons expressing insoluble tau would be detected with pFTAA, and whether it was the fixation that induced a conformation that allowed the binding of pFTAA to P301S tau, 2-day cultures of DRG neurons from 5-month-old mice were incubated with 3 μM pFTAA (rather than the higher concentration used in fixed cultures in order to minimize background fluorescence) for 30 min and then fixed, or fixed first and then stained with the same pFTAA solution. **Figure 5** shows that pFTAA stained similar profiles of HT7+ve neurons in live cultures before fixation as those that were fixed prior to staining. Hence, the conformation of tau detected by

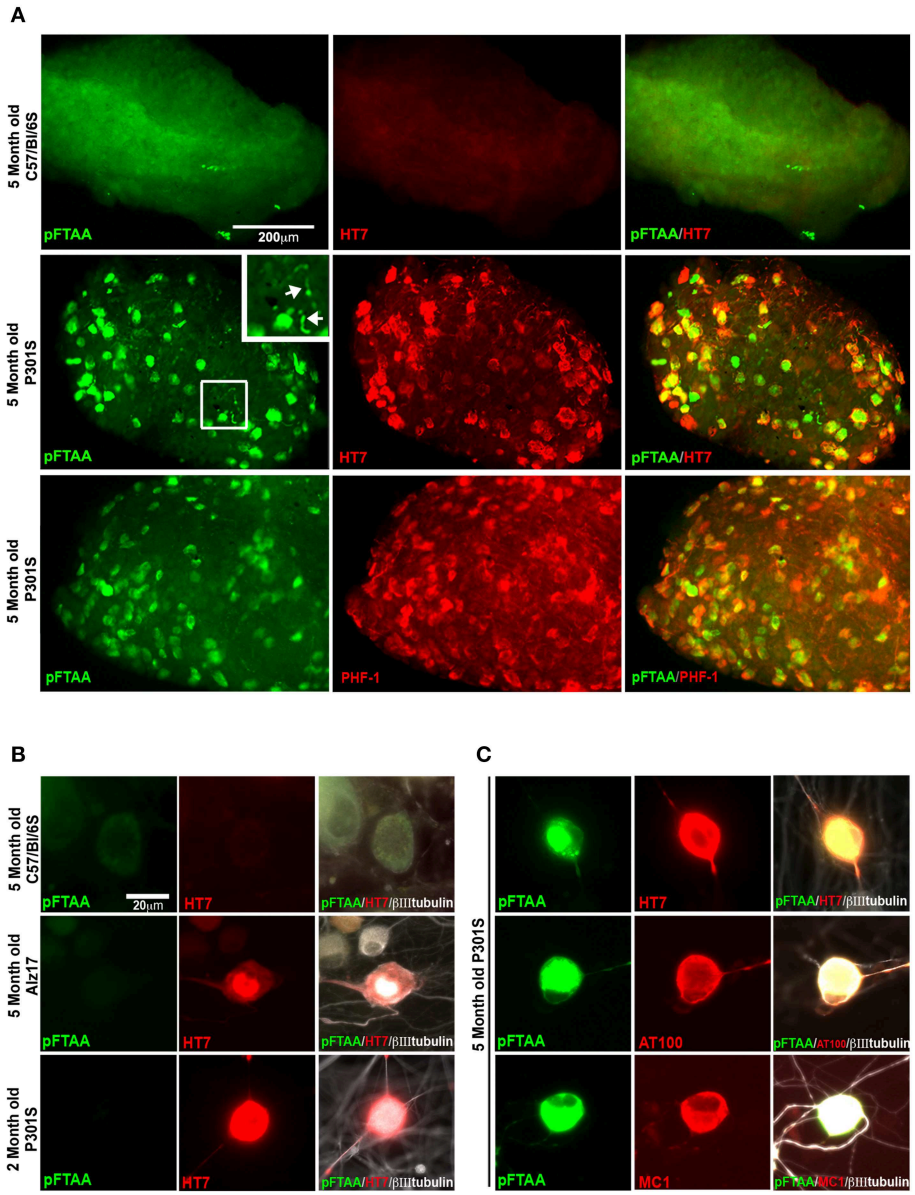

FIGURE 2 | pFTAA selectively labels fibrillar tau in DRG neurons in intact DRG *ex vivo* and cultured dissociated DRG neurons from 5-month-old P301S tau transgenic mice. (A) Intact DRG from 5-month-old C57BL/6 OlaHsd (wt, top row) or P301S tau mice (middle row) were incubated with 15 μM pFTAA at 4°C for 15 h, then fixed in 95% ethanol, rehydrated, and immunolabeling (red) with the HT7 antibody. Bottom row shows immunolabeling with the PHF-1 antibody (which recognizes phospho-serines396/404). Magnification box identifies pFTAA+ve threads visible in the axons of whole ganglia (arrows). Non-specific signal in wt intact ganglia is a-cellular debris carried over because of re-use of the dye. **(B)** DRG neurons were cultured from 5-month-old C57BL/6 OlaHsd wild type mice, or Alz17 mice transgenic for the longest isoform of human tau (2N4R), or 2-month-old P301S tau mice for 2 days. After ethanol fixation, cultures were labeled with pFTAA and immunolabeled with HT7. Note absence of pFTAA staining but presence of human tau in the neurons from Alz17 and 2-month-old P301S tau mice. (**C**) Dissociated DRG neurons from 5-month-old P301S tau mice were labeled after ethanol fixation by pFTAA (green), as well as for AT100 or MC1 (red). Images are representative of 133–224 pFTAA+ve cells across 3 biological replicates. Scale bar, 200 μm in **(A)** 20 μm in **(B,C)**.

pFTAA is preformed in the neurons and is not induced after – or by – fixation, as also demonstrated after delivering pFTAA *in vivo* or in ganglia stained by pFTAA before fixation.

Prior to the use of pFTAA, neurons had to be fixed and stained with antibodies to detect those neurons containing advanced forms of aggregated tau. This meant that the same field of live neurons that had been studied, for example, to follow mitochondrial transport, had to be retraced after fixation and antibody staining (Mellone et al., 2013). Given the positive detection of neurons containing fibrillar tau using pFTAA, we

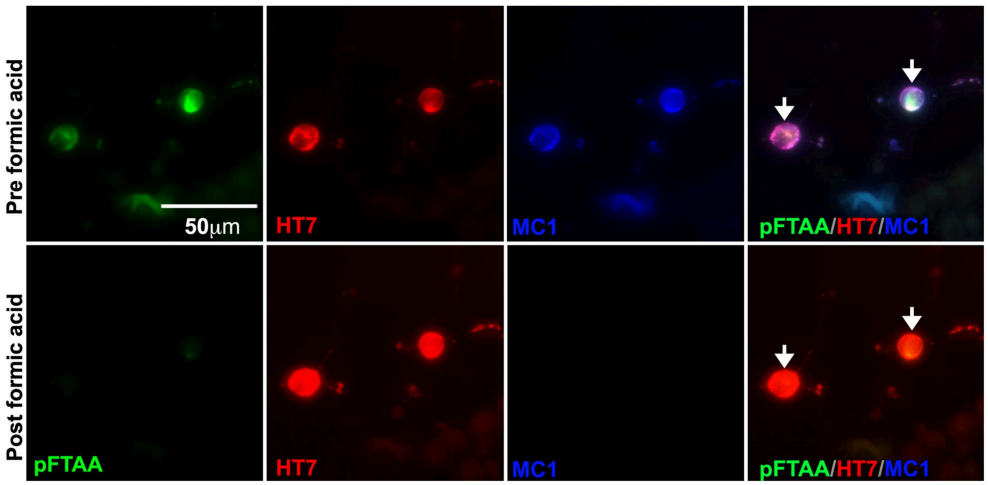

FIGURE 3 | Formic acid treatment solubilizes fibrillar tau, but not souble tau, and eliminates the binding of pFTAA. DRG neurons cultured from 5-month-old P301S tau mice for 2 days were fixed with 95% ethanol. Top row shows pFTAA+ve neurons immunolabeled with HT7 (red) and MC1 (blue) antibodies. Bottom row shows the same set of neurons re-stained with pFTAA and antibodies after a 15 min treatment with 90% formic acid. Note that formic acid treatment prevented the re-binding of pFTAA and the MC1 antibody whereas the interaction with HT7, which binds to soluble and insoluble tau, was restored. Scale bar, 50 μm.

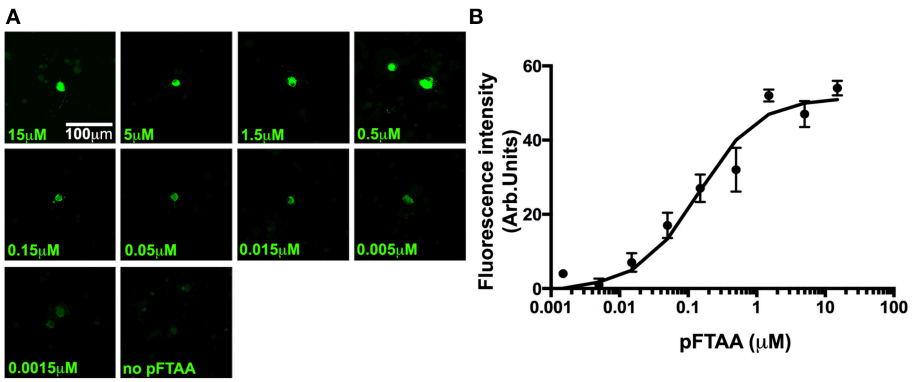

FIGURE 4 | A dose response of pFTAA demonstrates a single, homogeneous binding site to neurons expressing filamentous tau. pFTAA was added at the concentrations indicated to ethanol-fixed DRG neurons cultured from 5-month-old P301S tau mice for 2 days. **(A)** Representative images of pFTAA binding intensities at the concentrations indicated. **(B)** Quantification of signal intensity. To determine the relationship between the concentration of pFTAA and fluorescence intensity, the fluorescence intensity values from 15 neurons per dose were averaged (circles) and the results were submitted to a least square curve fitting analysis according to an equation that predicts a single, saturable binding site (line) from which a half saturation constant of 0.142 μM ($R^2 = 0.983$) was derived. Error bars indicate sem values. Scale bar, 100 μm.

asked whether we could visualize the progression of processes that occur in these neurons over longer periods of time. One such process of interest is the mechanism of cell death. As mentioned above, there is an ongoing debate as to tau+ve neurofibrillary tangles induce cell death or whether these protein aggregates are benign or even protective. We reported the loss of AT8 and AT100+ve neurons in long-term cultures of DRG neurons from P301S tau mice, but could not establish whether this was due to intrinsic properties or to do with post-fixation handling, as neurons that die tend to detach and disappear. To examine whether P301S tau+ve neurons with fibrillar tau were dying at a measurable rate in long-term cultures, we labeled neurons with pFTAA and used PI to track cell death in the labeled population over 25 days. **Figure 6** shows that identified pFTAA+ve neurons lost plasma membrane integrity and/or disappeared from the cultures over this period while the majority of the unlabeled cells remained PI-negative, except for the occasional appearance of a PI+ve cell, as shown in the image from $t = 2$ days. The number of surviving cells was significantly different ($p < 0.0144$) from day 10 *in vitro* (3 days from the time pFTAA was added) onwards. It is unlikely that pFTAA was toxic, as the percentage of surviving pFTAA-ve cells identified by phase contrast at day 25 was significantly higher than that of pFTAA+ve cells ($p = 0.0318$, paired t-test).

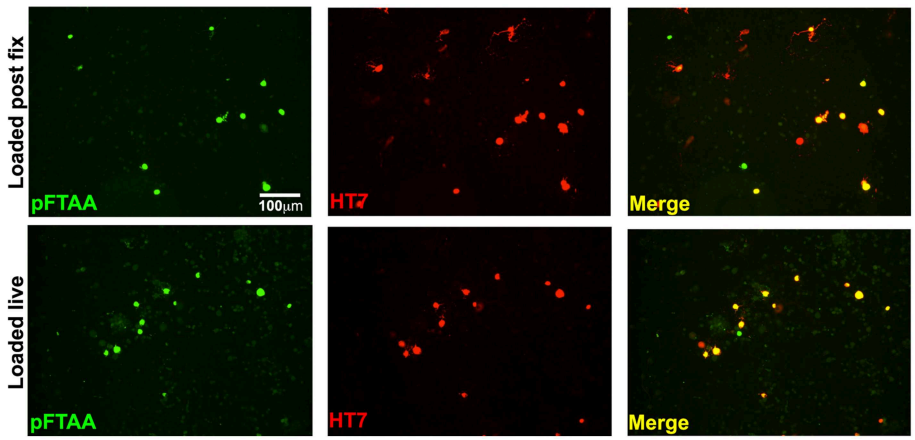

FIGURE 5 | Labeling of insoluble tau is not an artifact of fixation. DRG neurons cultured from 5-month-old P301S tau mice for 2 days were either incubated with 3 μM pFTAA for 30 min at room temperature before fixation in 95% ethanol, or fixed before labeling with pFTAA using the same solution and conditions. Cultures were immunolabeled with the HT7 antibody (red). Loading of pFTAA into either live or fixed dissociated DRG neurons produced equivalent signal and co-localization with HT7. Note that some HT7+ve neurons are not labeled with pFTAA because HT7 neurons with insoluble tau only represent about 44% of all the HT7+ve neurons. Scale bar, 100 μm.

Discussion

Misfolded and hyperphosphorylated tau is now known to be at the fulcrum of many neurodegenerative diseases and it is thus of major interest to understand how it exerts its toxic effects. One of the limitations of current studies aimed at understanding the actions of NFTs (and other abnormally misfolded proteins) in neurodegenerative diseases is the lack of dyes that detect aggregates and fibrils in living neurons. There are many dyes that stain protein preparations of fibrillar tau but few that have been useful for detecting fibrillar tau in living neurons in animal models (Velasco et al., 2008) or in culture (Bandyopadhyay et al., 2007; Lira-De Leon et al., 2013) without toxicity. Kuchibhotla et al. (2014) reported that Methoxy X-04 delivered via the eye vein rapidly detects NFTs in the visual cortex of P301L tau transgenic mice but noted the dye's near-full depletion from the brain within 24 h. Because of its rapid reversibility, they could not corroborate that the dye stained exclusively insoluble NFTs by using antibodies, as they did using the well-established dye Thioflavin S in fixed tissue. We developed the DRG neuron culture system because it can be established from adult mice, the neurons develop pathological forms of tau at the same rate as those that develop in CNS neurons, and some of these properties develop and are maintained during long-term cultures (Mellone et al., 2013).

The present work shows that pFTAA is a non-toxic, high affinity, rapid-acting, and highly specific dye for detection of fibrillar (but not soluble) tau in living neurons *in vivo, ex vivo* and in dissociated cultures of neurons. That the binding of pFTAA is specific to fibrillar tau is shown by the high degree of overlap between pFTAA-labeled neurons and neurons that express the AT100 and MC1 epitopes. Moreover, solubilization of fibrillar tau with formic acid disabled pFTAA binding and MC1 immunolabeling whilst soluble P301S tau detected by HT7 immunolabeling was still abundant in the neurons. pFTAA stained not only the cell bodies of neurons but also the axons, exemplified in the images of intact DRG ganglia (**Figure 2A**) and in the cultures (**Figure 2B**), coinciding with immunolabeling for AT100 or MC1.

To our knowledge the affinity of dyes that bind to fibrillar tau in primary neurons has not been reported previously. In neuroblastoma SH-SY5Y cells, uptake and cell viability of the most commonly used pro-aggregating anionic dyes Congo red (CR), Thioflavin S (ThS) and Thiazine Red (TR) were measured after 7 days of treatment (Lira-De Leon et al., 2013). Congo red stained the highest number of cells (55% at 5 μM) and no toxicity was reported. However, EC50 values for ThS and TR were around 75 and 45 μM, respectively, but viability was commensurately reduced even at the lowest concentrations (5 μM). Moreover, these compounds induced aggresome-like structures rather than reporting on their presence, whereas pFTAA is a reporter without evident inducing or dispersing activity. Bandyopadhyay and colleagues (Bandyopadhyay et al., 2007) reported that 10 μM Congo red induced NP40-resistant aggregates in cell pellets from HEK-293T cells expressing human tau, but no uptake of ThS or TR was detected. However, CR also induced considerable toxicity. Due to its high affinity to fibrillar tau, it is tempting to speculate that higher concentrations pFTAA might be used to dissociate NFTs or perturb NFT formation. However, Wegenast-Braun and colleagues showed that repeated weekly intravenous injection of pFTAA at concentrations similar to those used in our study for up to 6 months produced no inhibitory effect on A-beta aggregate formation (Wegenast-Braun et al., 2012). Furthermore, over 25 days *in vitro* we did not observe any instances of living pFTAA+ve neurons that subsequently became pFTAA-ve despite having added a concentration of pFTAA that is 20-fold higher than the half saturation constant of the dye. More work using even higher concentrations will have to be conducted to assess

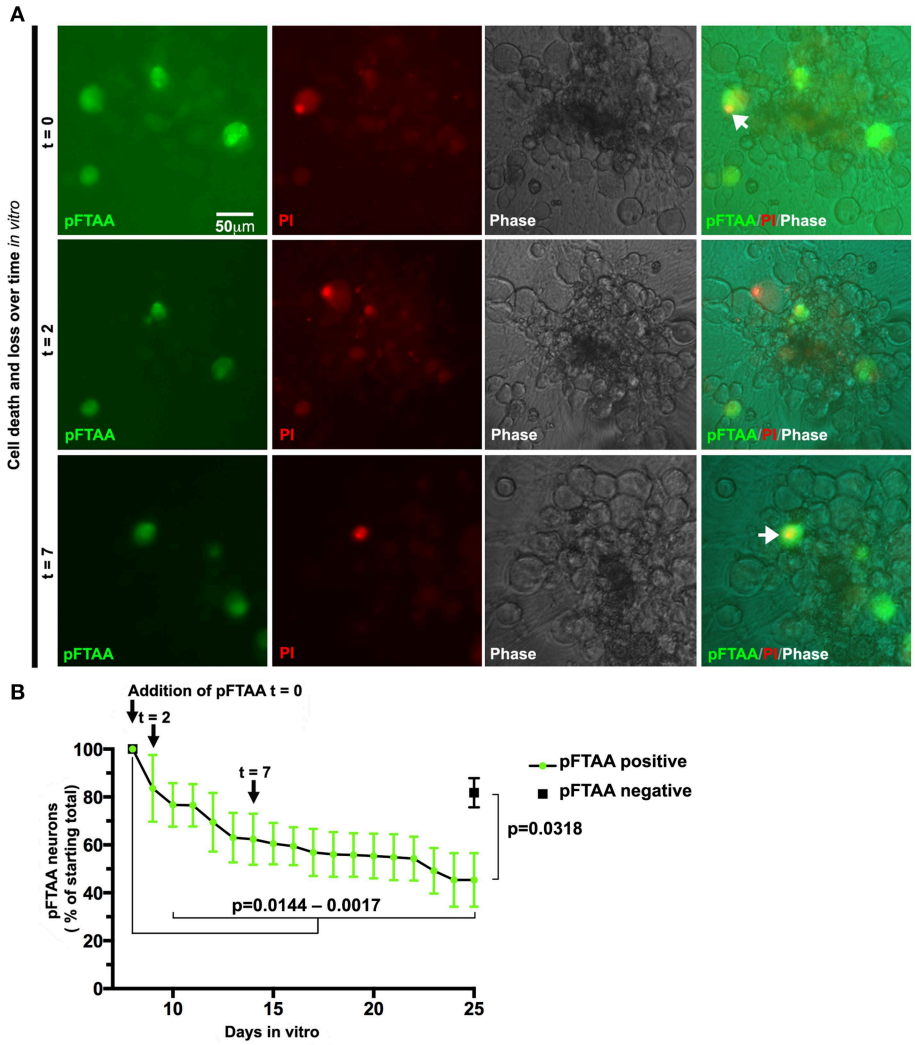

FIGURE 6 | Slow death of defined DRG neurons expressing fibrillar tau can be followed continuously after labeling with pFTAA. DRG neurons were cultured from 5-month-old P301S tau mice for 7 days before labeling with 3 μM pFTAA, (t = time in days after addition of pFTAA). Medium was replaced with PI-containing medium without pFTAA every 3 days. **(A)** Images of the same set of neurons at 0, 2, and 7 days after addition of pFTAA showing appearance of PI+ve neurons and their subsequent disappearance from the cultures. **(B)** The loss of pFTAA+ve neurons as a percentage of total neurons originally labeled is 45% after 25 days *in vitro*. The loss of pFTAA+ve neurons becomes significant at 10 days *in vitro* ($p = 0.0144$) and continues to increase until 25 days *in vitro* ($p = 0.0017$). Black square denotes percentage survival of pFTAA-ve neurons in the same culture after 25 days (mean ± sem from the three biological replicates, $p = 0.0318$, paired *t*-test). Scale bar, 50 μm.

whether pFTAA-bound fibrillar tau remains stable over longer times.

We measured the affinity of pFTAA to fixed DRG neurons from P301S tau mice, finding a half saturation constant of 0.142 μM with a remarkably high correlation coefficient of 0.983 after fitting the data to a binding curve that assumes a single saturable binding site. Even though fibrillar tau may contain more than one binding site per molecule, and several binding sites per cell, it appears that there is no interaction between these individual binding sites (otherwise the curve would show positive or negative cooperativity). It will be interesting to compare this value to that of binding of pFTAA to pure fibrils of tau to determine the relationship between the macroscopic binding to cells and the microscopic binding parameters of the individual fibrils. Unfortunately at present it is not possible to form fibrils of pure tau without resort to fibril-initiating compounds such as polyanions or seeds of fibrillar tau. Hence this data is the first of its kind to probe binding parameters of dyes that are specific to preformed fibrillar tau in neurons.

Along with the relatively high affinity of pFTAA to the fibrils, we found that pFTAA is rapid acting: the shortest time between adding 3 μM pFTAA to the neurons and inspecting them under the microscope was 10 min and already by this time, the intensity of labeling the neurons appeared to be maximal compared to the values obtained 30 min later. Unlike Methoxy X-04 (Kuchibhotla et al., 2014) pFTAA labeling in our mice remained extensive

after 48 h and is still detectable 6 months later in fixed brain slices despite extensive washing. Having found that pFTAA labels fibrillar tau in living neurons, we utilized the dye to follow a defined set of neurons over 25 days to examine whether they undergo cell death. We used PI to label dying cells as it is not toxic to living neurons, but because necrotic cells tend to detach from the substrate a short time after they die, we also recorded loss of pFTAA+ve neurons. We found that the rate of cell death appeared to follow two phases; a more rapid phase that lasted over 14 days, and a slower phase that lasted until the end of the study. The reason for this biphasic response is not clear. Since the neurons were cultured for 7 days prior to onset of labeling, it is unlikely that the faster-dying neurons were damaged due to culture preparation. However, unpublished work from our lab indicates that there is high variability in the oxidative status of P301S tau-expressing neurons, which may sensitize a stochastic subset of neurons to death. Nevertheless, it is clear that a sizeable population of the neurons containing fibrillar tau can survive 25 days in culture. Previously, Wegenast-Braun et al. (2012) injected pFTAA to label β-amyloid in APP23 and tau in P301S mice and found no discernable toxicity (Wegenast-Braun et al., 2012). Data presented here corroborates this observation, since several pFTAA+ve neurons survived for 25 day. Additionally, significantly more pFTAA-ve neurons remained viable after 25 days, indicating that the cell death we observed was not due to toxicity but was associated with fibrillar tau. Elucidating the properties of the relatively resistant neurons, which can now be distinguished by using pFTAA, may provide a clue to the factors that are required to resist the possible toxicity of fibrillar tau.

There are some limitations to the use of pFTAA that are worth noting. One proviso was that some neurons appeared to be stained without apparent presence of P301S tau. In some cases, on closer inspection using higher intensity illumination, there was a small remnant of HT7 labeling though the pFTAA still appeared to label a larger part of the neuronal cell body. However, pFTAA also labeled a few *a priori* dead neurons in the culture but these were readily distinguishable under light microscopy as phase dark and grainy. We did not remove the stained dead cells from consideration when we calculated the percentage of pFTAA+ve neurons that were AT100 or MC1+ve, thus accounting for the small percentage of neurons that were recorded as pFTAA+ve but were antibody-negative. Another aspect worth noting is the longevity of pFTAA binding. Although it has been retained without notable fade over several months in the brain sections and ganglia after *in vivo* labeling, in the living cultures, the intensity of pFTAA staining waned over time, though it was still discernable 25 days later. Although we did not replenish the pFTAA in these experiments, adding fresh pFTAA to another set of neurons renewed the staining to the same intensity as that which was recorded after first application. The fade was unlikely to be due to photobleaching, since a continuous 60-min exposure of fixed pFTAA+ve neurons under continuous illumination at the same intensity we use to image pFTAA in the neurons produced minimal loss of fluorescence (see graph here: https://www.dropbox.com/s/1xwyq5bbgoebvmm/pFTAA%20photobleaching.tiff?dl=0). The cumulative intensity x time of exposure in this experiment is 300-fold higher than the intensity the neurons would have been exposed to during the experiment. A more careful quantitative analysis of pFTAA binding intensity together with periodic addition of another vital dye that differentiates neurons with newly-formed fibrillar tau from those stained initially may help inform on the mechanisms of formation and turnover of fibrils in living neurons.

Identifying living neurons containing fibrillar tau with pFTAA opens up many research opportunities. Of utmost interest is the understanding of mechanisms by which neurons with advanced forms of tau pathology die. Although PI labels cells whose plasma membrane is ruptured, the neurons may have followed one of several cell death pathways that culminate in necrosis (Green and Victor, 2012). We can now study these processes by intervening in key steps of each putative death pathway in these identified neurons without having to sample all the neurons and identify those affected after fixation. The strong binding of pFTAA to fibrillar tau over long periods of time coupled to evidence that the dye will dissociate when the fibrils are solubilized will allow us to monitor disaggregation when testing anti-aggregation drugs, and follow at the same time other cellular processes that may participate in the disaggregation process. Likewise, it will be possible to monitor the events leading up to fibrillization as detected by pFTAA and determine the intracellular processes that contribute to the fibrillization process. Finally, we can now purify neurons with fibrillar tau from, for example, the cortex of P301S tau mice, allowing us to develop a biochemical approach to elucidate the mechanisms underlying dysfunction and death of neurons with fibrillar tau. Ultimately, elucidating the events that lead to fibrillar tau formation and neuronal death in our models may have direct relevance to other models of human neurodegenerative diseases, and to the human system in particular.

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REVIEW
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Imaging Multimodalities for Dissecting Alzheimer's Disease: Advanced Technologies of Positron Emission Tomography and Fluorescence Imaging

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The rapid progress in advanced imaging technologies has expanded our toolbox for monitoring a variety of biological aspects in living subjects including human. *In vivo* radiological imaging using small chemical tracers, such as with positron emission tomography, represents an especially vital breakthrough in the efforts to improve our understanding of the complicated cascade of neurodegenerative disorders including Alzheimer's disease (AD), and it has provided the most reliable visible biomarkers for enabling clinical diagnosis. At the same time, in combination with genetically modified animal model systems, the most recent innovation of fluorescence imaging is helping establish diverse applications in basic neuroscience research, from single-molecule analysis to animal behavior manipulation, suggesting the potential utility of fluorescence technology for dissecting the detailed molecular-based consequence of AD pathophysiology. In this review, our primary focus is on a current update of PET radiotracers and fluorescence indicators beneficial for understanding the AD cascade, and discussion of the utility and pitfalls of those imaging modalities for future translational research applications. We will also highlight current cutting-edge genetic approaches and discuss how to integrate individual technologies for further potential innovations.

Keywords: Alzheimer's disease, biomarkers, multimodality, PET, fluorescence

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INTRODUCTION

Progressive neuronal loss and dysfunction in specific brain circuits are common neurological features of neurodegenerative disorders. The fundamental mechanism of neurodegenerative onset is still largely unknown, but accumulating evidence has indicated that deposition of filamentous and/or soluble oligomeric aggregates of specific proteins with significant neurotoxicity generally serves as the initial trigger for the sequential cascade of disease-related pathophysiology. In brain of Alzheimer's disease (AD) patients, senile plaques (SPs), and neurofibrillary tangles (NFTs) have been defined as two major pathological hallmarks, suggesting that the formation process of the resulting abnormal lesions is tightly linked to the pathogenic mechanism of AD. SPs are extracellular deposits composed of hydrophobic 38–49 amino acid peptides termed amyloid β (Aβ) and, since the discovery of the *in vitro* neuronal toxicity of aggregated Aβ species, numerous studies have indicated that brain accumulation of Aβ aggregates is a major causative risk factor for AD

pathogenesis (Selkoe, 1991, 2001; Hardy and Selkoe, 2002). This "amyloid cascade hypothesis" is also strongly supported by the fact that Aβ metabolism is genetically linked to the familial AD causative genes β-amyloid precursor protein (APP) and Presenilins (PSs), adding major impetus to diverse therapeutic attempts to attenuate brain Aβ abnormality over the last two decades. However, in spite of these various advances in knowledge, no therapeutic approaches targeting Aβ have as yet successfully survived a Phase III trial (Huang and Mucke, 2012). This situation has now brought us to a turning point, as we need to re-evaluate our understanding of the consequences of AD pathophysiology and to carefully consider the direction of future therapeutic approaches so as to finally gain advantage over these complicated neurological disorders.

NFTs are composed of intra-neuronal and glial inclusions of hyperphosphorylated microtubule-associated protein Tau (MAPT), and they are widely observed in diverse tauopathies including progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD), suggesting their importance for general neurodegenerative abnormalities (reviewed in Lee et al., 2001). In the pathological course of AD, NFTs begin to appear in the entorhinal cortex and then spatially spread through a wide range of brain areas including the hippocampus, limbic system, and neocortex (Braak and Braak, 1991; Braak et al., 2011). The severity of NFT lesions is well-correlated with synaptic dystrophy and neuronal loss, followed by brain atrophy, and test results of cognitive and memory impairment also revealed correlation with brain accumulation of tau (Arriagada et al., 1992). Importantly, pathological mutations in the genetic locus of *Tau* gene have been identified in the family of frontotemporal dementia with Parkinsonism linked to *MAPT* on chromosome-17 (FTDP-17-*MAPT*) (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). Furthermore, recent studies have demonstrated that transgenic mouse models overexpressing human Tau transgene harboring pathogenic FTDP-17-*MAPT* mutations exhibit robust NFTs formation followed by neuronal loss and memory impairment (reviewed in Sahara et al., 2011). These findings suggest that abnormal aggregation and/or physiological malfunction of Tau protein has more potential significance for neurodegeneration, and indicate that the severity of tau pathology is also a potent biomarker for the early clinical diagnosis of AD.

As proposed in Braak's model, the pathological severities of SPs and NFTs in post-mortem AD brains can be categorized into progressive stages (stages A-C for SPs and I-VI for NFTs), providing fundamental criteria for a definitive diagnosis of AD (Braak and Braak, 1991, 1997). Hopeful attempts by AD researchers to establish visible biomarkers for tracing the real time course of Aβ and tau lesions in brain of living patients have followed a long, sometimes frustrating journey, as so far, histological findings at autopsy have usually suffered from technical limitations during the post-mortem procedure. In this regard, an *in vivo* imaging approach is particularly important for assessing the consequence of clinical symptoms and spatial dynamics of pathological lesions simultaneously. For this purpose, non-invasive neuroimaging techniques represented by positron emission tomography (PET) and magnetic resonance imaging (MRI) have a major significance, as they allow us to monitor both functional and structural circumstances in brain of AD patients (Mori et al., 2012; Jack and Holtzman, 2013). Aβ PET radiotracers have been developed and approved for clinical use, and they have added in shedding more light on the process of the early detection of AD (Yang et al., 2012). More recently, several laboratories including our group demonstrated the potential utility of tau-specific PET radiotracers to validate the spatiotemporal kinetics of the pathological stage of NFTs (reviewed in Ariza et al., 2015; Villemagne et al., 2015). These consistent advances of radiological imaging technologies are really just a beginning stage, and further accumulations of new insights and important knowledge for constructing biomedical databases will be required for future translational research. It is important to realize that there is still a lot of room to manipulate and improve the capability of PET radiotracers in terms of binding specificity and chemical properties that can critically improve the diagnostic interpretation of PET neuroimaging. To visualize the brain status of AD more precisely, complimentary imaging approaches will be essential for assessing the detailed kinetics and fast dynamics of target molecules. Fluorescence-based imaging has great potential as a key technology to satisfy this aim. Although the utility of fluorescence is still restricted to animal models at this time, the major advantages of the fluorescence system are a variety of applications based on a wide range of probe design strategies including molecular genetics for dissecting the detailed molecular pathophysiology of neurodegenerative diseases. In this review, we will specifically introduce current imaging multimodalities beneficial for understanding the AD cascade and discuss how to integrate the respective modalities to the view of providing significant advances toward future translational research.

PET IMAGING FOR DIAGNOSIS OF ALZHEIMER'S DISEASE

General Usage for Brain PET Imaging

Radiological techniques supply a variety of applications for *in vivo* brain imaging, becoming a most effective approach in terms of the clinical diagnosis of neurological disorders. PET technology is especially adept at monitoring the behavior of neuronal molecules in living subjects including rodent, monkey, and human. Although the spatial resolution obtained by MRI, which is superior for morphometric analysis, is much higher than that of PET imaging, the performance of PET is greatest in the quantification of the kinetics of neuronal receptor molecules and chemical probe interaction in intact brain. In principle, chemical probes are labeled by positron emitting radionuclides such as ^{11}C and ^{18}F, and PET scan identifies the spatial location of these probes as radiotracers with high detection sensitivity and superior quantitative dynamic range (Piel et al., 2014). Therefore, we can directly obtain a densitometric 3D map of the accumulation level of radiotracers binding to targets. A number of small chemical compounds with high affinity to target molecules can be screened as potential

candidates of PET radiotracers without disrupting their parental structure and physicochemical properties. Only a small amount of radiation emitted from the tracer can be enough to allow detection, without any diffusion, and relatively fast decay of the radioisotope ($t_{1/2}$ = ∼20 min for ^{11}C, ∼110 min for ^{18}F) minimizes the radiation risk for patients (Piel et al., 2014). Furthermore, PET scan also provides important information about the detailed disposition and tissue circulation of the radiotracers themselves in brain, facilitating the design and synthesis of effective chemical derivatives that can successfully pass through the blood brain barrier (BBB) and penetrate brain tissue without obvious neurotoxicity (Piel et al., 2014). This basic feature of PET imaging makes this technology ideal for clinical use, and it also provides various potential utilities for scientific research.

PET Tracers for Detecting Aβ and Tau Pathologies

Since the initial discovery of the β-sheet enriched common structure in the aggregates of Aβ and tau, various β-sheet binding dyes such as congo-red and thioflavin have been isolated as candidate compounds for PET imaging, and their use for visualizing SPs and NFTs in animal models and human subjects has been attempted. Representative and currently available Aβ and tau PET tracers are listed in **Table 1**. The earliest PET studies identified the capability of [^{18}F]FDDNP, a fluorine-modified derivative of the lipophilic compound DDNP, as Aβ PET tracer (Barrio et al., 1999; Small et al., 2006). However, this tracer has not become widely used for *in vivo* imaging because it demonstrates a relatively low signal-to-noise ratio, and it also recognizes NFTs, which makes interpretation difficult (Shoghi-Jadid et al., 2002; Thompson et al., 2009; Smid et al., 2013). A superior Aβ-specific PET tracer termed [^{11}C]Pittsburgh Compound-B ([^{11}C]PiB) was screened as a derivative of thioflavin T, and it is now the most widely characterized radio ligand with improved sensitivity and high specificity against Aβ aggregate (Klunk et al., 2004). A ^{18}F-labeled analog of PiB, termed [^{18}F]3′-FPiB (Flutemetamol; Vizamyl, GE Healthcare), was developed for diagnostic application and was approved by the US Food and Drug Administration (Yang et al., 2012). In parallel, other Aβ PET tracers, [^{18}F]AV-45 (florbetapir; Amyvid, Eli Lily) and [^{18}F]AV-1 (florbetaben; Neuraceq, Piramal Imaging), have also been independently engineered from diaryl alkenes and are currently available for clinical diagnosis (Herholz and Ebmeier, 2011; Vallabhajosula, 2011). For the purpose of standardizing the difference of these Aβ PET tracers, several groups have investigated direct comparison of PET imaging data obtained by sequential scanning using several tracers in the same cohort (Vandenberghe et al., 2010; Wolk et al., 2012; Landau et al., 2013, 2014). As for the cortical retention ratio of [^{11}C]PiB, both [^{18}F]3′-FPiB and [^{18}F]AV-45 demonstrate a highly significant ability to discriminate between control subjects and AD patients, with a high linear correlation to the data obtained by [^{11}C]PiB. However, it has been pointed that the retention level in white matter varies among the tracers, which makes it more challenging to precisely measure the cortical retention ratio to the reference brain region such as the brain stem, which contains a large amount of white matter (Landau et al., 2014). Hence, it would still be a worthwhile investment to improve and characterize the capability of Aβ PET tracers (Kudo et al., 2007; Yousefi et al., 2011; Cselenyi et al., 2012). The development race of Tau PET tracers is just heating up (reviewed in Ariza et al., 2015; Villemagne et al., 2015). Three lines of chemical probes have been independently screened and characterized for their potential clinical utility (**Table 1**). [^{18}F]THK523 was originally engineered as a derivative of quinoline, and it demonstrates high affinity and selectivity for tau aggregates in *in vitro* assay (Fodero-Tavoletti et al., 2011). Although human PET imaging using [^{18}F]THK523 indicated its specificity not for Aβ but for NFTs, a relatively high background of this radiotracer in white matter was initially anticipated for its further clinical use (Fodero-Tavoletti et al., 2011; Harada et al., 2013). Improved novel radiotracers labeled [^{18}F]THK5105,

TABLE 1 | Reference list for Aβ and Tau PET radiotracers.

Target	Radiotracer for PET imaging of SPs or NFTs	Approval for clinical use	References
Aβ/SPs	[^{18}F]FDDNP		Barrio et al., 1999; Shoghi-Jadid et al., 2002; Small et al., 2006; Thompson et al., 2009; Smid et al., 2013
Aβ/SPs	[^{11}C]PiB		Klunk et al., 2004
Aβ/SPs	[^{18}F]3′-FPiB	Flutemetamol; Vizamyl, GE healthcare	Yang et al., 2012
Aβ/SPs	[^{18}F]AV-1	Florbetaben; Neuraceq, Piramal imaging	Rowe et al., 2008
Aβ/SPs	[^{18}F]AV-45	Florbetapir; Amyvid, Eli Lily	Choi et al., 2009
Tau/NFTs	[^{18}F]THK523		Fodero-Tavoletti et al., 2011; Harada et al., 2013
Tau/NFTs	[^{18}F]THK5105		Okamura et al., 2014
Tau/NFTs	[^{18}F]THK5117		Okamura et al., 2014
Tau/NFTs	[^{18}F]THK5351		Villemagne et al., 2014
Tau/NFTs	[^{18}F]T807		Xia et al., 2013
Tau/NFTs	[^{18}F]T808		Zhang et al., 2012
Tau/NFTs	[^{11}C]PBB3		Maruyama et al., 2013

[^{18}F]THK5117 and [^{18}F]THK5351, 2-arylquinoline derivatives, were recently engineered by the same group and demonstrated superior affinity and specificity in *in vitro* assays, making these tracers promising candidates for tau PET imaging (Okamura et al., 2014; Tago et al., 2014; Villemagne et al., 2014). [^{18}F]T807 and [^{18}F]T808, also screened as benzimidazole pyrimidine derivatives, demonstrated specific selectivity to tau aggregates with nano-molar affinity (Zhang et al., 2012; Xia et al., 2013). Human PET studies using these radiotracers revealed significant association between clinical severity and their cortical retention, and this observation was also followed by a good correlation with post-mortem pathological NFT lesions (Chien et al., 2013). [^{11}C]PBB3 was developed as a phenyl/pyridinyl-butadienyl-benzothiazoles/benzothiazolium (PBB) derivative that possesses superior affinity and specificity for NFTs, much higher than for SPs, by radioautography using brain sections from both rodent models and AD patients (Maruyama et al., 2013). [^{11}C]PBB3 PET analysis of AD patients and non-AD tauopathies revealed that the severity of tau accumulation correlates well with clinical scores and follows the pathological staging model proposed by Braak. Because of it being a relatively new topic, as well as the highly competitive nature of this research field, a side-by-side direct comparison of the capability of the respective tau PET tracers in the same individuals has not yet been achieved. However, recent studies based on *in vitro* binding assays indicate that those various tracers demonstrate preferential binding capability to distinctive tau pathologies observed in AD and non-AD, suggesting each tracer potentially recognizes the different structural status of tau aggregates (Maruyama et al., 2013; Marquie et al., 2015). Overall, current tau PET imaging is promising, and detailed characterization and improvement of independent radiotracers will provide better clinical insight and shed greater light on the detection of AD progression.

Non-Amyloid PET Imaging for Detecting Disease Progression

In addition to Aβ and Tau PET imaging, various disease-associated pathophysiologies such as aberration of energy metabolism, glial inflammation, dysfunction of calcium homeostasis, and imbalanced neuronal activity can also be targeted as potential biomarkers of AD progression. The benefits of characterizing these additional biological parameters for AD diagnosis are the following: (i) accumulating a repertory of biomarkers will eventually contribute to establishing a PET biomarker database that will allow us to conduct more precise diagnoses and case-by-case categorizations with unbiased criteria by the combinations of several parameters; (ii) the signal-to-noise ratio of current amyloid PET imaging is still being developed and is not yet sufficient to detect early stages of pathological lesions; (iii) some of the biomarkers can be useful for the diagnosis of other neurodegenerative disorders as common pathophysiological abnormalities. [^{18}F]fluordeoxy glucose ([^{18}F]FDG) PET scan has been conducted to identify glucose hypometabolism in AD patients, and it is generally selected as the initial choice for the clinical diagnosis of AD (McGeer et al., 1986; Mosconi, 2005). However, the difficulty in identifying a specific and precise time window of the AD status has been a matter of debate, as [^{18}F]FDG hypometabolism reflects various mixture effects including less neuronal excitability and neuronal loss, which are widely observed in various neurodegenerative disorders (Jack and Holtzman, 2013). Brain inflammatory response with reactive gliosis is the other potential target for staging AD progression (Heppner et al., 2015). A considerable amount of evidence has indicated that activated reactive astrocytes contribute to clearing SPs in the early stage of AD. Since the inflammation is massively amplified by the paracrine neuroprotective response, it would be one of the key candidates as an early-stage biomarker that can detect subtle changes indicating pathological abnormality. In fact, using a tau transgenic mouse model, it was demonstrated that microglial activation proceeds to NFTs formation (Yoshiyama et al., 2007), suggesting its potential utility as a much earlier-stage diagnostic biomarker. This evidence supports the concept of the potential utility of several inflammation-related molecules for early clinical diagnosis. On the other hand, it has been debated whether inflammatory response represents both aspects of neuroprotective and neurodegenerative signals (Higuchi et al., 2010; Heneka et al., 2015; Heppner et al., 2015). Particularly, there is evidence that prolonged microglial activation accelerates the neurodegenerative process (Maeda et al., 2007; Ji et al., 2008), suggesting a potential difficulty for interpreting inflammatory parameters as early-stage biomarkers. In any case, it should be feasible to target a molecule that can segregate astrocytic and microglial activations. Based on this concept, TSPO (Translocator Protein 18 kDa, also known as Peripheral Benzodiazepine Receptor; PBR) has been targeted for imaging nueroinflammation (reviewed in Jacobs et al., 2012). Since increased TSPO expression in activated microglia has been associated with a number of neurological diseases (reviewed in Venneti et al., 2006), several PET tracers for TSPO (e.g.,; [^{11}C]*(R)*-PK11195, [^{11}C]PBR28, [^{11}C]DAA1106, [^{11}C]AC5216, and [^{18}F]DPA-714) have been studied to visualize the microglial activation in animal models and AD patients (Jacobs et al., 2012; Mori et al., 2012; Golla et al., 2015). However, there are several limitations of current TSPO ligands such as selectivity to microglia and different affinity in polymorphisms (reviewed in Venneti et al., 2013; Turkheimer et al., 2015). Regardless, imaging neuroinflammation will offer one of diagnostic tools for neurological diseases.

Pitfalls of PET Imaging

PET imaging is an extremely powerful tool for the purpose of non-invasive clinical diagnosis. However, it is still not easy to install these imaging systems into a single laboratory; it would be a costly and space-consuming challenge to establish a special radiation control facility requiring a high level of expertise for the maintenance and manipulation of radioactive molecules. In addition, there are also technical limitations in regard to spatial and temporal resolution, and there is not yet a suitable approach for evaluating binding specificity and molecular dynamics of the tracer, pivotal criteria for reliable imaging interpretation. For instance, despite the development of newly screened radiotracer candidates based on considerable effort and time for synthesis

and radiolabeling, *in vitro* and *ex vivo* binding assays such as autoradiography under low magnification are recognized as so far being the sole method for verifying the capability of the tracer. An additional weakness of PET technique is the inability to obtain new biological insight into the structural and/or environmental status of target molecules, meaning that the solid radiation signal we monitor during PET scans completely lacks that kind of molecular-based information. Therefore, an ideal approach would be to evaluate the biological validity and capability of the tracer by complementary technologies side-by-side, and this would comprehensively enhance the developmental productivity by mutual complementary and synergistic effect of different technologies. In the next section, we will discuss the utility of fluorescence imaging in the effort to lessen the gap in PET imaging.

FLUORESCENCE IMAGING TO DISSECT MOLECULAR PATHOPHYSIOLOGY

Utility of Fluorescence for *In vivo* Brain Imaging

During the rapid evolution of optical microscope technologies over the last several decades, fluorescence imaging has become a powerful tool for current modern neuroscience (Giepmans et al., 2006; Wilt et al., 2009). A variety of microscope systems with combinations of many available fluorescence probes supply a wide range of applications including single molecular analysis, live cell imaging, and conventional histological analysis in animal brain. In particular, the recent availability of the multi-photon excitation technique has broken down the limitation of optical scattering in thick tissues and expanded the utility of fluorescence for *in vivo* brain imaging in animal models (reviewed in Svoboda and Yasuda, 2006). In this system, only target fluorophores in a restricted volume can be excited by simultaneous photon absorption using a long-wavelength pulse laser, allowing us to illuminate molecular dynamics up to a depth of ∼1 mm. To conduct a long period of efficient excitation and detection (at least 1 month), setting of a chronic cranial window onto the bone of the skull is generally used (Tomita et al., 2005). Furthermore, current progress of fluorescence-based microendoscopy using optical fibers has enabled us to conduct cellular level imaging within the deep areas of brain tissue such as hippocampus and striatum in freely moving animals (reviewed in Mehta et al., 2004; Oh et al., 2013). In spite of mechanical inflexibility and physical invasiveness caused by surgical insertion of submillimeter-diameter fiber optic devices, it offers potential applications for longitudinal studies of disease progression (Barretto et al., 2011). At this stage, these approaches are specifically limited to usage in rodent models and is technically still far removed from any availability for human diagnosis. However, the combinatorial use of the AD mouse model with multi-color fluorescence probes should represent a beneficial approach for the monitoring of the progressive course of the AD cascade simultaneously, relying on fast temporal kinetics and high spatial resolution.

In vivo Fluorescence Imaging for Detecting Aβ and Tau Pathologies

As most of the β-sheet binding chemical compounds engineered for PET radiotracers possess their own fluorescence derived from a polycyclic aromatic hydrocarbon backbone structure, these small chemical compounds have ideal multimodalities for side-by-side *in vivo* imaging comparison by both fluorescence and PET techniques (Hawe et al., 2008). So far, studies focusing on the beneficial usage of *in vivo* fluorescence approaches have been published from only a few laboratories (**Table 2**). As pioneer studies, SPs in APP transgenic mouse, which demonstrates massive Aβ pathology, were first successfully visualized by the hydrophilic chemical compound Thioflavin S or by anti-Aβ antibody conjugated to fluorescein by two-photon microscopy (Bacskai et al., 2001; Christie et al., 2001). Since these reagents cannot penetrate BBB, the authors injected them directly into the imaging area of the brain surface, demonstrating that the size and morphology of SPs are rapidly cleared by immunization with Aβ peptide during the course of a few days (Bacskai et al., 2001). The relatively hydrophobic Congo-Red derivative Methoxy-X04 was next developed and used to visualize Aβ pathology in brain of APP transgenic mouse by intraperitoneal bolus administration (Klunk et al., 2002). In addition, importantly, it was also verified that Pittsburgh Compound-B (PiB) successfully visualized SPs in mouse brain parenchyma by intravenous administration, opening a new possibility for the simultaneous characterization of a chemical tracer for both fluorescence and PET imaging (Bacskai et al., 2003). The advantage of these compounds is their ability to pass through BBB and therefore provide very important information on the kinetics and tissue distribution of Aβ pathology with great spatiotemporal resolution (∼μm for spatial and ∼sec for temporal). Although initial studies have suggested a relatively stable appearance of existing SPs over several months, more recent studies indicated that both SPs and cerebral amyloid angiopathy (CAA), the cerebrovascular Aβ deposition in arterial vessels, grow at a consistent rate (Robbins et al., 2006; Garcia-Alloza et al., 2007; Hefendehl et al., 2011). It has also been demonstrated that newly developed SPs grow rapidly for 24 h and dynamically change their morphology, suggesting that steady-state Aβ pathology undergoes flexible regulation by various environmental factors in living animal. Similar approaches to visualize tau pathology in Tau transgenic mouse model have also been used. By direct application of Thioflavin S, NFTs with neuronal loss in brain of rTg4510 tau transgenic mice were first evaluated by multi-photon microscopy (Spires-Jones et al., 2008). X-34, a Congo-Red derivative, and PBB3 were next independently developed by different groups and used for successful visualization of NFTs with great spatiotemporal resolution by intravenous injection in living mouse models (de Calignon et al., 2010; Maruyama et al., 2013). Although the detailed *in vivo* time course of NFTs formation has not yet been well-characterized, these studies promise the dual utility of the compounds for both radiological and fluorescence *in vivo* imaging applications. Importantly, both local abnormalities of Aβ and Tau pathology can be detected by multi-photon excitation at a very early stage, indicating that the fluorescence approach

TABLE 2 | Reference list for *in vivo* fluorescence imaging in living mouse model.

Mouse model	Reagents and application for *in vivo* visualization of SPs or NFTs	Fluorescence reporters for *in vivo* visualization of AD pathophysiology	References
PDAPP (APP V717F)	Thioflavin S, anti-Ab w/Fluorescein (intracerebral injection)		Bacskai et al., 2001
Tg2576 (APPsw)	Thioflavin S, anti-Ab (10D5) conjugated Cy3 (intracerebral injection)		Christie et al., 2001
PS-APP (PS1M146L vs. Tg2576)	Methoxy-X04 (i.p.)		Klunk et al., 2002
PDAPPTg2576PS-APP	Thioflavin T, Thioflavin S, PiB (i.v.)		Bacskai et al., 2003
Tg2576	Methoxy-X04 (i.p.)		Robbins et al., 2006
PS1dEx9/APPsw	curcumin (i.v.) Methoxy-X04 (i.p.)		Garcia-Alloza et al., 2007
PS1L166P/APPsw	Methoxy-X04 (i.p.)		Hefendehl et al., 2011
3xTg-ADAPPdutch/iowaTg2576	–	fluo-4 AM	Takano et al., 2007
PS1dEx9/APPsw	Methoxy-X04 (i.p.)	YC3.60 camereon (AAV, intracerebral injection)	Kuchibhotla et al., 2008
PS45(PS1G384A)/APP23(sw)	Thioflavin S (intracerebral injection)	Oregon green BAPTA	Busche et al., 2008
PS1dEx9/APPsw	Methoxy-X04 (i.p.)	Oregon green BAPTA SR-101 for astrocyte	Kuchibhotla et al., 2009
PS45/APP23	Thioflavin S (intracerebral injection)	Fluo-8 AM	Busche et al., 2012
PS1dEx9/APPsw	Methoxy-X04 (i.p.)	CaMKII 5′UTR-EGFP/Venus-3′UTR (AAV, intracerebral injection)	Meyer-Luehmann et al., 2009
PS1dEx9/APPsw	Methoxy-X04 (i.p.)	Crossbreeding to Arc/dVenus mice	Rudinskiy et al., 2012
Tg2576	Methoxy-X04 (i.p.)	Crossbreeding to B6C3-YFP mice Crossbreeding to Cx3CR1/GFP mice	Meyer-Luehmann et al., 2008
PDAPP	Methoxy-X04 (i.p.)	Crossbreeding to Cx3CR1 mice	Koenigsknecht-Talboo et al., 2008
rTg4510 (Tau P301L)	Thioflavin S (intracerebral injection)	FLICA (intracerebral injection)	Spires-Jones et al., 2008
rTg4510 (Tau P301L)	Thiopflavin S (intracerebral injection) X-34 (i.v.)	FLICA (intracerebral injection)	de Calignon et al., 2010
PS19 (Tau P301S)	PBB3 (i.p., i.v.)		Maruyama et al., 2013
rTg4510 (Tau P301L)	Thioflavin S (intracerebral injection)	YC3.60 camereon (AAV, intracerebral injection)	Kopeikina et al., 2013
rTg4510 (Tau P301L)	Thioflavin S (intracerebral injection)	YC3.60 camereon (AAV, intracerebral injection)	Kuchibhotla et al., 2014

has a greater advantage for identifying beneficial molecular clues linking early pathological change in AD model animals.

Utility of Fluorescence Biosensors of AD Pathophysiology

Recently, unique functional fluorescence biosensors to monitor biophysical properties were developed for dissecting the detailed physiological status of animal brain. Since Green fluorescent protein (GFP) was discovered from jellyfish *Aequorea victoria* by Shimomura et al., many unique native fluorecent proteins emitting diverse colors have been isolated from multiple marine organisms (Shimomura et al., 1962). The improved fluorescent proteins in terms of wavelength, brightness, photostability, and less oligomeric property were successfully engineered by random mutagenesis analysis, and the fluorescent protein color palette has been extensively matured from blue to a near-infrared spectrum (Giepmans et al., 2006). Engineered fluorescent proteins with enhanced unique original properties (e.g., photo activation/conversion, environment sensitivity, etc. …) have also been created during this process, opening a new window to the utility of fluorescent proteins as molecular biosensors generally termed "genetically encoded fluorescence indicators" (GEFIs) to monitor a wide variety of biological parameters including calcium, voltage, ATP, cAMP, glutamate, and kinase activities (Knopfel, 2012; Miyawaki and Niino, 2015). In addition, approaches to the use of virus-mediated gene delivery or conventional animal genetics with specific promoter-dependent regulation allow us to manipulate specific subpopulations of neurons or glial cells, and GEFIs are certain to become a powerful and broadly used tool for dissecting the molecular pathophysiology of neurodegenerative disorders.

With the combination of fluorescence reporters and biosensors, attempts have been made to illuminate the more complicated AD-related pathophysiology by multi-photon excitation techniques (**Table 2**). Abnormal dendritic curvature and spine loss associated with SPs in brain of APP transgenic mouse were first illuminated in living neurons expressing GFP by either Adeno associated virus (AAV)-mediated manipulation or by crossbreeding with fluorescence reporter mouse (Spires et al., 2005). Using AAV-mediated overexpression of YC3.6 calcium sensor proteins, Aβ pathology-related aberrant overloading of neuronal calcium in the somatosensory cortex was then

detected with great temporal resolution (Kuchibhotla et al., 2008). Interestingly, local administration of Oregon-Green BAPTA (OGB), a small-molecule calcium dye, also revealed hyperactivity in neurons and disrupted synchronous calcium oscillation in the astrocyte network associated with Aβ pathology (Busche et al., 2008; Kuchibhotla et al., 2009). In addition, inhibition of Ca^{2+}/calmodulin-dependent protein phosphatase 2B (Calcineurin) attenuated the calcium dysregulation and structural abnormality of neurites. These findings suggest that dysfunction of calcium homeostasis is one of the key environmental parameters related to amyloid pathology. In addition to these observations, cross-breeding of APP transgenic mice with Cx3CR1 mice, which specifically express GFP in microglial cells, identified robust microglial activation associated with Aβ pathology (Meyer-Luehmann et al., 2008). Importantly, this immune response has been constantly observed during both the development of SPs and anti-Aβ immunization, suggesting that inflammation plays an essential role in the regulation of the dynamics of Aβ pathology (Koenigsknecht-Talboo et al., 2008). As an additional unique fluorescence reporter, transgenic mouse expressing destabilized Venus under control of immediate early gene Arc promoter was generated to identify disrupted orchestrated neuronal network activity in an APP transgenic mouse model (Rudinskiy et al., 2012). Taken together, these studies strongly indicate the power of the fluorescence system in combination with the current genetics approaches to provide major insights into the AD pathophysiology. Using the fluorescence indicator of caspase activation (FLICA), it was also demonstrated that the majority of caspase-positive neurons contain NFTs in brain of rTg4510 tau transgenic mouse (Spires-Jones et al., 2008). Interestingly, infrequent NFTs-negative neurons demonstrated rapid suppression of caspase activity followed by NFTs formation within 1 day (de Calignon et al., 2010). This indicates that NFTs formation potentially has a neuroprotective role against caspase-mediated neuronal apoptosis. The other surprising observation was that rTg4510 tau transgenic mouse demonstrated normal calcium dynamics despite massive synaptic loss in both sensory and visual cortex, suggesting that tau-related synaptic abnormality was induced by a calcium-independent mechanism (Kopeikina et al., 2013; Kuchibhotla et al., 2014). Dissection of the brain status of tau transgenic mouse model by *in vivo* fluorescence imaging is still at a developmental stage, and therefore additional studies with fluorescence reporter can be expected to further expand the future potential and contribution of the examination of the tau-related pathophysiology of AD.

Limitations of Fluorescence Imaging

As we summarize in this section, fluorescence-based optic imaging has become an attractive approach to simultaneously monitor the interaction between target molecules and disease-related pathophysiology with high spatiotemporal kinetics, and to offer solid molecular-based insight for dissecting the *in vivo* mechanism underlying the progression of the neurodegenerative process in AD model animals. It also offers a relatively simple and inexpensive system that can be relatively easy to install into laboratory space, and potentially provide powerful biomedical applications such as *in vivo* high-throughput drug screening. However, considering future *in vivo* applications, there are still many technical challenges and space that will require solutions. First, the issue of optical diffusion tissue *in vivo* still strongly restricts the visualization of fluorophore in only the surface area even in mouse brain (reviewed in Svoboda and Yasuda, 2006). Second, the potential invasiveness and toxicity caused by both the surgery process and local genetic manipulation limit the usage of these techniques in animals, although not in human (reviewed in Hillman, 2007). Third, it is also important to note that overexpression of fluorescent reporter proteins potentially has undesirable photostability, toxicity, and mislocalization (reviewed in Shaner et al., 2005). Based on an understanding of these advantages and disadvantages, we will then consider how to integrate each of the imaging modalities in the future as described in the last section.

PERSPECTIVES FOR FUTURE IMAGING MULTIMODALITY

PET Imaging for Early Detection of AD Pathology

The current successful establishment of Aβ and tau PET radiotracers in combination with the available biomarkers promises the availability of PET imaging for the clinical diagnosis of AD. The next challenge for future PET neuroimaging is the design of more advanced chemical probes that specifically recognize the distinct conformational and/or modification status of aggregates. In fact, it is known that PiB efficiently detects SPs in AD brain but not in mouse brain, suggesting that a modification process in different biological species can influence the *in vivo* binding capability of the tracer (Klunk et al., 2005). Recent studies suggest that not filamentous but soluble oligomeric aggregates of Aβ and tau are toxic species during the pathological course of AD (Cheng et al., 2013). In particular, tau protein undergoes multiple post-translational modifications such as phosphorylation, nitration, or truncation, which are predicted to associate with the pathogenic mechanism(s) of AD (Marcus and Schachter, 2011). Using *in vitro* aggregation assay, several groups have successfully identified unique chemical compounds that preferentially bind to intermediate aggregated species of Aβ and tau (Wischik et al., 1996; Bolognesi et al., 2010; Smith et al., 2010; Carrasco-Gallardo et al., 2012; Jameson and Dzyuba, 2013; Teoh et al., 2015). More recently, pentameric formyl thiophene acetic acid (pFTAA) was screened to successfully visualize the conformation specific status of tau aggregates in living dorsal root ganglion neurons isolated from tau transgenic mouse model (Brelstaff et al., 2015). Therefore, some chemical compounds that potentially have specific binding affinity to toxic oligomer or modified species of Aβ and tau might be more suitable tracers for the early detection of AD pathology. Various intermediates of tau aggregates have been isolated during the pathological course of NFTs formation, and it has been highly debated whether soluble oligomeric aggregates of tau are toxic species (Makrides et al., 2003; Maeda et al., 2006; Sahara et al., 2007; Peterson et al., 2008; Lasagna-Reeves et al., 2010; Bader et al., 2011; Patterson et al., 2011). It has also been hypothesized that the formation process of intracellular tau inclusion potentially has a trophic role against

tau toxicity (Spires-Jones et al., 2011; Cowan and Mudher, 2013). In this regard, to segregate the early pathological tau species that are toxic vs. those that are more neuroprotective would be a more challenging, but also a more attractive investment for advanced preclinical diagnosis. A fluorescence-based imaging approach will have a great advantage in addressing this fundamental issue. Current elegant studies have also demonstrated a cell-based fluorescence system that can detect the very early stage of tau aggregates isolated from juvenile tau transgenic mouse brain by fluorescence resonance energy transfer (FRET), pointing out its potential complementary application as a more physiological drug screening system (Holmes et al., 2014). Taken together, it is clearly important to expand multimodal imaging approaches to characterize the basic capability of chemical compounds, and this will eventually improve the screening efficiency and open attractive directions for more sophisticated molecular diagnoses with appropriate data interpretation.

Genetic Manipulation of Animal Models

To follow this multimodal approach, a key component would be advancing technical innovation for genetic manipulation. Many transgenic/knock-in mouse models overexpressing mutant APP, PSs, and/or Tau have been established during the last two decades, and a tremendous amount of fundamental knowledge about the AD cascade has been acquired. However, these classical genetic systems still have many limitations, as follows. First, it is quite difficult to reproduce the spatiotemporal dynamics of the AD pathological progression in these classical models because transgene overexpression is controlled by specific promoter activity. Second, it is still expensive and time-consuming to generate mouse lines, and technical innovation to improve productivity will be required. Third, rodent models may in fact represent the ultimate limitation to the reproduction of the human brain circumstances because of the species barrier. As an advanced mouse model, a drug inducible Cre-loxP system, or a Tet on/off system, has been incorporated into mouse genetics to achieve more precise conditional control of transgene expression (Santacruz et al., 2005). In recent studies, P301L mutant Tau was strictly expressed in Layer II/III neurons in the entorhinal cortex by neuropsin promoter in combination with a tetracycline inducible system (de Calignon et al., 2012; Liu et al., 2012). An interesting finding was that this region-specific expression of tau causes progressive spreading of NFTs pathology along the tri-synaptic entorhinal-hippocampus circuit in an anterograde manner, suggesting the presence of toxic tau species that can be transmitted via trans-synaptic communication. This prion-like propagation theory is further supported by studies with *in vivo* administration of fibrillar tau aggregates into mouse brains resulting in the spread of tau pathology to synaptically connected distant brain regions (Clavaguera et al., 2009, 2013; Lasagna-Reeves et al., 2012; Iba et al., 2013). As an alternative approach, the AAV system has become a widely used technique to achieve spatiotemporal manipulation of transgene expression. In fact, circuit-dependent pathological spreading of tau pathology was also recently achieved by local administration of AAV-encoding mutant tau into the entorhinal cortex of adult rodent (Siman et al., 2013). Therefore, a very fascinating approach would be to generate a tau mouse model genetically modified in the local cell population of a specific neuronal circuit, and to trace the *in vivo* spatiotemporal consequence of tau propagation in the brain of the same individual living animal by fluorescence and PET simultaneously, which would potentially facilitate further understanding of non-cell autonomous toxic mechanisms shared among various neurodegenerative disorders.

Very recently, an artificial nuclease-mediated gene edition presented as the CRISPR/CAS9 system has rapidly expanded its utility for the generation of animal models from insects to a primate system (Sander and Joung, 2014). Using such innovative tools, the establishment of genetic imaging reporter animals is one of the attractive directions for future AD research. Several studies have demonstrated that herpes simplex virus type 1 thymidine kinase (HSV1-TK)-mediated phosphorylation and intracellular accumulation of nucleoside analogs such as 9-(4-fluoro-3-hydroxymethylbutyl) guanine (FHBG) is a useful PET reporter system for assessing gene expression, protein-protein interactions, and enzymatic activity (Gambhir et al., 2000; Luker et al., 2002; Massoud et al., 2010). Current advances in chemical biology are also creating the potential availability of multiple new technologies such as advanced affinity tag and light manipulation for various *in vivo* imaging applications (Kramer et al., 2013). Therefore, the combination of these genetic manipulations is certain to contribute to the efficient development of advanced animal model systems, and will allow us to conduct simultaneous multimodal imaging comparisons to monitor the sequential pathophysiology of AD progression.

CONCLUDING REMARKS

Multimodal neuroimaging may open an attractive direction for future translational research, and allow us to simultaneously monitor the actual behavior of target molecules of interest in living subjects from a microscopic to a macroscopic view. Based on the current progress of PET and the fluorescence system, there are still a lot of areas and issues to be addressed, and integration of these very different techniques will still present some challenging hurdles. At this moment, all we need is to sincerely evaluate both the advantages and disadvantages of individual imaging systems, and to consider ways to complement these very different techniques by seeking even small improvements in a step-by-step manner. Eventually, this steady work and effort will result in a breakthrough in the area of advanced biomedical research including future diagnostics.

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Researching the data for the article: MS and NS. Drafting of the manuscript: MS. Discussion of the content: MS, MH, TS, and NS. Review and editing of the manuscript: MH, TS, and NS.

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Alternative neural circuitry that might be impaired in the development of Alzheimer disease

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It is well established that some individuals with normal cognitive capacity have abundant senile plaques in their brains. It has been proposed that those individuals are resilient or have compensation factors to prevent cognitive decline. In this comment, we explore an alternative mechanism through which cognitive capacity is maintained. This mechanism could involve the impairment of alternative neural circuitry. Also, the proportion of molecules such as Aβ or tau protein present in different areas of the brain could be important.

Keywords: Alzheimer disease (AD), cognitive decline, tau proteins, abeta, tauopathies

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Introduction

Loss of episodic memory is the most well known feature of Alzheimer disease (AD). Braak and Braak (1996), suggested that damage of the connections between the entorhinal cortex (EC) and hippocampal area could play an important role in the memory impairment of AD (Gomez-Isla et al., 1996). Within the hippocampus, other studies have suggested that the CA1 hippocampal subregion could be the minimal region that is required for acquisition of episodic memory (Zola-Morgan et al., 1986; Volpe et al., 1992; Tsien et al., 1996; Shimizu et al., 2000; Bendel et al., 2005; Buenz et al., 2006; Bueters et al., 2008). There are several works indicating the possible (and different) pathways that connect the EC with CA1 (for a review see Moser et al., 2014 and **Figure 1**). Some of these pathways go through the dentate gyrus, in which adult neurogenesis could be involved in the formation of new memories (Zola-Morgan et al., 1986; Deng et al., 2010).

Damage to the EC and hippocampal region in AD is associated with the appearance of senile plaques and neurofibrillary tangles, aberrant structures first described by Alzheimer (1907). Indeed, it is typically considered that a proper diagnosis of AD is only really complete when, at autopsy, the appearance of plaques and tangles are observed in the brain of the patient (Khachaturian, 1985).

However, the causal nature of the correlation between appearance of plaques and loss of memory (or cognitive impairment) is not clear. Thus, it has been proposed that the disease should be treated, not the lesions (Zhu et al., 2007), because the pathology may be a protective, possibly antioxidant response to the primary pathogenesis. Furthermore, studies on familial AD (FAD) cases reveal an asymptomatic phase in which there are plaques without cognitive impairment (Bateman et al., 2012), although it may also suggest that the presence of plaques is a first step that could favor the subsequent onset of the disease and that their presence is not immediately related to cognitive impairment (Bateman et al., 2012).

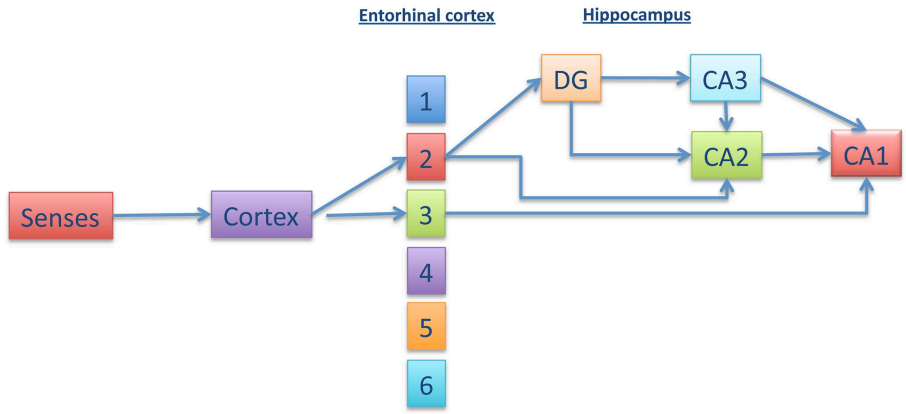

FIGURE 1 | Different pathways connecting entorhinal cortex with CA1. Information from the senses is transmitted to the cortex and, afterwards, to the enthorhinal cortex (EC) (upper layers). From EC, the information could go directly to CA1 or, indirectly, through dentate gyrus (DG), CA3 (or CA2), and CA1. This trisynaptic pathway involves adult neurogenesis.

Plaques and Cognitive Impairment

There is controversy surrounding a total correlation between the presence of plaques and the presence of cognitive impairment. Some time ago, Katzman et al described cases of cognitively normal people bearing plaques (Katzman et al., 1989). In a more recent study, analyzing those people without cognitive impairment, it was found that some people met the criteria for high likelihood AD, based on the presence of plaques (Bennett et al., 2006), indicating that AD pathology can be found in the brain of those without cognitive impairment (Aizenstein et al., 2008). Some functional magnetic resonance analysis have also reported the presence of Aβ aggregates in people without cognitive impairment (Dickerson et al., 2004; Sperling et al., 2009; Mormino et al., 2012).

More recently, Elman et al reported that some older people may maintain normal cognition despite the presence of plaques observed by positron emission tomography (PET) analyses (Elman et al., 2014). There are at least two additional explanations from those discussed above, a possible resilience, based on personal characteristics such as having a higher cognitive reserve (Xu et al., 2014), or compensation of a degenerated pathway by using an alternative functional pathway (Elman et al., 2014). In addition, it can be suggested that failure of more than one neuronal circuit can be needed for cognitive impairment.

This led us to consider another alternative to that involving the connections from EC to CA1, indicated in **Figure 1**, an alternative that might prevent cognitive decline.

In the work of Elman et al. (2014), there are explanations that involve different types of connections, based on fMRI studies, suggesting that CA1 can connect with the cortex without going through the EC. In those studies, Elman et al. (2014) took advantage of the system by analyzing two networks that were previously identified. One of them links those areas that respond to a specific cognitive activity, like visualization of a photograph (Fox et al., 2005; Xu et al., 2014). This is the task-positive network (TPN) (Fox et al., 2005). The other one indicates the regions that are activated in the resting state (Buckner et al., 2008). This is the default mode network (DMN). The DMN can be disrupted in some neurodegenerative disorders (Buckner et al., 2008).

In human subjects, with normal cognition and no plaques, engaging in a task activates the TPN while the DMN shuts down. However, Elman et al. (2014) found that in the same conditions, people with plaques and without cognitive impairment also displayed higher TPN activity but—critically—DMN was less deactivated. The authors focused on this decrease of DMN shut down to try to explain the maintenance of cognition. Indeed, there are several works, using fMRI analysis, or other image techniques, indicating the connection between dysfunction of DMN and cognitive decline. We include some examples of those works (Petrella et al., 2011; Wang et al., 2013; Garces et al., 2014; Gardini et al., 2015).

Alternative Pathways to CA1

The activated areas in DMN include prefrontal cortex and posterior cingulate cortex (Buckner et al., 2008). Cingulate cortex is connected to other structures involved in memory, including the mammillary bodies (MB) (Vann and Aggleton, 2004; Shah et al., 2012). A connection between MB and CA2, which in turn is connected to CA1, has also been found (Haglund et al., 1984; Kohara et al., 2014). Thus, we propose this MB-CA2-CA1 circuit as an alternative pathway through which information from the cerebral cortex can reach CA1 and avoid the EC (**Figure 2**).

We suggest that, for severe cognitive impairment to occur, disruption of both circuits, that involving EC-CA1 and that involving cingulate cortex-CA1, is needed. Indeed, abnormal connectivity between the posterior cingulate and hippocampus has been described in patients with cognitive impairment (Zhou et al., 2008). In addition, it has been also indicated that a decrease in the functional connectivity at the posterior cingulate cortex can be critical for the conversion from mild cognitive impairment to AD (Bozzali et al., 2014). Also, in the pioneering works of Stern (2002, 2006) on resilience (cognitive reserve) or compensation,

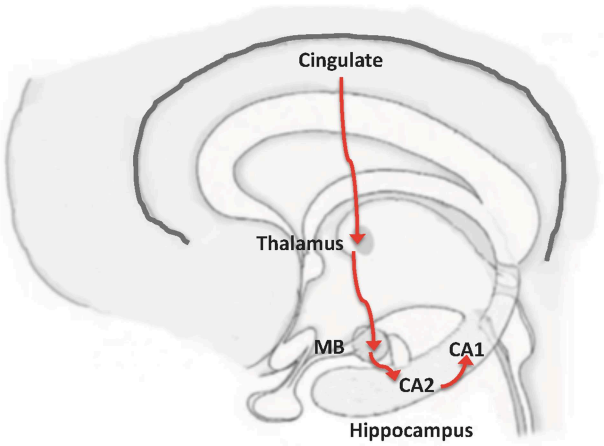

FIGURE 2 | From neocortex to hippocampus, by-passing entorhinal cortex: the MB-CA2-CA1 pathway. A possible pathway to connect the cortex with CA1, without transmission through EC, could be based on a proposed connection between mammillary bodies (MB) to CA2, and from CA2 to CA1 (Haglund et al., 1984; Kohara et al., 2014). In this way, the previously known connection shown in the Figure could be complemented by the following pathway: Cortex ➔ cingulum ➔ mammillary bodies ➔ CA2 ➔ CA1.

the loss of connectivity at the posterior cingulate cortex was already proposed. In this comment, our main focus is not on resilience (see on that subject the recent work of Pereira et al., 2014), but on compensation.

If, indeed, there is first a loss of connectivity at the hippocampal area (location of the dentate gyrus), followed by a loss of connectivity at the posterior cingulate cortex (Zhou et al., 2008), risk factors, such as aging, could also play a role in this process. It has been recently reported that there is a brain network that links development, aging, and vulnerability to AD (Douaud et al., 2014). This link is based on the so-called Ribot's law indicating that the destruction of memories progresses in reverse order to that of their formation (Douaud et al., 2014). Brain structures, like dentate gyrus, that play a role in recent memory, are assembled very late in development but can lose their functionality very early in the neurodegeneration process.

We suggest that, it is possible that both mechanisms, that involving EC-CA1 and that involving posterior cingulate cortex-CA1, could be damaged and are needed to develop a severe cognitive impairment and AD. Thus, both mechanisms should be needed for disease progression.

Distribution of Aβ and Tau in Cingulate Gyrus and Enthorinal Cortex

As previously indicated, the brain of AD patients contain senile plaques, composed of amyloid-beta peptide (Aβ) and neurofibrillary tangles, containing tau protein polymers. By looking at the causes of FAD, genetic analyses have indicated that mutations in APP (amyloid precursor protein), PSEN-1 (presenilin 1) or PSEN-2 (presenilin 2) genes are the cause of the different types of familial disease. The main consequence of these mutations is an increased production of Aβ. This observation resulted in the proposed amyloid cascade hypothesis of AD. This hypothesis indicates that Aβ accumulation in brain is the primary factor driving AD pathology (Selkoe, 1991; Hardy and Higgins, 1992).

Afterwards, it was shown that the presence of tau protein is essential to the amyloid-beta induced neurotoxicity, occurring in AD (Rapoport et al., 2002; Roberson et al., 2007; Ittner et al., 2010). Based on these and other reports, it was suggested that Aβ could initiate the pathological process but the presence of tau is needed for the progression of the process. In this way, the cell co-localization of Aβ and tau could increase the possibility of neuronal damage.

At the molecular level, a double dissociation in regional distribution of tau and amyloid-beta has been reported when comparing cingulate gyrus and enthorhinal cortex in post-mortem Alzheimer's brains (Shukla and Bridges, 1999). It was described that tau load was almost twice as great in the enthorinal cortex than elsewhere in the brain, whereas Aβ levels were much higher in the cingulate gyrus compared to enthorinal cortex (Shukla and Bridges, 1999). It should be known if those differences may play a role in the development of the disease, mainly within the cingulate cortex, although we know that EC and posterior cingulate cortex could be interconnected and that modified tau could spread from EC to the cingulate cortex (Yassa, 2014). That transport time of modified tau from EC to posterior cingulate cortex may determine the progression time of the disease during the transition from MCI to AD.

In many transgenic animal models, there are no differences in the expression of tau or Aβ at specific locations of the brain. This is because, sometimes, the expression of tau or APP mRNAs is under strong promoters and it may facilitate the expression of a protein throughout the whole brain in a non-physiological way. Thus, in these animal models the progression of degeneration may take place in a different fashion than that occurring in the human disease.

If both, Aβ and tau aggregates, play a common, important role in the pathogenesis of AD, it will be of interest to determine the localization and overlap of those aggregates in different brain regions, given that the presence of plaques in the absence of tau, in some regions, might not be sufficient for cognitive decline. In this way, decreasing the amount of tau at specific brain regions could have a therapeutic function if, as indicated, the common presence of Aβ and tau aggregates is needed for the progression of the disease. Determining Aβ and tau overlap may, however, be complex as clear differences in the amount of tau can be found even between subregions of memory-related brain areas, e.g., hippocampus CA1 (high tau amount), vs. CA3 and CA2 (low tau amount) (our preliminary results). In addition, the presence (or absence) of other molecules related to Aβ or tau pathology could play a role in the development of neurodegeneration. A recent example of this is the telomerase protein TERT that apart from being protective against oxidative damage has a protective role against tau pathology (Spilsbury et al., 2015). Previously it was reported that a coordinated expression of tau and heme oxygenase 1 may play a pivotal role in the cytoprotection of neuronal cells (Takeda et al., 2004). The oxidative imbalance in AD has been extensively reviewed (Zhu et al., 2005; Mondragon-Rodriguez et al., 2013).

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REVIEW
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Chronic Glutamate Toxicity in Neurodegenerative Diseases—What is the Evidence?

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Together with aspartate, glutamate is the major excitatory neurotransmitter in the brain. Glutamate binds and activates both ligand-gated ion channels (ionotropic glutamate receptors) and a class of G-protein coupled receptors (metabotropic glutamate receptors). Although the intracellular glutamate concentration in the brain is in the millimolar range, the extracellular glutamate concentration is kept in the low micromolar range by the action of excitatory amino acid transporters that import glutamate and aspartate into astrocytes and neurons. Excess extracellular glutamate may lead to excitotoxicity *in vitro* and *in vivo* in acute insults like ischemic stroke via the overactivation of ionotropic glutamate receptors. In addition, chronic excitotoxicity has been hypothesized to play a role in numerous neurodegenerative diseases including amyotrophic lateral sclerosis, Alzheimer's disease and Huntington's disease. Based on this hypothesis, a good deal of effort has been devoted to develop and test drugs that either inhibit glutamate receptors or decrease extracellular glutamate. In this review, we provide an overview of the different pathways that are thought to lead to an over-activation of the glutamatergic system and glutamate toxicity in neurodegeneration. In addition, we summarize the available experimental evidence for glutamate toxicity in animal models of neurodegenerative diseases.

Keywords: glutamate receptors, glutamate transporters, system x_c^-, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, neurodegeneration, excitotoxicity

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INTRODUCTION

L-glutamate (L-glu) is the major excitatory neurotransmitter in the brain and is functionally involved in virtually all activities of the nervous system. In this section, we will first outline the general principles of L-glu signaling in the brain. Then, we will broaden this scheme by expanding the model to (a) different pools of extracellular glutamate (synaptic, perisynaptic, and extrasynaptic) resulting from either vesicular and non-vesicular sources or atypically located glutamate receptors outside of synapses and (b) other molecules present in the brain that have the ability to activate glutamate receptors and discuss their possible physiological functions.

Glutamate Signaling in the Brain

The human brain contains 6–7 μmol/g wet weight of L-glu (Perry et al., 1971; Lefauconnier et al., 1976). Thus, L-glu along with glutamine is the most abundant free amino acid in the central nervous system. More than five decades ago, Curtis et al. demonstrated that L-glu has an excitatory action

on nerve cells (Curtis et al., 1960). Since then, its role as an excitatory neurotransmitter as well as its cerebral metabolism have been studied in detail (reviewed by Fonnum, 1984; Hertz, 2006; Marmiroli and Cavaletti, 2012; Zhou and Danbolt, 2014).

L-glu is concentrated in synaptic vesicles in the presynaptic terminal by the action of vesicular glutamate transporters (vGLUT; Takamori, 2006). In addition, some of the L-glu in the vesicles might be generated by a vesicle-associated aspartate amino transferase from 2-oxoglutarate using L-aspartate (L-asp) as the amino group donor (Takeda et al., 2012). Upon depolarization of the presynaptic membrane, L-glu is released into the synaptic cleft and binds to ionotropic glutamate receptors (iGluRs) at the postsynaptic membrane (**Figure 1**). iGluRs are ligand-gated ion channels and include receptors of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, and N-methyl-D-aspartic acid (NMDA) types (reviewed in Lodge, 2009). While AMPA and kainate receptors primarily mediate sodium influx, NMDA receptors have high calcium conductivity. Activation of NMDA receptors plays an important role in synaptic plasticity and learning (reviewed in Miyamoto, 2006). In contrast to the other iGluRs, the activity of NMDA receptors is inhibited by a so-called Mg^{+2} block at the regular membrane potential but the ion channel is readily de-blocked by membrane depolarization, which removes Mg^{+2} from the pore (Vargas-Caballero and Robinson, 2004). NMDA receptors are tetramers containing two NR1 subunits and two NR2 or NR3 subunits (Paoletti and Neyton, 2007).

In addition to iGluRs, eight isoforms of metabotropic glutamate receptors (mGluRs; Spooren et al., 2010) exist, which belong to the family of G-protein-coupled receptors and do not form ion channels but rather signal via various second messenger systems (reviewed in Spooren et al., 2010). L-glu-induced depolarization leads to a postsynaptic excitatory potential which facilitates the generation of an action potential at the axon hillock. The glutamatergic synapse is ensheathed by astrocytic processes that express high levels of excitatory amino acid transporters (EAATs; Chaudhry et al., 1995). Five different EAATs exist, EAAT1-5, of which EAAT1 and 2 are the primary astrocytic EAATs, whereas EAAT3 shows a predominantly neuronal expression (Zhou and Danbolt, 2013). About 90% of the L-glu transport is mediated by EAAT2 (aka GLT-1 in rodents). These transporters co-transport 2 or 3 molecules of Na^+ and a proton with each molecule of L-glu (or L-asp) in conjunction with the counter-transport of a K^+ ion (Zerangue and Kavanaugh, 1996). Thus, by using the electrochemical gradient of these ions across the plasma membrane as an energy source, the transporters are capable of effectively accumulating L-glu and L-asp in cells against their steep intra- to extracellular concentration gradients. This allows the brain to maintain a very low extracellular L-glu concentration in the low micromolar range (Baker et al., 2003; De Bundel et al., 2011). It is generally thought that L-glu taken up by astrocytes is converted to glutamine by the enzyme glutamine synthetase, the glutamine is then released, taken up by neurons and converted to L-glu and used once again for neurotransmission (Coulter and Eid, 2012).

The Role of Extrasynaptic Glutamate in the Brain

Apart from the role of L-glu as the principal excitatory neurotransmitter released from glutamatergic presynapses as described above, it has become apparent that L-glu receptors outside the synaptic cleft also play an important role in brain physiology (Papouin and Oliet, 2014). In the cerebellum, it was demonstrated by analyzing AMPA receptor-mediated currents in Bergmann glia that synaptically released L-glu concentrations can reach extrasynaptic concentrations of up to 190 μM, while concentrations in the synaptic cleft exceed 1 mM (Dzubay and Jahr, 1999). Moreover, some mGluRs have been shown to exhibit a distinct localization in proximity to the postsynaptic density that would allow them to readily detect L-glu leaking from the synaptic cleft (Luján et al., 1997; **Figure 1**). However, in recent years, evidence has accumulated that iGluRs, especially of the NMDA type, are also present at extrasynaptic sites in the neuronal cell membrane (Papouin and Oliet, 2014). Using light and electron microscopy, Petralia et al. showed that extrasynaptic NMDA receptors cluster at distinct sites of close contact of the dendritic shaft with either axons, axon terminals, or astrocytic processes (Petralia et al., 2010). The proportion of extrasynaptic NMDA receptors was estimated to be as high as 36% of the dendritic NMDA receptor pool in rat hippocampal slices (Harris and Pettit, 2007). Although extrasynaptic NMDA receptors were associated with similar scaffolding proteins as synaptic NMDA receptors (Petralia et al., 2010), an *in vitro* study suggested that extrasynaptic and synaptic NMDA receptors may activate different downstream signaling pathways with contrasting results: suppression of CREB activity by extrasynaptic NMDA receptor activation but activation by synaptic NMDA receptors (Hardingham et al., 2002). Functionally, NMDA receptors localized extrasynaptically on dendritic shafts bind extrasynaptic L-glu and mediate Ca^{2+} influx upon relief of the Mg^{+2} block by dendrite depolarization upon backfiring of action potentials (Wu et al., 2012). Angulo et al. showed that L-glu release from astrocytes can activate so-called slow inward currents via extrasynaptic NMDAR receptors in CA1 neurons which thereby can be synchronized (Angulo et al., 2004). Thus, the mechanisms through which glial cells release L-glu as well

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; ADME, absorption, distribution, metabolism, and excretion; ALS, amyotrophic lateral sclerosis; ALS/PDC, Guamian amyotrophic lateral sclerosis/Parkinson-dementia complex; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANA, anthranilic acid; APP, amyloid precursor protein; Asc-1, alanine-serine-cysteine amino acid transporter 1; L-asp, aspartate; BMAA, β-*N*-methylamino-L-alanine; BOAA, β-*N*-oxalylamino-L-alanine; Cef, ceftriaxone; CREB, cyclicAMP response element-binding protein; CSF, cerebral spinal fluid; CNS, central nervous system; DAO, D-amino acid oxidase; DIV, days *in vitro*; EAAT, excitatory amino acid transporter; GFAP, glial fibrillary acidic protein; iGluR, ionotropic glutamate receptor; L-glu, glutamate; 3HANA, 3-hydroxyanthranilic acid; 3-HAO, 3-hydroxyanthranilate oxidase; L-HCA, homocysteate; HD, Huntington's disease; 3HK, 3-hydroxykynurenine; Htt, huntingtin protein; IDO, indoleamine 2,3-dioxygenase; KAT, kynurenine aminotransferase; KMO, kynurenine monoxygenase; KYN, kynurenine; KYNA, kynurenic acid; MAPK, mitogen activate protein kinase; mGluR, metabotropic glutamate receptors; MSN, medium spiny neurons; NMDA, N-methyl-D-aspartic acid; QUIN, quinolinic acid; SOD1, superoxide dismutase 1; SSZ, sulfasalazine; TBI, traumatic brain injury, Tg, transgenic, TSC, tuberous sclerosis complex; vGLUT, vesicular glutamate transporters; YAC, yeast artificial chromosome.

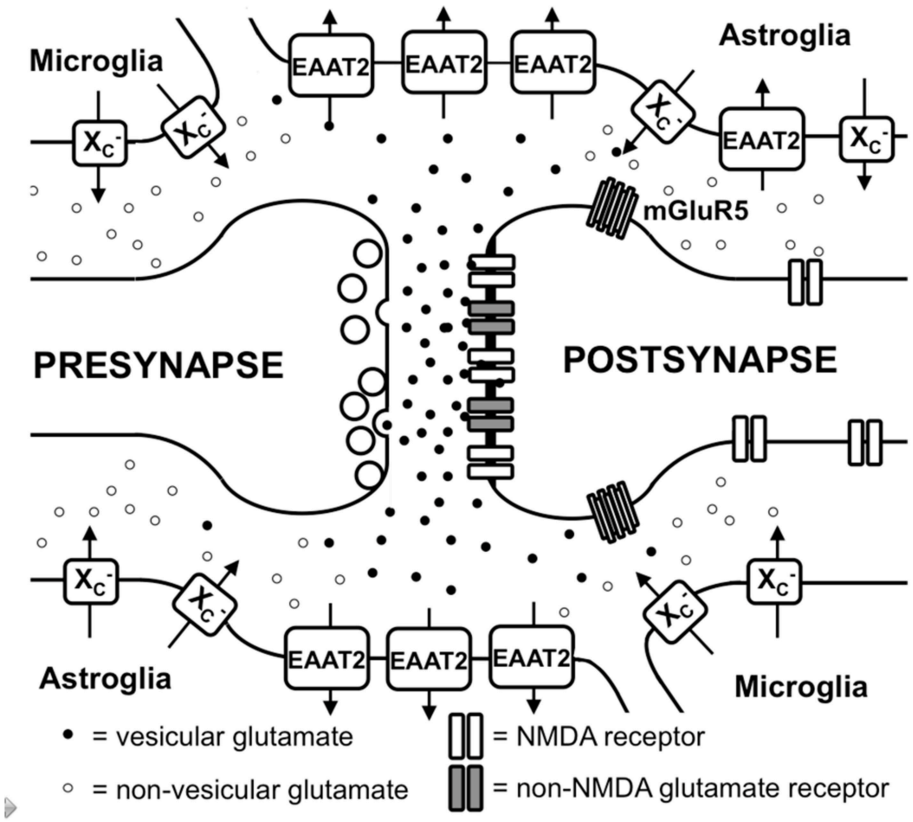

FIGURE 1 | Glutamate metabolism in the brain. Vesicular glutamate is synaptically released and binds to ionotropic glutamate receptors at the postsynapse. Glutamate is rapidly taken up by astrocytic glutamate transporters, especially EAAT2. Spillover from the synaptic cleft can activate perisynaptic mGluR5. Migroglia and astrocytes non-vesicularly release glutamate into the extrasynaptic extracellular space via system x_c^- (x_c^-) where it can activate extrasynaptic NMDA receptors.

as how the extrasynaptic L-glu concentrations are regulated are pivotal to understanding how the activity of extrasynaptic NMDA receptors are regulated.

Different mechanisms through which astrocytes can release L-glu have been proposed: vesicular L-glu release (Adak et al., 2000) and non-vesicular release via anion channels (Wang et al., 2013) and connexin hemichannels (Stehberg et al., 2012) as well as release via the cystine/glutamate antiporter system x_c^- (Massie et al., 2015). Data by Wang et al. strongly suggest that vesicular release from astrocytes plays a minor role, as the Ca^{+2}-mediated release of L-glu was still present in astrocytes derived from dominant-negative SNARE mice (Wang et al., 2013) where vesicular release can be blocked by doxycycline withdrawal (Pascual et al., 2005). System x_c^- is a cystine/glutamate antiporter which belongs to the class of heterodimeric amino acid transporters, consisting of xCT as the specific subunit and 4F2hc as the promiscuous heavy chain (Sato et al., 1999). This transporter is expressed in the brain, especially in astroglial and microglial cells (Fogal et al., 2007; Mesci et al., 2015; **Figure 1**). The fact that extrasynaptic L-glu levels in different areas of the brain are downregulated by approximately 60–70% in xCT knock out mice (De Bundel et al., 2011; Massie et al., 2011) indicates that system x_c^- releases L-glu into the extrasynaptic space and demonstrates that this transporter is important in the regulation of extrasynaptic L-glu levels. This is further supported by the observation that when measured by *in vivo* microdialysis, the rise in extrasynaptic L-glu induced by EAAT inhibitors is neutralized by blocking system x_c^- while blocking neuronal vesicular L-glu release is ineffective (Baker et al., 2002; Melendez et al., 2005).

Taken together, glutamatergic neurotransmission not only occurs via classical excitatory synapses but also via extrasynaptic L-glu receptors (**Figure 1**). Moreover, the levels of extrasynaptic L-glu are determined, at least in part, by glial non-vesicular L-glu release (**Figure 1**). However, the regulation of extrasynaptic L-glu levels as well as its temporal-spatial dynamics and its impact on neuronal function, neurodegeneration, and behavior are far from being fully understood.

Other Molecules that are Physiologically Present in the Brain and May Activate Glutamate Receptors

Very early studies indicated that L-asp, like L-glu, has an excitatory action on neurons (Curtis et al., 1960). L-asp co-localizes with L-glu in the synaptic vesicles of asymmetric excitatory synapses (Gundersen et al., 1998). However, the total concentration in the brain (0.96–1.62 μmol/g wet weight)

(Perry et al., 1971; Lefauconnier et al., 1976), the extracellular concentrations in the cortex as measured by microdialysis (1.62 μM for L-asp and 9.06 μM for L-glu) and its distribution as determined by immunohistochemistry (Gundersen et al., 1991) indicate that L-asp is less abundant that L-glu. However, L-asp is a potent agonist on NMDA receptors but not other iGluRs with an EC_{50} only eight-fold higher than that of L-glu (Patneau and Mayer, 1990). EAATs that play an important role in the uptake of vesicularly released L-glu in the CNS (Tanaka et al., 1997; Petr et al., 2015) also avidly take up L-asp (Arriza et al., 1994). Thus, L-asp is probably not as important as L-glu with respect to the total excitatory tone mediated by iGluRs but must not be forgotten in this context. In addition to its role as a neurotransmitter, as mentioned above, L-asp is also required as a substrate for aspartate amino transferase that converts 2-oxoglutarate to L-glu for transport into the synaptic vesicles of glutamatergic neurons (Takeda et al., 2012) and thus might indirectly increase L-glu release.

One feature that distinguishes NMDA receptors from other iGluRs is that activation of NMDA receptors requires the binding of a co-agonist to the glycine binding site of the receptor (Johnson and Ascher, 1987). Within the spinal cord and the retina, the source of glycine might be spillover from glycinergic inhibitory synapses (Ahmadi et al., 2003; Kalbaugh et al., 2009). However, in other parts of the brain with high NMDA receptor expression, like the hippocampal formation (Monyer et al., 1994), responses mediated by strychnine-sensitive glycine receptors are absent, at least in adult neurons, indicating the lack of glycinergic inhibitory neurotransmission (Ito and Cherubini, 1991). However, glycine is present in the extracellular fluid in the hippocampus at baseline levels of approximately 1.5 μM (Yamamoto et al., 2010), which is near the saturation of the glycine binding site of the NMDA receptor (Johnson and Ascher, 1987), but can be dynamically up- and down-regulated (Yamamoto et al., 2010). The source of extracellular glycine in the hippocampus might be neurons, which release glycine via the alanine-serine-cysteine amino acid transporter 1 (asc-1; Rosenberg et al., 2013). However, glycine release by astrocytes, which is stimulated by depolarization and kainate, has also been reported (Holopainen and Kontro, 1989).

Even very early studies of the NMDA receptor and its co-activation by glycine showed that D-amino acids, especially D-serine, are almost as effective as glycine (Kleckner and Dingledine, 1988). Only a few years later, it became apparent that D-serine is present in rat and human brains at approximately one third of the concentration of L-serine with an absolute concentration of more that 0.2 μmol/g brain tissue (Hashimoto et al., 1992, 1993). Using an antiserum specific for D-serine, Schell et al. demonstrated that D-serine in the brain is exclusively localized in astrocytes and that its distribution matches the expression of NMDA receptors (Schell et al., 1995). Moreover, the same authors showed that D-serine is released from cultured astrocytes upon exposure to L-glu or kainate (Schell et al., 1995). The abundance of D-serine is determined by both the degrading enzyme D-amino acid oxidase (DAO), which shows high expression in the hindbrain, where D-serine levels are low, and the synthetic enzyme serine racemase that generates D-serine from L-serine (Schell et al., 1995; Wolosker et al., 1999). D-Serine seems to be stored in cytoplasmic vesicles in astrocytes and is released by exocytosis (Mothet et al., 2005). Long-term potentiation depends on D-serine release from astrocytes in hippocampal slices, indicating that this amino acid indeed plays an important role in glutamatergic neurotransmission via NMDA receptors (Henneberger et al., 2010). Also in hippocampal slices, Papouin et al. determined, using D-serine and glycine degrading enzymes, that D-serine serves as a co-transmitter for synaptic NMDA receptors on CA1 neurons whereas glycine serves as the endogenous co-agonist for extrasynaptic NMDA receptors (Papouin et al., 2012). This, however, is not a general rule as synaptic NMDA receptors of dentate gyrus neurons use glycine instead of D-serine as the co-agonist (Le Bail et al., 2015).

Taken together, multilayered evidence suggests that not only L-asp acting as an agonist on NMDA receptors but also glycine as well as D-serine play important roles in glutamatergic neurotransmission in the brain. However, other molecules have also been proposed to be biologically relevant modulators of glutamatergic neurotransmission.

L-homocysteate (L-HCA) shares structural similarities with L-glu. This non-protein amino acid is an oxidation product of homocysteine (Frauscher et al., 1995) which is biosynthesized from methionine by the removal of its terminal methyl group and is an intermediate of the transsulfuration pathway through which methionine can be converted to cysteine via cystathionine (McBean, 2012). Early studies showed that this amino acid can induce calcium influx in cultured neurons as efficiently as L-glu (Berdichevsky et al., 1983). Indeed, L-HCA showed a high affinity for NMDA receptors as compared to other iGluRs in binding assays which correlated with its ability to induce NMDA receptor antagonist-inhibitable excitotoxicity and sodium influx (Pullan et al., 1987). In addition, L-HCA is able to activate mGluR5 as effectively as L-glu (Shi et al., 2003). L-HCA is present in the brain although the concentrations were found to be about 500-fold lower than those of L-glu and 100-fold lower than those of L-asp in different areas of the rat brain (Kilpatrick and Mozley, 1986). Upon potassium-induced stimulation, L-HCA release is induced from brain slice preparations as observed for L-asp and L-glu although the absolute release of HCA is about 50-fold lower (Do et al., 1986). Interestingly, HCA is a highly effective competitive inhibitor of cystine and L-glu uptake via the cystine/glutamate antiporter system x_c^- (Bannai and Ishii, 1982; Patel et al., 2004), the activity of which regulates the extracellular extrasynaptic L-glu concentrations in the brain (De Bundel et al., 2011). Thus, the effect of L-HCA on the activation of NMDA and other L-glu receptors (Yuzaki and Connor, 1999) might also depend on the L-HCA-induced release of L-glu via system x_c^-. In summary, L-HCA may only play a limited role in the overall stimulatory input on L-glu receptors. However, this may change dramatically under some conditions, e.g., in patients with high-dose methotrexate therapy, an anticancer drug that, by inhibiting dihydrofolate reductase, inhibits the tetrahydrofolate-catalyzed recycling of methionine from homocysteine. Here, L-HCA concentrations of more than 100 μM have been documented in the cerebrospinal fluid whereas L-HCA was undetectable in control subjects (Quinn et al., 1997).

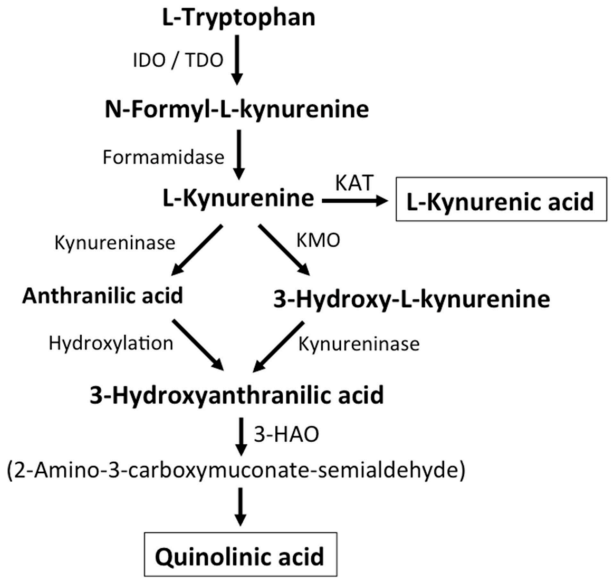

FIGURE 2 | Kynurenine metabolism. By either indoleamine-2,3-dioxygenase (IDO) or tryptophan-2,3-dioxygenase (TDO), tryptophan is converted to N-formyl-L-kynurenine, which is metabolized to L-kynurenine by formaminidase. L-kynurenine may be metabolized to kynurenic acid by kynurenine aminotransferase (KAT), to 3-hydroxy-L-kynurenine by kynurenine-3-monooxygenase or to anthranilic acid by kynureninase. 3-Hydroxy-L-kynurenine and anthranilic acid can both be converted to 3-hydroxyanthranilic acid. 3-Hydroxyanthranilate oxidase (3-HAO) metabolizes 3-hydroxyanthranilic acid to 2-amino-3-carboxymuconate-semialdehyde which is non-enzymatically converted to quinolinic acid. Inhibition of KMO results in increased production of kynurenic acid while the generation of quinolinic acid is inhibited (modified from Vécsei et al., 2013).

Additional endogenous small molecules that are thought to interfere with L-glu signaling are several intermediates of tryptophan metabolism (Vécsei et al., 2013; **Figure 2**). Via the action of indoleamine 2,3-dioxygenase (IDO) or tryptophan 2,3-dioxygenase (TDO), tryptophan is converted to N-formyl-L-kynurenine, which is then converted to kynurenine (KYN) by formamidase. From here three pathways, two of which merge at a later step, lead to further metabolism. First, via the action of kynurenine aminotransferase (KAT), KYN is converted to kynurenic acid (KYNA). KYN also can be converted to 3-hydroxykynurenine (3HK) by kynurenine monooxygenase (KMO), which can then be used as a substrate by kynureninase for the synthesis of 3-hydroxyanthranilic acid (3HANA). In addition, using KYN as a substrate, kynureninase produces anthranilic acid (ANA), which by non-specific hydroxylation can also be converted to 3HANA. 3HANA finally serves a substrate for the generation of quinolinic acid (QUIN).

The tryptophan concentration in rat brain is about 25 nmol/g wet weight and thus about 400-fold less than L-glu and 100-fold less than L-asp (Gál and Sherman, 1978; Kilpatrick and Mozley, 1986). The reported brain concentrations of kynurenines are even lower with 0.4–1.6 nmol/g for QUIN (Wolfensberger et al., 1983), 0.01–0.07 nmol/ml for KYNA (Moroni et al., 1988), and 0.016 nmol/g for 3HANA (Baran and Schwarcz, 1990). About 40% of brain KYN is locally synthesized (Gál and Sherman, 1978). The different metabolites of tryptophan show differential binding to plasma proteins and their transport via the blood-brain barrier is very different. KYN and 3HK are transported via the large neutral amino acid carrier system L (Fukui et al., 1991). Other kynurenines seem to enter the brain by passive diffusion. In addition, ANA, 3HANA and especially KYNA avidly bind to serum proteins, which limit their diffusibility across the blood-brain barrier.

As early as 1982, it was found that QUIN, when ionophoretically applied to rat cortex, induced neuronal firing that was inhibited by an NMDA receptor antagonist (Birley et al., 1982), indicating that QUIN may act as an NMDA receptor agonist. However, the EC_{50} for QUIN to activate NMDA receptor currents has been shown to be approximately 1000-fold higher than the EC_{50} of L-glu (Patneau and Mayer, 1990). Of note, intracerebral injection of QUIN has been shown to induce ultrastructural, neurochemical, and behavioral changes very similar to those induced by NMDA receptor agonists (Schwarcz et al., 1983). However, the fact that QUIN concentrations are about 5000- to 15,000-fold lower than cerebral L-glu concentrations makes it highly unlikely that modulation of NMDA receptor signaling by QUIN plays a significant role. KYNA was reported to act as an NMDA receptor antagonist (Ganong et al., 1983; Ganong and Cotman, 1986). However, although infusion with the KMO inhibitor Ro 61-8048 increased cerebral extracellular KYNA concentrations 10-fold, this did not lead to an inhibition of NMDA-mediated neuronal depolarization, a finding that challenges the notion that KYNA at near physiological levels directly modulates NMDA receptors (Urenjak and Obrenovitch, 2000). In contrast, increased KYNA in the brain induced by the KMO inhibitor JM6 decreased the extracellular cerebral L-glu concentration (Zwilling et al., 2011). In addition, KYNA levels in the extracellular cerebral fluid highly correlated with L-glu levels indicating that even at physiological or near physiological levels KYNA modulates L-glu metabolism (Zwilling et al., 2011). Both activation of the G-protein-coupled receptor GPR35 (Berlinguer-Palmini et al., 2013) and inhibition of presynaptic α7 nicotinic acetylcholine receptors have been implicated in the KYNA-induced reduction in L-glu release (Carpenedo et al., 2001). To summarize, although L-HCA and QUIN are present in the brain, their low concentrations speak against them having prominent roles in regulating glutamatergic neurotransmission. In contrast, although the pathways still have to be defined in more detail, evidence supports the view that KYNA can modulate L-glu release and thereby L-glu levels and neurotransmission.

THE CONCEPTS OF ACUTE AND CHRONIC GLUTAMATE TOXICITY

The term excitotoxicity was first used by Olney (1986) to describe the ability of L-glu, as well as structurally related amino acids, to kill nerve cells, a process that has been proposed to take place not only in acute but also chronic diseases of the central nervous system (for reviews Choi, 1988; Meldrum

and Garthwaite, 1990). Excitotoxicity results from the excessive activation of iGluRs and leads to a characteristic loss of post-synaptic structures including dendrites and cell bodies. There is a significant level of variation in the sensitivity of different nerve cells to the various iGluR agonists which is related to both the specific receptors expressed on the nerve cells as well as their distinct metabolisms. Moreover, the susceptibility of neurons to excitotoxicity can change tremendously with age. Acute excitotoxic nerve cell death is thought to occur in response to a variety of severe insults including cerebral ischemia, traumatic brain injury (TBI), hypoglycemia, and status epilepticus (Meldrum and Garthwaite, 1990; Meldrum, 1994). But what about neurodegenerative diseases where nerve cell death occurs over an extended period of time? Does chronic excitotoxicity also exist? In other words, could exposure of nerve cells to low but above normal concentrations of L-glu (or glutamatergic neurotransmission as a sum of the input via the various molecules involved as discussed above) over an extended period of time also eventually lead to nerve cell death?

Excitotoxicity was originally studied in animals but in order to understand the mechanisms underlying this process, cell culture models were developed (for review Choi, 1988). The basic cell culture model of acute excitotoxicity involves treating primary neurons in culture with L-glu or a specific iGluR agonist for a short time period (min) (e.g., Choi, 1992; Schubert and Piasecki, 2001) and then assessing downstream events at the time point that is most relevant for the study. For example, cell death is often determined after 24 h. While these types of studies have been shown to be very useful for understanding the pathways involved in acute excitotoxicity, it has proven much more difficult to study chronic excitotoxicity in culture partly because it is not entirely clear how to define "chronic" in the context of cell culture. Does chronic mean a low dose given for 24 h rather than a higher dose given for 5–10 min or is it more complicated than that? In one of the few studies that attempted to develop a model of chronic excitotoxicity (Ha et al., 2009), it was shown that it is indeed more complicated with acute and chronic excitotoxicity appearing to be distinct processes. In this study, the authors used pure cultures of primary cortical neurons prepared from day 14 mouse embryos and treated them after 7 and 14 days in culture (DIV; Ha et al., 2009). For chronic excitotoxicity, the neurons were exposed to L-glu or NMDA for 24 h and for acute excitotoxicity for 10 min. In both cases, cell death was measured after 24 h. Surprisingly, the EC_{50}s for the toxicity of L-glu were lower for acute toxicity, especially in the 7 DIV cultures, as compared to the EC_{50}s for chronic toxicity. In addition, it was found that a high cell culture density increased the sensitivity of the cells to acute but not chronic excitotoxicity. Further studies suggested that the lower sensitivity of the neurons to L-glu in the chronic excitotoxicity paradigm was due to the activation of mGluR1, consistent with earlier data on the neuroprotective effects of mGluR1 activation (Sagara and Schubert, 1998).

An alternative approach to studying chronic glutamate toxicity utilized organotypic spinal cord cultures in combination with L-glu uptake inhibitors (Rothstein et al., 1993). These spinal cord cultures, which were prepared from 8-day-old rat pups, can be maintained in culture for up to 3 months. Chronic inhibition of L-glu uptake with two different uptake inhibitors resulted in a persistent elevation of L-glu in the cell culture medium and time- and concentration-dependent motor neuron cell death. The highest concentration of uptake inhibitor increased extracellular L-glu levels at least 25-fold and began to kill the cells within 1 week whereas a five-fold lower concentration raised extracellular L-glu levels eight-fold and cell death only began after 2–3 weeks of treatment. The toxicity was blocked by non-NMDA but not NMDA receptor antagonists as well as by inhibitors of L-glu synthesis or release. These experiments suggest that even moderately increased L-glu concentrations can induce toxicity.

In vivo approaches to studying chronic excitotoxicity have mainly relied on an approach analogous to that used with the spinal cord cultures. In the majority of these studies, one or more EAATs were transiently or permanently genetically eliminated and the effects on brain function examined. In the first of these studies that used rats (Rothstein et al., 1996), chronic intraventricular administration of antisense RNA was used to eliminate each of the three main EAATs (EAAT1, EAAT2, and EAAT3). The loss of either of the glial L-glu transporters (EAAT1 and EAAT2) but not the neuronal transporter (EAAT3) resulted in large increases in extracellular L-glu concentrations in the striatum after 7 days as determined by microdialysis (EAAT2, 32-fold increase; EAAT1, 13-fold increase). Treatment with either the EAAT1 or EAAT2 antisense oligonucleotides caused a progressive motor impairment whereas the EAAT3 antisense oligonucleotide mainly produced epilepsy. The loss of any of the three transporters produced clear evidence of neuronal damage in the striatum and hippocampus after 7 days of treatment although the effects of the EAAT1 and EAAT2 antisense oligonucleotides were much more dramatic, consistent with the large increases in extracellular L-glu brought about by treatment with these antisense oligonucleotides.

Quite different results were obtained with homozygous mice deficient in EAAT2 (Tanaka et al., 1997) or EAAT1 (Watase et al., 1998). Mice deficient in EAAT2 displayed spontaneous and generally lethal seizures with 50% dead by 6 weeks of age (Tanaka et al., 1997). Approximately 30% of the mice showed selective neuronal degeneration in the hippocampal CA1 region at 4–8 weeks of age. L-glu levels in the CA1 region of the hippocampus measured by microdialysis were ∼three-fold higher in the mutant mice as compared with the wild type mice (Mitani and Tanaka, 2003). In contrast, heterozygous EAAT2 knock-out mice have a normal lifespan and do not show hippocampal CA1 atrophy (Kiryk et al., 2008). However, they display some behavioral abnormalities suggestive of mild glutaminergic hyperactivity. While mice deficient in EAAT1, which is highly expressed in cerebellar astrocytes, did not show changes in cerebellar structure or obvious symptoms of cerebellar impairment such as ataxic gait, they were unable to adapt to more challenging motor tasks such as quickly running on the rotorod (Watase et al., 1998). Taken together, these results suggest that disruptions in glutamatergic homeostasis have a much greater impact when they occur in the adult animal rather than when they are present from conception.

Tuberous sclerosis complex (TSC) is a multi-system genetic disease caused by mutation of either the *TSC1* or *TSC2* genes and characterized by severe neurological symptoms. Mice with inactivation of the *TSC1* gene in glia have a >75% decrease in the expression and function of EAAT1 and EAAT2 and develop seizures (Zeng et al., 2007). At 4 weeks of age, prior to the development of seizures in these mice, there was an ∼50% increase in extracellular L-glu in the hippocampus of these mutant mice, as determined by microdialysis, which correlated with increases in markers of cell death in neurons in both the hippocampus and cortex. Using hippocampal slices from 2 to 4 week old mice, impairments in long-term potentiation were identified, which translated into functional deficits when juvenile mice were tested for spatial and contextual memory in the Morris water maze and fear conditioning assays, respectively.

In most of the studies described above, there was a large increase in extracellular L-glu which, when examined, led, in most cases to negative impacts on the function of specific neuronal populations. To determine the long-term effects of more moderate increases in extracellular glutamate, Bao et al. (2009) created transgenic (Tg) mice with extra copies of the gene for *Glud1* specifically in neurons. The GLUD1 protein is a mitochondrial enzyme that converts L-glu to 2-oxoglutarate. Mitochondrial 2-oxoglutarate is transported to the cytoplasm of nerve terminals where it is converted back into L-glu and stored in synaptic vesicles thereby contributing to the pool of synaptically releasable L-glu (Palaiologos et al., 1988; Takeda et al., 2012). Nine month old Glud1 Tg mice showed an ∼10% increase in L-glu in the hippocampus and striatum relative to wild type mice as determined using magnetic resonance spectroscopy. In addition, evoked L-glu release in the striatum was increased by ∼50%. At 12–20 months of age, the Glud1 Tg mice showed significant decreases in the numbers of neurons in the CA1 region of the hippocampus and granule cell layer of the dentate gyrus as well as an age-dependent loss of both dendrites and dendritic spines in the hippocampus. There was also a decrease in long-term potentiation following high frequency stimulation in hippocampal slices from the Tg mice as compared to the wild type mice. However, whether these alterations resulted in behavioral impairments was not examined. Analysis of the transcriptome of the Glud1 Tg mice as compared to wild type mice suggested that long-term moderate increases in extracellular L-glu result in both accelerated aging at the level of gene expression coupled with compensatory responses that protected against stress and/or promoted recovery (Wang et al., 2010).

In summary, increases in extracellular L-glu *in vivo* can affect nerve cell survival and brain function. The consequences seem to be highly dependent on the degree of L-glu increase but even a 10% increase appears to affect nerve cell structure and survival particularly in the context of aging suggesting that chronic excitotoxicity may be particularly relevant to age-related neurodegenerative diseases.

Plant-based toxins have provided additional evidence for chronic excitotoxicity. These include domoic acid, the cause of amnesiac shellfish poisoning, from the alga *Pseudo-nitzschia* (Grant et al., 2010), β-*N*-oxalylamino-L-alanine (BOAA), the cause of lathyrism, from the plant *Lathyrus sativus* (Ludolph and Spencer, 1995) and possibly β-*N*-methylamino-L-alanine (BMAA), a postulated cause of Guamian amyotrophic lateral sclerosis/Parkinson-dementia complex (ALS/PDC), from the cycad *Cycas circinalis* (Karamyan and Speth, 2008). Based on evidence from human, and in some cases animal, exposures that resulted in significant neurological damage, further studies on these toxins showed that they all act on L-glu receptors and induce both acute and chronic neurotoxicity.

Domoic acid binds with high affinity to AMPA-, kainate-, and NMDA-type iGluRs (Qiu et al., 2006). Both acute, high-dose toxicity, which can include seizures and death as well as lower dose, chronic toxicity of domoic acid have been described in both humans and animals that consumed shellfish and other sea life that eat the alga and concentrate the toxin (Grant et al., 2010). Domoic acid-related neuropathology occurs mainly in the hippocampus and results in memory deficits which may be permanent (Grant et al., 2010). Studies in animals suggest that domoic acid toxicity can be progressive over time with a significant period of good health after exposure before the manifestation of delayed injury (Grant et al., 2010). Moreover, the toxicity appears to be exacerbated by increasing age (Qiu et al., 2006).

Lathyrism is a neurological condition that develops only after several months of ingestion of the grass pea and only when it constitutes at least 30% of a nutritionally-poor diet (Paleacu et al., 1999). BOAA binds preferentially to non-NMDA-type iGluR. In contrast to domoic acid, BOAA specifically and selectively targets the upper motor neurons resulting in spastic paraparesis (Ludolph and Spencer, 1995). Lathyrism is not associated with any cognitive problems (Paleacu et al., 1999).

BMAA, which is closely related to BOAA, binds to both NMDA-type and non-NMDA-type iGluRs (Lobner et al., 2007). In addition, it can both inhibit cystine uptake into cells via system x_c^- thereby promoting oxidative stress and, as it is a substrate inhibitor, promote L-glu release from cells via the same transporter, which can enhance excitotoxicity through activation of mGluR5 (Liu et al., 2009). While the evidence that it could be responsible for Guanamian ALS/PDC is still debatable after almost 50 years (Karamyan and Speth, 2008), oral administration of BMAA to macaques dose-dependently caused a motor system disorder with involvement of both the upper and lower motor neurons as well as the extrapyramidal system after 2–12 weeks of treatment (Spencer et al., 1987). Histological examination showed that the motor cortex was most affected followed by the spinal cord and then the substantia nigra, which was mostly spared. While these symptoms resembled the early stages of Guanamian ALS/PDC, the severe cognitive deficits associated with the disorder were not seen. However, this could be related to the relatively short treatment period, the relatively young age of the macaques and/or the nutritional adequacy of their diet.

In summary, several toxins that bind to iGluRs and have been shown to induce excitotoxicity in cell culture can cause slowly developing neurological problems in both humans and animals. Interestingly, each toxin appears to target a specific type of neuron, an effect that might be related to both the pharmacokinetic and ADME properties of the toxins, which have not yet been studied to any great extent. However, the data from

these toxins supports the idea that chronic excitotoxicity exists in humans and could play a role in multiple neurological disorders.

Since iGluRs are found both in the synapse as well in extra-synaptic locations, there has been a great deal of effort devoted to determining if the location of the receptors impacts the toxicity of glutamate and related molecules. An influential study using primary neuronal cultures (Hardingham et al., 2002) suggested that synaptic and extrasynaptic NMDA receptors have counteracting effects on cell fate with nerve cell death being mainly mediated by extrasynaptic NMDA receptors. However, these results have not been reproduced in brain slices or *in vivo* (reviewed in Papouin and Oliet, 2014). Furthermore, several more recent studies using the same primary neuronal culture preparation protocol as the earlier study found either no difference between synaptic and extrasynaptic NMDA receptors in promoting excitotoxicity (reviewed in Papouin and Oliet, 2014) or found that both receptors were required for cell death (Zhou et al., 2013). Moreover, a number of studies that supported the idea that extrasynaptic NMDA receptors promote excitotoxicity relied on the NMDA receptor inhibitor memantine which was originally thought to specifically act on extrasynaptic NMDA receptors (Xia et al., 2010). However, more recent studies have shown that memantine can inhibit both synaptic and extrasynaptic NMDA receptors (Emnett et al., 2013). Together these results strongly suggest that both synaptic and extrasynaptic NMDA receptors can contribute to excitotoxicity but that the precise contribution of each may depend on the experimental and/or pathological conditions.

EVIDENCE FOR GLUTAMATE DYSREGULATION AND EXCITOTOXICITY IN DIFFERENT NEURODEGENERATIVE DISEASES

Excitotoxicity in Acute Diseases of the CNS

As mentioned above, excitotoxicity was initially defined as an acute insult to nerve cells that leads to cell death by excessive activation of iGluRs. Acute excitotoxicity is known to play an important role in specific CNS disorders including cerebral ischemia, TBI, and status epilepticus. However, the mechanisms underlying acute excitotoxicity differ slightly among these different disorders as described below.

During brain ischemia, the initiation of L-glu- (or L-asp-) mediated excitotoxicity occurs within minutes due to the rapid increase in extracellular cerebral L-glu (and L-asp; reviewed in Dirnagl et al., 1999). The sudden loss of the energy supply due to the shut down of blood flow to the brain leads to a breakdown of the neuronal and astroglial membrane potentials as the maintenance of these is energy-dependent. In neurons, the subsequent membrane depolarization leads to vesicular L-glu release. In addition, energy depletion and disruption of ionic homeostasis inhibits EAAT activity in astrocytes and may even induce a reversal in their action thereby leading to non-vesicular L-glu and L-asp release. The release of L-glu/L-asp (Graham et al., 1990) from these different sources leads to excitotoxicity, mostly by over-activation of iGluRs of the NMDA type as shown by the efficacy of NMDA antagonists in the acute phase in animal models of transient cerebral ischemia (Park et al., 1988; Bielenberg and Beck, 1991; Katsuta et al., 1995).

In TBI, the mechanical tissue damage and disruption of the blood-brain barrier is the initiator of acute secondary neurodegeneration, which, in addition to neuroinflammation and oxidative stress, is mediated by L-glu release from intracellular compartments and thus by acute excitotoxicity (reviewed in Freire, 2012). Accordingly, acute administration of the NMDA antagonist MK801 following TBI ameliorates neuronal loss and long-term behavioral abnormalities (Sönmez et al., 2015).

In status epilepticus, ongoing synchronized activity of excitatory neuronal networks with concurrent breakdown of inhibitory mechanisms is the primary source of increased L-glu (and L-asp) release. As the intensity of synchronous activity is dependent on the integration of a nerve cell into a specific neuronal network and the ability of a nerve cell to withstand excessive glutamatergic input depends, among other properties, on the expression pattern of iGluRs, a rather restricted and maturation-dependent degeneration of neuronal populations is induced by prolonged epileptic seizures (Sankar et al., 1998). The relevance of excitotoxicity in status epilepticus is demonstrated by the fact that NMDA antagonists like ketamine reduce neuronal loss (Loss et al., 2012).

Chronic Excitotoxicity during Progressive Long-Term Neurodegeneration

As compared to acute insults to the CNS, in chronic neurodegenerative diseases the situation is much more complex. First, although compromised mitochondrial function has been repeatedly described in several neurodegenerative diseases (reviewed in Johri and Beal, 2012), the resulting impairments in energy supply are not nearly as severe as the energy failure in ischemic stroke. Thus, if excitotoxicity contributes to neurodegeneration, a very different time course of chronic excitotoxicity has to be assumed. In the following paragraphs, we will summarize what is known about the different pathways that might contribute to excitotoxicity in neurodegenerative diseases. We will focus on amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD) and Huntington's disease (HD) as important examples with sufficiently validated animal models.

Amyotrophic Lateral Sclerosis

ALS is a neurodegenerative disease where the degeneration of motor neurons dominates the clinical presentation and the course of the disease, which is fatal within a few years from onset. It is hypothesized that L-glu excitotoxicity plays a role in the motor neuron death in ALS as these cells express high levels of calcium-permeable AMPA receptors (Carriedo et al., 1996) and low levels of calcium binding proteins (Leal and Gomes, 2015). In contrast to the application of AMPA and kainate, as well as L-HCA, to the lumbar spinal cord of rats, treatment with NMDA spared motor neurons, indicating that NMDA excitotoxicity might not play a prominent role in ALS (Ikonomidou et al., 1996). However, NMDA receptor-mediated excitotoxicity in motor neurons was documented in chick embryo organotypic

slice cultures (Brunet et al., 2009). Electrophysiological studies indicated that there is a transient hyperexcitability of motor neurons in the presymptomatic stage of ALS in mice transgenic for the G93A mutation of human SOD1 that is associated with hereditary ALS (Fuchs et al., 2013). In addition, a cortical hyperexcitability has been documented in familial and sporadic ALS patients that precedes the onset of clinical symptoms in familial ALS mutation carriers (Vucic et al., 2008). Finally, the only approved drug to treat ALS, which increases survival by 2–3 months (Miller et al., 2012), acts as an inhibitor of NMDA and kainate receptors (Debono et al., 1993) as well as rapidly upregulating EAAT activity in synaptosomes (Azbill et al., 2000).

In autopsied spinal cord from patients with ALS, several groups reported a pronounced reduction of EAAT2 but not EAAT1 protein expression in the gray matter in areas with significant motor neuron loss (Sasaki et al., 2000). In addition, both L-glu uptake as well as EAAT2 immunoreactivity, as analyzed by Western blotting, were shown to be quantitatively reduced in postmortem tissue of ALS patients especially in the spinal cord, the tissue that is most affected by the disease (Rothstein et al., 1992; Lin et al., 1998). Moreover, it was reported that as a potential consequence of EAAT2 downregulation, L-glu levels are increased in the CSF in patients with ALS (Rothstein et al., 1990). However, this finding could not be replicated by others (Perry et al., 1990).

The downregulation of EAAT2 in human ALS is recapitulated in several animal models of ALS including transgenic mice expressing human SOD1 containing the G93A mutation that causes hereditary ALS (Bendotti et al., 2001) or transgenic rats expressing the same mutation (Howland et al., 2002). Interestingly, whereas Bendotti found a late decrease in EAAT2 expression at the time when the mice have already become symptomatic (Bendotti et al., 2001), Howland et al. reported changes in EAAT2 expression in the presymptomatic stage (Howland et al., 2002). The β-lactam antibiotic ceftriaxone (Cef) induces EAAT2 in cultured murine spinal cord slices (Rothstein et al., 2005) and in neuron/astrocyte co-cultures (Lewerenz et al., 2009). It also induced EAAT2 expression in the spinal cords of wild-type and mutant G93A mSOD1 Tg mice, which was associated with a decrease in motor neuron loss, weight loss, and other ALS-like symptoms and an increase in survival (Rothstein et al., 2005), compatible with the idea that EAAT2 loss contributes to chronic excitotoxicity in this mouse model. Just recently, a prominent reduction in EAAT2 immunoreactivity was reported in an independent rodent model for ALS, rats expressing ALS-inducing mutant TAR DNA binding protein 43 in astrocytes only (Tong et al., 2013). Interestingly, Alexander et al. found that, when measured by microdialysis, the extracellular L-glu and L-asp concentrations are increased and the L-glu clearance capacity is decreased in the cerebral cortex of G93A mSOD1 Tg mice although this area does not show overt pathology nor downregulation of EAAT1 and 2 when analyzed by Western blotting (Alexander et al., 2000).

Taken together these publications support the view that there is a downregulation of EAAT2 in both human ALS patients and animal models of ALS. However, while some animal studies suggest that EAAT2 downregulation occurs prior to motor neuron loss, others are compatible with the hypothesis that the downregulation of EAAT2, the astroglial expression of which depends on the presence of neurons (Morel et al., 2013), is a consequence of neuronal degeneration.

Whereas, EAATs decrease extracellular L-glu, extracellular cerebral L-glu is upregulated in different brain regions by the cystine/glutamate antiporter system x_c^- (De Bundel et al., 2011; Massie et al., 2011). xCT, the specific subunit of system x_c^-, was reported to be differentially regulated in mouse models of ALS. Albano et al. (2013) reported that the uptake of radiolabelled cystine was upregulated in spinal cord slices of presymptomatic G93A mSOD1 Tg mice at the age of 70 days but not at 55 or 100 days and not in symptomatic 130 day-old mice and confirmed that the upregulation of cystine uptake at day 70 was due to system x_c^- activity using the system x_c^- inhibitor sulfasalazine (SSZ). It has to be kept in mind, however, that cystine can also be transported by EAATs (Hayes et al., 2005). Thus, as data about the SSZ-sensitivity of the cystine uptake were not presented for days 100 and 130, the lack of differential cystine uptake found in this study at the older ages might rather be a consequence of decreased EAAT activity. In contrast, Mesci et al. (2015) using rtPCR, demonstrated a robust increase in xCT mRNA levels in G37R mSOD1 Tg mice upon the onset of symptoms, which was further increased as symptoms progressed (Mesci et al., 2015). Moreover, it was demonstrated that xCT was predominantly expressed in spinal cord microglia. When acutely purified from spinal cord, microglia already showed xCT mRNA upregulation in the presymptomatic phase. Taken together, these findings indicate that system x_c^- is upregulated in animal models of ALS. However, evidence is lacking about whether this is also true for human cases of ALS. Nevertheless, Mesci demonstrated that the mRNA levels of CD68, a marker of microglial activation, correlated with xCT mRNA expression in postmortem spinal cord tissue of patients with ALS, suggesting that neuroinflammation in humans is associated with xCT upregulation (Mesci et al., 2015).

Beyond the dysregulation of L-glu and L-asp levels by EAAT downregulation or system x_c^- upregulation, pathways that indirectly modulate glutamatergic neurotransmission have also been proposed to be involved in motor neuron degeneration in ALS. D-Serine levels have been shown to be progressively increased in the spinal cord of G93A mSOD1 Tg mice (Sasabe et al., 2007, 2012). Beginning at disease onset and continuing during the course of the symptomatic phase, D-serine augments NMDA excitotoxicity in motor neurons (Sasabe et al., 2007, 2012). The upregulation of D-serine in the spinal cord was replicated by others (Thompson et al., 2012). Downregulation of the D-serine metabolizing enzyme DAO in the reticulospinal tract was identified as the main mechanism for D-serine upregulation in the spinal cord in ALS mice (Sasabe et al., 2012). In addition, genetic inactivation of DAO in mice leads to motor neuron degeneration (Sasabe et al., 2012) and a deficiency in the D-serine generating enzyme serine racemase prolonged survival in G93A mSOD1 Tg mice although it paradoxically hastened disease onset (Thompson et al., 2012). A heterozygous mutation of DAO has been shown to segregate with the ALS phenotype in a large family with hereditary ALS (Mitchell et al., 2010).

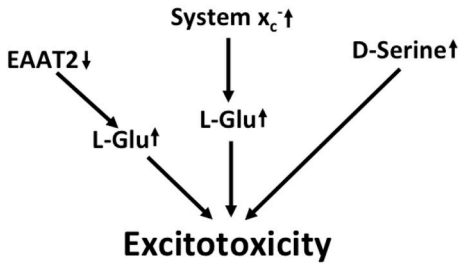

FIGURE 3 | Potential mechanisms that lead to excitotoxicity in ALS. EAAT2 downregulation and upregulation of system x_c^- cause increased activation of glutamate receptors. Moreover, signaling via NMDA receptors is potentiated by the upregulation of the co-activator D-serine.

However, this remains the only family identified so far where a DAO mutation may be linked to the disease (Millecamps et al., 2010).

Regarding the other amino acid co-agonist of the NMDA receptor, glycine, an increase in the CSF levels in patients with ALS was reported by one group (de Belleroche et al., 1984) but could not be replicated by others (Perry et al., 1990; Rothstein et al., 1990). Ilzecka reported that KYNA levels are upregulated in the CSF of bulbar ALS patients and those in end stage disease (Ilzecka et al., 2003). Independently, it was described that tryptophan and KYN levels are increased in the CSF from ALS patients as compared to controls (Chen et al., 2010). In addition, IDO was shown to be expressed in spinal cord microglia and neurons from patients with ALS, indicating that microglial activation could increase the conversion of tryptophan to KYN in ALS.

In summary, multilayered evidence suggests that increased glutamatergic neurotransmission is present in ALS and might contribute to neurodegeneration (**Figure 3**). Downregulation of EAAT2 in astrocytes and upregulation of system x_c^- in the context of microglial activation has been repeatedly documented. Increased co-activation of NMDA receptors by D-serine might also play a role in glutamatergic dysregulation. In addition, the kynurenine pathway seems to be activated in ALS, most likely in the setting of neuroinflammation.

Alzheimer's Disease

AD is the leading cause of dementia in the aging population. Neuropathologically, AD is defined by neurodegeneration with the presence of extracellular senile plaques consisting of β amyloid (Aβ) and intraneuronal neurofibrillary tangles consisting of aggregated tau (Grundke-Iqbal et al., 1986; Ingelsson et al., 2004), which first appear in the hippocampus and then spread as the disease progresses. Prominent microglial activation is also a hallmark of AD. Hereditary forms of AD are caused by mutations in the Aβ precursor protein, AβPP, or in the presenilins, which are part of the multi-protein complex involved in Aβ generation (Ringman et al., 2014). The pathophysiology of AD is complex and many pathways are involved in the synaptic and cellular degeneration in AD including abnormalies in signaling pathways via glycogen synthase kinase-3 beta or mitogen-activated protein kinases, cell cycle re-enty (reviewed by Majd et al., 2015), oxidative stress (reviewed by Niedzielska et al., 2015), or decreased transport of trophic factors and mitochondrial dysregulation (reviewed by Overk and Masliah, 2014). However, multiple lines of evidence suggest that L-glu dysregulation also plays a role in AD.

Grilli et al. showed that primary neurons from transgenic mice overexpressing mutant presenilin are more sensitive to excitotoxic stimuli *in vitro* (Grilli et al., 2000). *In vitro*, aggregated Aβ enhances both NMDA and kainate receptor-mediated L-glu toxicity, most likely by interfering with neuronal calcium homeostasis (Mattson et al., 1992). Others have shown that Aβ can increase neuronal excitability by impairing the ability of glycogen synthase kinase 3β inhibition to reduce NMDA receptor-mediated currents (Deng et al., 2014). Moreover, soluble Aβ oligomers were found to induce L-glu release from astrocytes eventually leading to dendritic spine loss via over-activation of extrasynaptic NMDA receptors (Talantova et al., 2013). Extracellular L-glu concentrations were found to be increased in a triple transgenic mouse model of AD, in which a 3-month treatment with the NMDA receptor inhibitor NitroMemantine rescued synapse loss (Talantova et al., 2013).

A number of mouse studies have shown impacts of AD-like pathology on EAAT expression and/or function. In acute hippocampal slice preparations, Aβ was reported to inhibit the clearance of synaptically released L-glu by decreasing membrane insertion of EAAT2, an effect probably mediated by oxidative stress (Scimemi et al., 2013). In aged AβPP23 mice, Schallier et al. observed the downregulation of EAAT2 expression in the frontal cortex and hippocampus, which in the frontal cortex was associated with an increase in xCT expression (Schallier et al., 2011). These changes were associated with a strong tendency toward increased extracellular L-glu levels as measured by microdialysis (Schallier et al., 2011). In triple transgenic AD mice expressing the amyloid precursor protein mutations K670N and M671L, the presenilin 1 mutation M146V and the tau P301L mutation (Oddo et al., 2003; Zumkehr et al., 2015), a strong and age-dependent reduction of EAAT2 expression was found (Zumkehr et al., 2015). Restoration of EAAT2 activity in these AD mice following treatment with the β-lactam antibiotic Cef was associated with a decrease in cognitive impairment as well as reduced tau pathology (Zumkehr et al., 2015). In human AD brains, decreased expression of EAAT2 protein as well as a decrease in EAAT activity was described (Li et al., 1997). However, this finding could not be replicated by others (Beckstrøm et al., 1999). Of note, on the transcriptome level, Scott et al. found exon-skipping splice variants of EAAT2 that decrease glutamate transport activity to be upregulated in human AD brains (Scott et al., 2011). In the CSF, some groups found an increase in glutamate concentrations (Pomara et al., 1992; Csernansky et al., 1996; Jimenez-Jimenez et al., 1998; Kaiser et al., 2010) in AD patients, whereas others found no change or even decreased levels (Basun et al., 1990; Martinez et al., 1993; Kuiper et al., 2000).

In vitro, Aβ induces L-glu release from primary microglia via upregulation of system x_c^- (Qin et al., 2006). Other found that it also induced L-glu release from astrocytes via activation of the α7 nicotinic acetylcholine receptor (Talantova et al., 2013). In

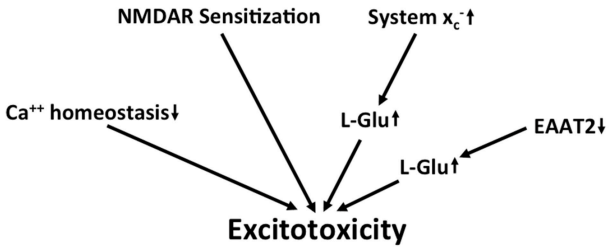

FIGURE 4 | Potential mechanisms that lead to excitotoxicity in AD. Altered calcium homeostasis and increased sensitization of NMDA receptors in AD renders neurons more sensitive to excitotoxicity. This is further amplified by the upregulation of extracellular glutamate via downregulation of EAAT2 and upregulation of system x_c^-.

addition, xCT, the specific subunit of system x_c^- is upregulated in the vicinity of senile plaques, probably in microglia, in Thy1-APP_{751} mice (TgAPP) expressing human APP bearing the Swedish (S: KM595/596NL) and London (L: V6421) mutations as well as after Aβ injection in the hippocampus (Qin et al., 2006). Semiquantitative immunoblot analysis revealed an upregulation of xCT protein expression in the frontal cortex in aged AβPP23 mice compared to wild-type controls (Schallier et al., 2011).

Postmortem studies indicate that KYN metabolism might also be modified in AD as increased concentrations of KYNA were found in the basal ganglia of AD patients (Baran et al., 1999). Using immunohistochemistry, Guillemin et al. found immunoreactivity for both IDO and QUIN upregulated in AD brains, especially in the vicinity of plaques (Guillemin et al., 2005). Of note, Aβ induces IDO expression in human primary macrophages and microglia (Guillemin et al., 2003). Indeed, systemic inhibition of KMO lead to increases in brain KYNA levels and ameliorated the phenotype of a mouse model of AD (Zwilling et al., 2011), indicating that an upregulation of KYNA might be an endogenous protective response. Also the IDO inhibitor, coptisine, reduced microglial, and astrocytic activation and cognitive impairment in AD mice (Yu et al., 2015).

Taken together, along with several other detrimental changes, there is evidence for chronic excitotoxicity in AD which may be driven by multiple factors including the sensitization of NMDA receptors, a decrease in L-glu and L-asp reuptake capacity and an increase in glutamate release via system x_c^- (**Figure 4**). Although the KYN pathway seems to be upregulated in AD, no specific conclusions can be drawn regarding glutamatergic neurotransmission from the reported upregulation of both neurotoxic QUIN and neuroprotective KYNA.

Huntington's Disease

HD is a dominantly inherited, fatal neurodegenerative disease caused by a trinucleotide (CAG) repeat expansion in the coding region of the *huntingtin* (htt) gene that leads to the degeneration of GABAergic medium-sized spiny neurons (MSN) in the striatum, although other brain regions are also affected as the disease progresses. HD presents as a movement disorder with co-morbid psychiatric and cognitive symptomatology (Nance, 1997). Both mutant htt RNA and the encoded protein that contains a polyglutamine repeat expansion are thought to lead to complex alterations in cellular metabolism culminating in mitochondrial dysfunction and oxidative stress (Ayala-Peña, 2013; Johri et al., 2013; Tsoi and Chan, 2014).

Early findings that suggested that excitotoxicity might play an important role in HD were based on the observation that injection of the KYN metabolite and NMDA receptor agonist QUIN, as well as L-glu and kainate, into the striatum of rats generated neuronal degeneration (Coyle and Schwarcz, 1976; Beal et al., 1986). Beal et al. reported that QUIN, as compared to NMDA and kainate, induces a rather selective degeneration of the MSNs rather than general neuronal death (Beal et al., 1986), thus strongly resembling the pathology of HD. Later, NMDA receptors were found to be hyperactive and striatal neurons from different HD mouse models, including those transgenic for a yeast artificial chromosome (YAC) that leads to over-expression of full-length htt with extended polyglutamine repeats (Zeron et al., 2002; Shehadeh et al., 2006), R6/2 mice over-expressing htt exon 1 with extended polyglutamine repeats as well as in knock-in mice with increased CAG repeats inserted in the mouse htt gene (Levine et al., 1999), were shown to be sensitized to excitotoxicity *in vitro*. Of note, *in vivo,* a sensitization to excitotoxin injection into the striatum was only observed in the transgenic YAC model of HD (Zeron et al., 2002), whereas mice over-expressing mutant htt exon 1, R6/1 and R6/2 mice, (Hansson et al., 1999, 2001b) or N171-82Q mice over-expressing mutant exon 1 and parts of exon 2 (Jarabek et al., 2004) or the so-called "shortstop" mouse expressing human N-terminal htt encoded by exon 1 and 2 with a 128 CAG repeat under the htt promoter (Slow et al., 2005) actually developed resistance to striatal excitotoxin injection during aging. However, this acquired neuroprotection is not specific for NMDA receptor agonists but extends to other neurotoxic insults (Hansson et al., 2001a; Petersén et al., 2001) and may represent an adaptive response to cellular stress.

Rat MSN express high levels of NR2A- and NR2B-containing NMDA receptors when compared to interneurons in the striatum (Landwehrmeyer et al., 1995). Correspondingly, NR1 and NR2B mRNA expression in the neostriatum of HD patients was found to be prominently decreased which correlated with the loss of the neurons (Arzberger et al., 1997). Moreover, NMDA receptor-mediated currents in MSN were demonstrated to be largely sensitive to the NR2B-specific inhibitor ifenprodil (Zeron et al., 2002). In HEK293 cells, over-expression of mutant htt increased NMDA receptor-mediated currents and exacerbated NMDA-induced cell death only when NR2B- but not when NR2A-containing NMDA receptors were co-expressed (Chen et al., 1999; Zeron et al., 2001). One possible explanation for the increase in NR2B-containing NMDA receptor expression in HD models is that an extended polyglutamine repeat in htt decreases its binding to PSD95, a postsynaptic density protein involved in NMDA and kainate receptor clustering, leading to increased interaction of PSD95 with the NR2B subunit (Sun et al., 2001; Fan et al., 2009). More recently, data have been presented that suggest that not only the subunit composition but also the localization of NMDA receptors might play an important role in the deleterious NMDA receptor activity in HD. Milnerwood et al.

(2010) showed that in acute striatal slice preparations from YAC transgenic mice with 128 CAG repeats, extrasynaptic NMDA receptors, especially those containing NR2B, are significantly increased compared to slices from wild-type mice and YAC mice expressing htt with 18 CAG repeats. As expected from *in vitro* studies (Hardingham et al., 2001), this change was associated with reduced CREB phosphorylation (Milnerwood et al., 2010). The increased proportion of NR2B-containing extrasynaptic NMDA receptors was demonstrated to be associated with an increased extrasynaptic localization of PSD95 (Fan et al., 2012). One pathway that might mediate the sensitization to excitotoxic stimuli downstream of the activation of extrasynaptic NMDA receptors was identified as activation of p38 MAPK (Fan et al., 2012). Taken together, multilayered evidence suggests that mutant htt leads to sensitization of MSN to glutamate excitotoxicity in part through a relative redistribution of NMDA receptors, especially those containing an NR2B subunit, from synaptic to extrasynaptic sites.

The activation of extrasynaptic NMDA receptors in acute striatal brain slices can be effectively induced in YAC mice with 128 CAG repeats by facilitating spillover of synaptic glutamate by inhibiting EAATs (Milnerwood et al., 2010). Consequently, it can be hypothesized that decreased EAAT expression might increase activation of extrasynaptic NMDA receptors. Interestingly, using *in situ*-hybridization, Arzberger et al. found a decrease in astrocytic EAAT2 mRNA expression in the neostriatum of HD patients (Arzberger et al., 1997). EAAT2 function was found to be decreased in striatal synaptosomes of YAC mice over-expressing human htt with 128 CAG repeats as compared to wild-type mice, however no changes in EAAT2 protein expression could be detected. The authors argued that a functionally relevant decrease in EAAT2 activity in the YAC model of HD was due to reduced palmitoylation of the transporter (Huang et al., 2010). In R6/2 mice, others found reduced EAAT2 mRNA and protein expression (Liévens et al., 2001; Behrens et al., 2002; Shin et al., 2005; Estrada-Sánchez et al., 2009) associated with decreased EAAT2 function in synaptosomes (Liévens et al., 2001) or acute cortico-striatal slices (Shin et al., 2005). However, extracellular striatal glutamate concentrations were found to be similar to those of wild-type control mice (Gianfriddo et al., 2004; Shin et al., 2005) and a reduced glutamate clearance capacity in the R6/2 mice could only be revealed by treatment with EAAT inhibitors or glutamate (Behrens et al., 2002; Estrada-Sánchez et al., 2009). A putative explanation for this finding could be a decrease in glutamate release via system x_c^- as recently a decrease in xCT, the specific subunit of system x_c^-, was demonstrated in the striatum of R6/2 mice at the mRNA and protein levels (Frederick et al., 2014).

As mentioned above, injection of the KYN metabolite QUIN at supraphysiological concentrations was used as an early animal model of HD (Beal et al., 1986). This led to further investigations of KYN metabolism in HD. Interestingly, the QUIN precursor 3HK exacerbates neurodegeneration in the QUIN HD model (Guidetti and Schwarcz, 1999), while KYNA is protective (Foster et al., 1984; Schwarcz and Pellicciari, 2002). Guidetti et al. found that in early-stage HD, as compared to control and end-stage HD, neostriatal 3HK and QUIN concentrations were significantly upregulated (Guidetti et al., 2004). Another study found KYNA levels decreased in autopsied HD striata as well as in the CSF of HD patients as compared to controls (Beal et al., 1992). The initial enzyme of the KYN pathway, IDO, is induced in the striatum of YAC mice with 128 CAG repeats (Mazarei et al., 2013a). Mice deficient in IDO are less sensitive to intrastriatal QUIN injection (Mazarei et al., 2013b). Analysis of KYN metabolites in different brain regions from three different mouse models of HD, R6/2 mice, YAC128 mice, and HdhQ92 and HdhQ111 knock-in mice, suggested age-dependent activation of the KYN pathway. However, the detailed pattern of metabolite changes differed somewhat among the models with increased 3HK in cortex, striatum, and cerebellum in R6/2 mice whereas mice expressing full-length mutant htt showed an additional cortical and striatal upregulation of QUIN (Guidetti et al., 2006). Moreover, treatment of R6/2 mice with a non-blood brain barrier permeable KMO inhibitor, JM6, which indirectly increased cerebral extracellular KYNA concentrations by ∼50%, was associated with a decrease in extracellular cerebral L-glu, decreased neurodegeneration and prolonged survival (Zwilling et al., 2011).

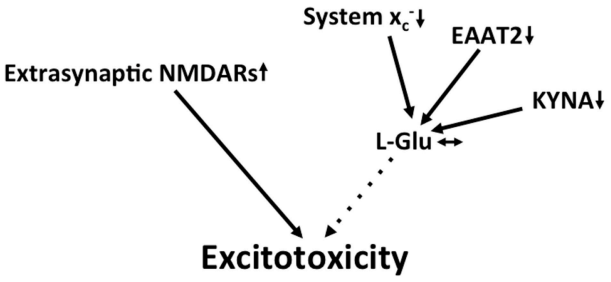

FIGURE 5 | Potential mechanisms that lead to excitotoxicity in HD. Increased redistribution of NMDA receptors to the extrasynaptic compartment is thought to be the prevailing mechanism that fosters excitotoxicity in HD. Although EAAT2 and the glutamate-lowering kynurenine metabolite kynurenic acid (KYNA) are downregulated, these changes might be compensated for by a decrease in system x_c^- expression.

Taken together, the published literature supports the view that in HD there is a redistribution of NMDA receptors, especially those containing NR2B, which might activate signaling pathways that foster neurodegeneration (**Figure 5**). There is no consistent evidence that extracellular cerebral L-glu levels are grossly increased in HD. This might be explained by the fact that although EAAT2 and KYNA might be downregulated, there is also a downregulation of system x_c^- activity. As only very high levels of QUIN activated NMDA receptors, this KYN metabolite is unlikely to directly contribute to the excitotoxic load.

PROOF OF CONCEPT EXPERIMENTS THAT SUPPORT THE HYPOTHESIS OF CHRONIC GLUTAMATE TOXICITY IN NEURODEGENERATION

As EAATs have been found to be down-regulated in many diseases of the nervous system (Sheldon and Robinson, 2007)

and hypothetically increased L-glu and L-asp clearance should dampen the excitotoxic component of these diseases, many researchers have set out to identify compounds that induce EAAT2, which is the principal EAAT in the brain and most frequently found to be downregulated (Sheldon and Robinson, 2007; Kim et al., 2011). This has led to the identification of many compounds that *in vitro* (Colton et al., 2010; Xing et al., 2011) or both *in vitro* and *in vivo* (Rothstein et al., 2005; Ganel et al., 2006; Kong et al., 2014) induce astrocytic EAAT2 expression. Some of these have proven to be protective in animal models of neurodegenerative diseases (Rothstein et al., 2005; Ganel et al., 2006; Kong et al., 2014). Cef is perhaps the best studied of these compounds and has been tested in models of AD (Zumkehr et al., 2015), HD (Miller et al., 2008), and ALS (Rothstein et al., 2005) with positive results. However, it has to be kept in mind that none of these compounds has been extensively screened for its ability to interact with other cellular pathways that might also be neuroprotective. Of note, Cef has been shown not only to induce EAAT2 expression but also to activate the transcription factor Nrf2 (Lewerenz et al., 2009), which induces the transcription of a plethora of genes involved in cytoprotection and antioxidant defense (Kensler et al., 2007). Since oxidative stress is assumed to play a role in many, if not all, neurodegenerative diseases (Bogdanov et al., 2001; Radi et al., 2014), this pathway may account for at least some of the neuroprotection induced by Cef. Indeed, xCT, which is one of the downstream targets of Nrf2, has been found to be upregulated by Cef *in vitro* and *in vivo* (Lewerenz et al., 2009; Knackstedt et al., 2010). Another *in vitro* EAAT2-inducing compound, MS-153, effectively protected against secondary neurodegeneration after traumatic brain injury, but through mechanisms other that EAAT2 upregulation (Fontana et al., 2015). Thus, proof of concept experiments that unequivocally demonstrate the pathophysiological role of a chronically increased excitotoxic input via iGluRs in neurodegenerative diseases require more specific manipulations of the neurotransmitter physiology.

As described in the Section on "The Concepts of Acute and Chronic Glutamate Toxicity," Glud1 Tg mice represent a model of chronic excitotoxicity mediated by increased synaptic L-glu release with limited neuronal loss (Bao et al., 2009). However, this animal model of increased glutamatergic neurotransmission has not yet been used to test whether Glud1 over-expression exacerbates the phenotype of mouse models of neurodegenerative diseases. Another genetically engineered model is the EAAT2-deficient mouse. As described in the Section on "The Concepts of Acute and Chronic Glutamate Toxicity," homozygous EAAT2 knock-out mice suffer from premature death due to epilepsy and show hippocampal and focal cortical atrophy (Tanaka et al., 1997; Kiryk et al., 2008; Petr et al., 2015). Heterozygous EAAT2 knock-out mice however develop normally and show only mild behavioral abnormalities (Kiryk et al., 2008). Consequently, this mouse model of mild glutamatergic hyperfunction has been used in a series of proof of principle studies that investigated the functional role of glutamate in animal models of neurodegenerative diseases. ALS mice that carry both the G93A mSOD1 mutation and a reduced amount of EAAT2 (SOD1(G93A)/EAAT2$^{\pm}$) exhibited an increase in the rate of motor decline accompanied by earlier motor neuron loss when compared with single mutant G93A mSOD1 Tg mice (Pardo et al., 2006). A modest reduction in survival was also noted in these double mutant mice. When crossed with transgenic mice expressing mutations of the human amyloid-β protein precursor and presenilin-1 (AβPPswe/PS1ΔE9), partial loss of EAAT2 unmasked spatial memory deficits in 6-month-old mice expressing AβPPswe/PS1ΔE9. These mice also exhibited an increase in the ratio of detergent-insoluble Aβ42/Aβ40 suggesting that deficits in glutamate transporter function contribute to early pathogenic processes associated with AD (Mookherjee et al., 2011). In contrast, the phenotype of the R6/2 HD mouse model was not altered in mice that contained only one EAAT2 allele (Petr et al., 2013).

As a complement to these studies, transgenic mice that over-express EAAT2 specifically in astrocytes via the GFAP promoter have also been developed (Guo et al., 2003). EAAT2/G93A mSOD1 double Tg mice showed moderate amelioration of the ALS-like phenotype with a statistically significant (14 days) delay in grip strength decline and loss of motor neurons as well as a reduction in other events including caspase-3 activation and SOD1 aggregation but not in the onset of paralysis, body weight decline or an extended life span when compared with monotransgenic G93A mSOD1 littermates (Guo et al., 2003). The same EAAT2 transgenic mouse model was used to test the effect of increased astrocytic L-glu (and L-asp) uptake by cross-breeding with an animal model of AD, AβPPswe/Indmice. Increased EAAT2 protein levels significantly improved cognitive function, restored synaptic integrity, and reduced amyloid plaques in these AD mice (Takahashi et al., 2015).

In mice in which genetically engineered deletion of xCT leads to deficiency in the glutamate/cystine antiporter system x_c^-, the prominent reduction of extrasynaptic L-glu is associated with a robust resistance of dopaminergic neurons against 6-hydroxydopamine-induced neurodegeneration (Massie et al., 2011), possibly as a result of decreased excitotoxicity. However, microglial activation has also been shown to be modulated by system x_c^- deficiency resulting in a more neuroprotective phenotype (Mesci et al., 2015) which provides an alternative explanation for the protective effect of xCT deletion in this context.

Thus, genetic models support the role of chronic excitotoxicity in neurodegenerative diseases, especially ALS and AD. Of note, all of these models represent life-long changes in glutamatergic neurotransmission. From the therapeutic perspective, these models cannot predict whether drugs that specifically ameliorate the glutamatergic tone during the neurodegenerative process are protective. To this end, either intensive testing of EAAT2-inducing drugs for their interaction with other signaling pathways or the development of inducible mouse models with dampened excitotoxic load are warranted.

SUMMARY

In summary, "glutamatergic" excitatory input on neurons is a sum of the interaction of several different activators including the direct activators, L-glu, and L-asp, and the co-activators,

glycine, and D-serine, on iGluRs and mGluRs. Other pathways including tryptophan metabolism and, especially, the tryptophan metabolite KYNA, modulate glutamatergic neurotransmission. Glutamatergic input on neurons is either via synaptically released L-glu (and L-asp) acting on synaptic iGluRs or by non-synaptically released L-glu acting at extrasynaptic L-glu receptors. The glutamate/cystine antiporter, system x_c^-, might be an important determinant of the extrasynaptic cerebral L-glu concentration. Chronically increased input via iGluRs, even if it is only moderate, has the propensity to induce neuronal degeneration, so-called chronic excitotoxicity. In many neurodegenerative diseases, including HD, AD, and ALS, multilayered evidence suggests that glutamatergic dysregulation is an important contributor to disease pathology although the molecular basis for this varies widely and might be distinct for each disease and most likely does not represent the only pathway that leads to neurodegeneration.

However, as specific pharmacological tools or inducible genetically engineered mouse models that allow manipulation of glutamatergic input are lacking, it is not known to what extent L-glu dysregulation contributes to disease progression in specific mouse models of different neurodegenerative diseases. Thus, while the idea that chronic excitotoxicity contributes to multiple neurodegenerative diseases is supported by many layers of scientific evidence, it is not clear that therapeutic interventions that re-establish glutamatergic homeostasis during ongoing neurodegeneration will be effective tools for stopping the disease process. Besides direct modulators of iGluR activity, strong candidates for future approaches to treating chronic excitotoxicity include specific inducers of EAAT2 to stimulate L-glu and L-asp uptake, inhibitors of system x_c^- to reduce L-glu release as well as compounds that aim to decrease extracellular L-glu by modulating KYN metabolism, e.g., KMO inhibitors. In addition, it has to be kept in mind that combinations of these interventions might be required to obtain clinically significant benefits without evoking adverse side effects.

AUTHOR CONTRIBUTIONS

JL provided the concepts for the review and wrote much of it. PM wrote a portion of the review and edited the entire review.

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Evidence for a neuroprotective microRNA pathway in amnestic mild cognitive impairment

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MicroRNAs (miRNAs) that regulate mRNA stability have been linked to amyloid production, tau phosphorylation, and inflammation in Alzheimer's disease (AD). However, whether cerebral miRNA networks are dysregulated during the earliest stages of AD remains underexplored. We performed miRNA expression analysis using frontal cortex tissue harvested from subjects who died with a clinical diagnosis of no cognitive impairment (NCI), amnestic mild cognitive impairment (aMCI, a putative prodromal AD stage), or mild AD. Analysis revealed that the miRNA clusters miR-212/132 and miR-23a/23b were down-regulated in the frontal cortex of aMCI subjects. Both miR-212/132 and miR23a/b are predicted to destabilize the message for sirtuin 1 (sirt1); hence, down-regulation of either miR-212/132 or miR-23a/b in frontal cortex should promote sirt1 mRNA expression in this region. qPCR studies revealed that frontal cortex levels of sirt1 were increased in aMCI. Given the ability of frontal cortex to respond to the onset of dementia by neuronal reorganization, these data suggest that miRNA-mediated up-regulation of the sirt1 pathway represents a compensatory response to the onset of the disease. By contrast, qPCR analysis of inferior temporal cortex, an area affected early in the progression of AD, showed no changes in miR-212/132, miR-23a/b, or sirt1 transcripts in the same aMCI subjects. *In vitro* mechanistic studies showed that coordinated down-regulation of miR-212 and miR-23a increased sirt1 protein expression and provided neuroprotection from β-amyloid toxicity in human neuronal cells. Taken together, these data suggest a novel miRNA-mediated neuroprotective pathway activated during the progression of AD that may be amenable to therapeutic manipulation.

Keywords: Alzheimer's disease, mild cognitive impairment, frontal cortex, microRNAs, sirtuin 1, miR-23a, miR-212, gene expression regulation

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INTRODUCTION

Progress in slowing the course of Alzheimer's disease (AD) has been confounded by a lack of disease modifying therapeutics. Given the vast complexity of this multisystem disorder, therapeutic development will likely depend on a deeper understanding of the intricate molecular mechanisms that regulate the maintenance and survival of selectively vulnerable neuronal populations during disease progression. In this regard, the presence of small non-coding microRNAs (miRNAs)

that negatively regulate mRNA stability (Lagos-Quintana et al., 2001) presents an underexplored mechanism for fine-tuning gene expression within complex cellular networks, which likely plays a pivotal role in the balance between health and disease (Nelson et al., 2008; Hébert and De Strooper, 2009). Select miRNAs regulate diverse brain functions including neurogenesis and differentiation, synaptic plasticity, and energy metabolism (Schratt et al., 2006; Aschrafi et al., 2008; Fineberg et al., 2009; Rajasethupathy et al., 2009). This widespread influence of miRNA regulation on neuronal physiology suggests that perturbations in miRNA function could be involved in the pathogenesis of complex neurodegenerative disorders including AD (Nelson et al., 2008; Hébert and De Strooper, 2009). Indeed, AD brains display altered expression of several miRNAs that regulate β-secretase BACE1, a key enzyme involved in the generation of amyloid-β (Aβ) plaque pathology (Hébert et al., 2008; Wang et al., 2008). In addition, miRNA dysregulation has been linked to tau phosphorylation (Hébert et al., 2010; Absalon et al., 2013; Banzhaf-Strathmann et al., 2014) and pro-inflammatory activity (Cui et al., 2010; Lukiw et al., 2010; Li et al., 2012). However, whether miRNA networks are dysregulated in the brains of people in the putative prodromal stages of AD such as amnestic mild cognitive impairment (aMCI) (Yaffe et al., 2006; Albert et al., 2011) and the extent to which these changes have physiologic consequences for the onset of AD remain unclear. To begin to address these knowledge gaps, we performed microarray and quantitative PCR (qPCR) studies to compare the levels of miRNAs isolated from frontal cortex (Brodmann area 10) and inferior temporal cortex (Brodmann area 20) tissue obtained postmortem from people who died with a clinical diagnosis of no cognitive impairment (NCI), aMCI, or AD. We report that two families of miRNAs, miR-212/132 and miR-23a/b, were down-regulated in frontal cortex in aMCI and AD compared to NCI, yet remained stable in inferior temporal cortex. Down-regulation of either miRNA family was predicted to up-regulate the deacetylase sirtuin 1 (sirt1), which is involved in mediating protective neuronal cell stress responses (Brunet et al., 2004; Qin et al., 2006; Bonda et al., 2011). Sirt1 mRNA levels were higher in frontal cortex of aMCI subjects but stable in inferior temporal cortex, suggesting a link between miR-212/132 and miR-23a/b down-regulation and reduced transcriptional repression of sirt1 target mRNA. Experimental down-regulation of miR-212 and miR-23a in cultured neurons up-regulated sirt1 and provided neuroprotection against Aβ toxicity. Given the relatively delayed involvement of frontal cortex in AD pathogenesis and the ability of this region to respond to the onset of dementia by neuronal reorganization (DeKosky et al., 2002; Counts et al., 2006; Bell et al., 2007; Williams et al., 2009; Bossers et al., 2010), these data suggest that miRNA-mediated up-regulation of sirt1 is a novel neuroprotective pathway activated during prodromal AD.

MATERIALS AND METHODS

Subjects

This study was exempt from IRB approval following guidelines for using de-identified postmortem tissue administrated by the Rush University Medical Center. Frontal and inferior temporal cortex tissue was obtained postmortem from 32 participants in the Rush Religious Orders Study (Bennett et al., 2002) who were clinically diagnosed within a year of death with NCI ($n = 12$), aMCI ($n = 10$), or AD ($n = 10$; see **Table 1**). Details of clinical evaluations and diagnostic criteria have been previously published (Mufson et al., 1999; Counts et al., 2006; Ginsberg et al., 2010). In addition to an annual clinical evaluation, subjects were administered the Mini Mental State Exam (MMSE) and a battery of 19 neuropsychological tests referable to multiple cognitive domains (e.g., episodic memory, perceptual speed, Mufson et al., 1999). A Global Cognitive Score (GCS), consisting of a composite z-score calculated from this test battery, was determined for each participant (Bennett et al., 2002). The MCI population was defined as subjects who exhibited cognitive impairment on neuropsychological testing but who did not meet the clinical criteria for AD recommended by the joint working group of the National Institute of Neurologic and Communicative Disorders and Stroke/AD and Related Disorders Association (NINCDS/ADRDA) (McKhann et al., 1984; Bennett et al., 2002). The aMCI diagnosis is based on impairments in episodic memory (Yaffe et al., 2006; Albert et al., 2011). These criteria are compatible with those used by experts in the field to describe subjects who are not cognitively normal but do not meet established criteria for dementia (Petersen et al., 2001). "Other dementia" (OD) neurologic controls ($n = 5$) used in the analysis included three multi-infarct dementia and two Lewy body dementia cases.

Tissue samples were accrued as previously reported (Mufson et al., 1999; Counts et al., 2006; Ginsberg et al., 2010). At autopsy, tissue from one hemisphere was immersion-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 for 24–72 h at 4°C. Tissue slabs from the opposite hemisphere were frozen at −80°C prior to biochemical analysis. Series of fixed tissue sections were prepared for neuropathological evaluation including visualization and quantitation of neocortical and hippocampal amyloid plaques and neurofibrillary tangles (NFTs) using antibodies directed against Aβ peptide (Aβ; 4 G8, Covance), tau (PHF1, a gift from Dr. Peter Davies) (Mufson et al., 1999; Bennett et al., 2002) as well as thioflavine-S histochemistry and a modified Bielschowsky silver stain. Additional sections were stained for Lewy bodies using antibodies directed against ubiquitin and α-synuclein. Exclusion criteria included argyrophilic grain disease, frontotemporal dementia, Lewy body disease, mixed dementias, Parkinson's disease, and stroke. A board certified neuropathologist blinded to the clinical diagnosis performed the neuropathological evaluation. Neuropathological criteria were based on NIA-Reagan, CERAD, and Braak staging (Braak and Braak, 1991; Mirra et al., 1991; Hyman et al., 2012). Amyloid burden and apolipoprotein E (ApoE) genotype were determined for each case as described previously (Mufson et al., 1999; Bennett et al., 2002).

miRNA Expression Profiling

A pilot miRNA microarray screen (Exiqon miRCURY LNA microarray v.11, ∼1360 array features including synthetic spike-in miRNA controls) was performed using RNA derived from

TABLE 1 | Clinical, demographic, and neuropathological characteristics by diagnosis category.

	Clinical Diagnosis				
	NCI (*N* = 12)	**aMCI (*N* = 10)**	**AD (*N* = 10)**	***P*-value**	**Pair-wise comparison**
Age (years) at death: Mean ± SD (Range)	83.0 ± 5.9 (67–92)	82.9 ± 4.9 (72–90)	88.6 ± 7.0 (76–97)	0.3[a]	–
Number (%) of males:	6 (50%)	5 (50%)	4 (40%)	0.4[b]	–
Years of education: Mean ± SD (Range)	16.8 ± 3.4 (12–20)	18.8 ± 2.7 (12–22)	18.2 ± 3.4 (15–23)	1.0[a]	–
Number (%) with ApoE ε4 allele:	0	3 (33%)	4 (40%)	0.007[b]	NCI < AD
MMSE: Mean ± SD (Range)	28.3 ± 1.7 (26–30)	28.0 ± 1.3 (26–30)	17.5 ± 8.1 (7–28)	<0.001[a]	(NCI, MCI) > AD
Global cognitive score: Mean ± SD (Range)	0.06 ± 0.3 (−0.3–0.7)	−0.03±0.4 (−0.6–0.2)	−1.3 ± 0.8 (−2.5–0.1)	<0.0001[a]	(NCI, MCI) > AD
Postmortem interval (hours): Mean ± SD (Range)	5.7 ± 2.4 (3.0–12.4)	5.8 ± 3.1 (2.0–10.6)	4.8 ± 3.2 (1.5–12.3)	0.5[a]	–
Distribution of Braak scores: 0 I/II III/IV V/VI	 0 5 7 0	 0 4 5 1	 0 1 3 6	<0.001[a]	(NCI, MCI) < AD
NIA Reagan diagnosis: No AD Low Intermediate High	 0 7 3 0	 0 5 5 0	 0 1 4 5	<0.001[a]	(NCI, MCI) < AD
CERAD diagnosis: No AD Possible Probable Definite	 3 2 4 1	 4 3 3 0	 0 0 5 5	0.008[a]	(NCI, MCI) < AD

[a] *Kruskal-Wallis test, with Bonferroni correction for multiple comparisons.*
[b] *Fisher's exact test, with Bonferroni correction for multiple comparisons.*

postmortem frozen frontal cortex tissue from three NCI [age = 86.7 ± 1.5 (mean ± *S.D.*) years, MMSE = 29.7 ± 0.6, PMI = 5.2 ± 1.5 h] and three AD (age = 87.7 ± 1.5, MMSE = 21.7 ± 2.5, PMI = 5.2 ± 1.7) subjects. Approximately 30% of the miRNAs on the array were proprietary and not available for analysis in public databases. Based upon the microarray pilot data, we performed qPCR validation for select transcripts using frozen frontal cortex and temporal cortex from each of the NCI, aMCI, AD, and OD neurologic control cases collected for the full study. Total RNA was extracted (miRvana, Ambion) and RNA integrity and concentration was verified using Bioanalysis (Agilent). Samples were assayed on a real-time PCR cycler (7900HT, Applied Biosystems) in 96-well optical plates as described previously (Counts et al., 2007; Ginsberg, 2008; Alldred et al., 2009). Target miRNAs of interest as well as the RNU48 artificial normalization control were amplified using specific Taqman hydrolysis probe sets (Applied Biosystems). In addition, Taqman probe sets specific for sirt1 and control glyceraldehyde 3-phosphate dehydrogenase were used to quantify sirt1 transcript levels in the same samples. The ddCT method was employed to determine relative expression levels of each amplicon (Counts et al., 2007; Ginsberg, 2008; Alldred et al., 2009). Variance component analyses revealed relatively low levels of within-case variability, and the average value of the triplicate qPCR products from each case was used in subsequent analyses.

Dual *in Situ* Hybridization/Immunohistochemical Localization of miR-23a and Sirt1

In situ hybridization to detect miR-23a was performed on 10 μm, cryostat-sectioned samples of frozen frontal cortex using a digoxin (DIG)-labeled hsa-miR-23a probe (Exiqon), adapting the protocol of Doné and Beltcheva (2014). Briefly, tissue sections were fixed in 10% neutral buffered formalin overnight at room temperature (RT). The next day, sections were treated with 20 μg/mL proteinase K for 10 min at 37°C followed by hybridization with 400 nmol hsa-miR-23a probe for 1 h at 55°C. The sections were then blocked with 2% sheep serum/1% bovine serum albumin for 15 min at RT

followed by incubation with alkaline phosphatase-conjugated sheep anti-DIG Fab fragments (1:500, Roche) for 1 h at RT. The sections were then incubated with the alkaline phosphatase substrates NBT (nitro blue tetrazolium)/BCIP (5-bromo-4-chloro-3-indolyl-phosphate; Roche) for 2 h at 30°C revealing a dark purple reaction product. Following miR-23a visualization, the sections were incubated overnight at 4°C with a rabbit anti-sirt1 monoclonal antibody (1:100, Origene) in Tris-buffered saline (TBS, pH 7.4)/0.25% Triton X-100/1% normal goat serum. Following TBS rinses, the sections were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antiserum (Vector Laboratories) for 1 h at RT. Sirt1 labeling was accomplished by serial incubations in ABC peroxidase reagent (Vector Laboratories) and 3, 3′diaminobenzidine tetrahydrochloride hydrate at RT to reveal a brown reaction product.

Neuronal Cell Culture

hNT neuronal cultures were derived from the human teratocarcinoma NT2 cell line (a gift from Dr. Virginia Lee, Univ. Penn) (Andrews et al., 1984; Counts and Mufson, 2010). NT2 cells were maintained in OptiMem (Invitrogen) with 5% fetal bovine serum (FBS). For differentiation, cells were seeded at 25,000/cm^2 into T75 flasks in 1:1 DMEM/F-12 media (Invitrogen)/10% FBS, treated twice a week with 10 μM all-*trans* retinoic acid (Sigma) for 4 weeks and then seeded to new T75 flasks at 650,000/cm^2 and treated with the mitotic inhibitors cytosine arabinoside (1 μM) and fluorodeoxyuridine (10 μM, Sigma) for 2 weeks. This resulted in a layer of phase-bright, post-mitotic neuronal cells loosely attached atop a monolayer of non-neuronal cells. Neuronal enrichment was achieved by gently trypsinizing the top neuronal layer and replating at 125,000/cm^2 onto 2% Matrigel (BD Biosciences) and 10 μM poly-D-lysine (Sigma)-coated black-walled 96 well plates (cell viability) or 60 mm dishes (western blotting) (BD Biosciences) in 1:1 DMEM/F-12 media/10% FBS (Counts and Mufson, 2010).

miRNA Inhibition and Functional Validation

hNT cultures were transfected with small miRNA inhibitors (miRCURY LNA inhibitors, Exiqon) specific for miR-212, miR-132, miR-23a, miR-23b, or an inhibitor negative control sequence (Exiqon) (n = 8/treatment group in three independent experiments). hNT neurons were plated at 20K/cm^2 and incubated with 50 nM inhibitor/1% Lipofectamine RNAiMAX (Life Technologies) in OptiMem for 18 h, then exchanged into 1:1 DMEM/F-12 media/10% FBS for 36 h prior to experimentation.

Quantitative Western Blotting

hNT neurons were harvested 36 h post-transfection and separated into nuclear and cytosolic fractions (NE-PER, Pierce) for quantitative immunoblotting. Nuclear proteins were solubilized in loading buffer and separated by SDS-PAGE, transferred to Immobilon-P membranes (Millipore), blocked in Tris buffered saline (pH 7.4) containing 0.1% Tween-20 and 2% nonfat milk, and then incubated overnight at 4°C with rabbit polyclonal antiserum to sirt1 (1:500; Chemicon) and a mouse monoclonal antibody to lamin A (1:500, Abcam) as a loading control for the nuclear fraction. Blots were then incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit (Bio-Rad; 1:5000) and anti-mouse (1:8000; Pierce, IL) IgG secondary antibodies and reactivity was quantified using Kodak 1D image analysis software (Perkin-Elmer). Each sample was analyzed on three different Western blots in independent experiments. Signals for sirt1 were normalized to lamin A for quantitative analysis (Counts et al., 2004, 2006).

Amyloid Toxicity Experiments

Following miRNA inhibitor or control transfections, differentiated hNT neurons were challenged with 10 μM $A\beta_{1-42}$ for 48 h in the presence or absence of the sirt1-specific inhibitor EX527 (100 nM, Tocris). $A\beta_{1-42}$ was dissolved in DMSO and pre-aggregated for 16 h at 37°C. Western blotting revealed an accumulation of SDS-soluble immunoreactive material migrating at ∼40–48 kDa reminiscent of oligomeric amyloid (Walsh et al., 1999). Neuronal viability was determined by propidium iodide (PI) retention (Counts and Mufson, 2010).

Statistical Analysis

miRNA levels quantified by qPCR were compared among the NCI, aMCI, AD, and OD neurologic controls via One-way ANOVA with Bonferroni *post-hoc* testing. The relationship between specific miRNA and mRNA levels was assessed by Spearman rank correlations. Quantitative Western blotting data of control and miR-treated samples were analyzed by unpaired *t*-tests. Finally, cell death comparisons in the different cell culture treatment groups were assessed by One-way ANOVA with Bonferroni *post-hoc* testing. The level of statistical significance was set at $\alpha = 0.05$ (two-tailed).

RESULTS

Subject Demographics

The clinical diagnostic groups did not differ by age, gender, years of education, or postmortem interval (**Table 1**). There were more subjects with an ApoE 4 allele in the aMCI (33%) and AD (44%) groups than in the NCI group (0%). AD cases had significantly lower MMSE scores compared to both aMCI and NCI cases ($p < 0.001$), whereas the latter two groups did not differ statistically (**Table 1**). GCS z-scores were significantly lower in AD compared to the NCI and aMCI groups ($p < 0.0001$). Subjects in the different clinical diagnostic groups displayed considerable heterogeneity with respect to pathological diagnostic criteria. Neuropathological examination revealed that 58% of NCI, 60% of aMCI, and 90% of AD cases were classified as Braak stages III–VI. Using NIA-Reagan criteria, 25% of NCI, 50% of aMCI, and 90% of AD cases were classified as intermediate to high likelihood of AD (**Table 1**). For CERAD diagnosis, 42% of NCI, 30% of aMCI, and 100% of AD cases received a diagnosis of probable/definite AD. Statistical analysis revealed that the AD group displayed significantly higher pathology than the NCI and aMCI groups based on Braak ($p < 0.001$), NIA-Reagan ($p < 0.001$) and CERAD ($p = 0.008$) diagnosis.

TABLE 2 | Top 100 miRNAs dysregulated in AD compared to NCI frontal cortex.

Annotation	*P*-value	Average		AD vs. NCI	
		NCI	AD	ΔLMR	Fold change
hsa-miR-1285	5.92E-04	0.04	−0.21	−0.25	0.84
hsa-miR-1296	2.00E-03	−0.09	−0.33	−0.24	0.85
hsa-miR-668	5.48E-03	−0.06	−0.57	−0.51	0.70
hsa-miR-551b*	6.07E-03	−0.45	−0.01	0.44	1.36
hsa-miR-1826	6.49E-03	−0.11	−0.53	−0.42	0.75
hsa-miR-518e*	7.48E-03	−0.44	0.15	0.59	1.50
hsa-miR-132	9.07E-03	0.53	−0.22	−0.74	0.60
hsa-miR-525-5p	1.08E-02	−0.09	0.15	0.24	1.18
hsa-miR-135b	1.40E-02	0.45	0.17	−0.29	0.82
hsa-miR-671-5p	2.23E-02	−0.24	0.00	0.24	1.18
hsa-miR-498	2.68E-02	−0.23	0.29	0.51	1.43
hsa-miR-423-3p	2.76E-02	0.05	−0.17	−0.22	0.86
hsa-miR-886-3p	2.95E-02	0.32	−1.62	−1.94	0.26
hsa-miR-518a-5p	3.76E-02	−0.29	0.07	0.36	1.29
hsa-miR-204	3.97E-02	0.41	0.19	−0.22	0.86
hsa-miR-886-5p	4.21E-02	0.02	−1.49	−1.50	0.35
hsa-miR-132*	4.25E-02	0.24	−0.67	−0.91	0.53
hsa-miR-129*	4.38E-02	0.20	−0.36	−0.56	0.68
hsa-miR-382	4.08E-02	0.16	−0.03	−0.19	0.88
hsa-miR-921	5.35E-02	−0.58	−0.13	0.45	1.36
hsa-miR-140-5p	5.67E-02	0.33	−0.18	−0.51	0.70
hsa-miR-330-5p	5.87E-02	0.38	0.23	−0.15	0.90
hsa-miR-510	6.01E-02	−0.17	0.12	0.28	1.22
hsa-miR-491-3p	6.15E-02	0.23	−0.35	−0.57	0.67
hsa-miR-320a	7.03E-02	0.29	−0.27	−0.56	0.68
hsa-miR-877	7.05E-02	−0.11	0.17	0.28	1.22
hsa-miR-320b	7.07E-02	0.29	−0.26	−0.55	0.68
hsa-miR-1252	7.13E-02	−0.43	0.10	0.53	1.44
hsa-miR-30e	7.40E-02	0.24	−0.31	−0.55	0.68
hsa-miR-129-3p	7.40E-02	0.21	−0.45	−0.65	0.64
hsa-miR-149	7.43E-02	0.14	−0.26	−0.40	0.76
hsa-miR-25*	7.49E-02	−0.21	0.15	0.36	1.28
hsa-miR-149*	7.57E-02	−0.02	0.36	0.38	1.30
hsa-miR-483-5p	7.63E-02	−0.29	0.18	0.47	1.39
hsa-miR-630	7.94E-02	−0.16	0.30	0.46	1.38
hsa-miR-1273	8.07E-02	−0.45	0.14	0.59	1.50
hsa-miR-1264	8.34E-02	0.20	−0.12	−0.32	0.80
hsa-let-7a*	8.46E-02	0.21	−0.10	−0.31	0.81
hsa-miR-505*	8.53E-02	−0.20	0.27	0.47	1.38
hsa-miR-377*	8.62E-02	0.00	0.15	0.15	1.11
hsa-miR-21	8.77E-02	0.39	−0.69	−1.08	0.47
hsa-miR-126	8.81E-02	0.14	−0.22	−0.36	0.78
hsa-miR-625	9.22E-02	−0.20	0.11	0.31	1.24
hsa-miR-320d	9.33E-02	0.17	−0.21	−0.39	0.77
hsa-miR-29b-2*	9.40E-02	−0.01	−0.33	−0.33	0.80
hsa-miR-29c*	9.41E-02	0.13	−0.15	−0.27	0.83
hsa-miR-30a	9.58E-02	0.14	−0.28	−0.41	0.75
hsa-miR-1265	9.70E-02	0.20	−0.03	−0.23	0.85
hsa-miR-135a	1.00E-01	0.51	−0.04	−0.54	0.69
hsa-let-7i	1.04E-01	0.15	−0.33	−0.48	0.72
hsa-miR-589	1.04E-01	−0.14	−0.46	−0.32	0.80
hsa-miR-1201	1.06E-01	−0.20	−0.57	−0.37	0.78
hsa-miR-1259	1.06E-01	0.12	−0.22	−0.34	0.79
hsa-miR-720	1.07E-01	0.18	−0.24	−0.42	0.75
hsa-miR-34b	1.09E-01	0.10	−0.21	−0.32	0.80
hsa-let-7c	1.09E-01	0.19	−0.27	−0.46	0.73
hsa-miR-320c	1.09E-01	0.23	−0.20	−0.43	0.74
hsa-miR-583	1.12E-01	−0.35	0.10	0.46	1.37
hsa-miR-206	1.12E-01	−0.07	0.25	0.32	1.24
hsa-miR-23a	1.12E-01	0.03	−0.44	−0.47	0.62
hsa-miR-181c	1.12E-01	−0.08	−0.44	−0.36	0.78
hsa-miR-769-5p	1.16E-01	0.11	−0.30	−0.41	0.75
hsa-miR-181b	1.17E-01	0.47	−0.46	−0.93	0.53
hsa-miR-1827	1.19E-01	−0.26	0.29	0.55	1.46
hsa-miR-1253	1.20E-01	−0.58	−0.32	0.26	1.19
hsa-miR-301a	1.20E-01	0.16	−0.38	−0.54	0.69
hsa-miR-184	1.21E-01	−0.17	0.15	0.32	1.25
hsa-miR-140-3p	1.21E-01	0.14	−0.46	−0.61	0.66
hsa-miR-33a	1.21E-01	0.31	−0.27	−0.58	0.67
hsa-miR-485-3p	1.22E-01	−0.18	0.16	0.34	1.26
hsa-miR-1274b	1.22E-01	0.21	−0.36	−0.56	0.68
hsa-miR-181a*	1.22E-01	0.07	−0.46	−0.53	0.69
hsa-miR-516a-5p	1.23E-01	−0.42	0.02	0.44	1.36
hsa-miR-30d	1.24E-01	0.24	−0.11	−0.35	0.78
hsa-miR-150	1.25E-01	−0.28	0.38	0.66	1.58
hsa-miR-874	1.25E-01	−0.03	−0.24	−0.20	0.87
hsa-miR-339-5p	1.27E-01	−0.03	−0.48	−0.45	0.73
hsa-miR-146b-5p	1.28E-01	0.29	−0.26	−0.55	0.68
hsa-let-7g	1.28E-01	0.10	−0.41	−0.50	0.70
hsa-miR-142-3p	1.28E-01	0.56	0.04	−0.51	0.70
hsa-miR-212	1.29E-01	−0.10	−0.53	−0.44	0.64
hsa-miR-518d-5p	1.30E-01	−0.12	0.08	0.19	1.14
hsa-miR-433	1.32E-01	0.03	−0.14	−0.18	0.89
hsa-miR-200b*	1.34E-01	−0.16	0.20	0.36	1.28
hsa-miR-491-5p	1.34E-01	0.02	−0.31	−0.32	0.80
hsa-miR-24	1.35E-01	0.16	−0.39	−0.54	0.69
hsa-miR-193b*	1.38E-01	−0.26	0.19	0.46	1.37
hsa-miR-130b*	1.38E-01	−0.13	0.18	0.31	1.24
hsa-miR-30c	1.40E-01	0.14	−0.22	−0.36	0.78
hsa-miR-23b	1.41E-01	0.13	−0.53	−0.66	0.63
hsa-miR-106b	1.42E-01	0.30	−0.32	−0.62	0.65
hsa-let-7b	1.42E-01	0.30	−0.14	−0.45	0.73
hsa-let-7d	1.43E-01	0.17	−0.43	−0.60	0.66
hsa-miR-647	1.43E-01	−0.29	0.04	0.33	1.26
hsa-miR-181a	1.43E-01	0.42	−0.95	−1.37	0.39
hsa-miR-130a	1.44E-01	0.16	−0.11	−0.28	0.82

(Continued)

**Star strand of miRNA.*

miRNA Expression Dynamics during the Progression of AD

A small pilot miRNA microarray screen performed on RNA isolated from frozen frontal cortex tissue from three NCI and AD cases showed that the expression levels of 30 miRNAs were significantly different between the two groups, along with miRNAs that showed high but non-significant magnitudes of expression change between the two groups. The top 100 miRNAs are listed in **Table 2**. We performed qPCR to measure select miRNAs in frontal cortex across all groups examined. Specifically, we tested the expression levels of 20 of the top 100 miRNAs based on significant differences, magnitude of expression level difference and/or interest based on a literature search (Cogswell et al., 2008; Hébert et al., 2013; Lau et al., 2013; Wong et al., 2013) (**Table 3**). Of the 20 candidate miRNAs evaluated, miR-498, and miR-150 were significantly up-regulated in AD frontal cortex; miR-150 was up-regulated in aMCI. Several miRNAs were also significantly down-regulated in AD frontal cortex, including miR-886-3p, miR-132, miR-21, miR-23a, miR-140-3p, miR-212, miR-23b, let-7d, and miR-181a (**Table 3**). However, two distinct clusters of these miRNAs, miR212/132, and miR 23a/23b, were also significantly down-regulated by ∼50% in the frontal cortex of aMCI subjects relative to NCI subjects (**Table 3**, **Figures 1A,B**). By contrast, qPCR analysis of temporal cortex revealed that miR-212 and miR-132 expression levels were decreased only in the AD group, whereas miR-23a and miR-23b were unchanged across the clinical diagnostic groups (**Table 4**). Since miRNAs negatively regulate transcript stability, down-regulation of the miR-212/132 and miR-23a/b clusters in frontal cortex would be predicted to promote the stability of their target mRNAs in aMCI. Given several lines of evidence that the frontal cortex undergoes neuroplastic, presumably neuroprotective remodeling in the face of mounting AD neuropathology during MCI (DeKosky et al., 2002; Counts et al., 2006; Bell et al., 2007; Williams et al., 2009; Bossers et al., 2010), we searched the miRanda and miRbase prediction algorithm databases (microRNA.org) for mRNA targets of these miRNAs that might mediate potentially compensatory pathways to promote neuronal viability. Surprisingly, all four miRNAs were predicted to target sirt1, a histone deacetylase involved in mediating neuronal cell stress responses (Brunet et al., 2004; Qin et al., 2006; Bonda et al., 2011). To determine whether target sirt1 transcripts were also differentially regulated in the frontal and temporal cortex in aMCI, we performed qPCR analysis of sirt1 expression levels in the same tissue samples used for miRNA analysis. This analysis revealed that sirt1 mRNA levels were significantly up-regulated by ∼40% in the frontal cortex of aMCI compared to NCI and AD subjects (**Figure 1C**). By contrast, sirt1 levels were stable in temporal cortex across the diagnostic groups (**Table 4**). Furthermore, increased sirt1 mRNA expression was significantly associated with decreased miR-212 levels frontal cortex levels, but this association was not found in the temporal cortex (**Figure 2**).

TABLE 3 | qPCR analysis of select miRNAs in frontal cortex of NCI, aMCI, and AD subjects.

miRNA	aMCI fold Δ vs. NCI	AD fold Δ vs. NCI
hsa-miR-668	0.87	0.85
hsa-miR-135b	0.76	0.84
hsa-miR-498	1.27	1.39*
hsa-miR-886-3p	0.85	0.51**
hsa-miR-132	0.58**	0.42**
hsa-miR-320a	0.98	0.91
hsa-miR-1252	1.17	1.14
hsa-miR-1273	1.03	1.22
hsa-miR-21	0.78	0.65*
hsa-miR-23a	0.53**	0.41**
hsa-miR-181b	0.88	0.93
hsa-miR-1827	1.27	1.21
hsa-miR-140-3p	0.85	0.62*
hsa-miR-33a	0.76	0.81
hsa-miR-150	1.43*	1.56**
hsa-miR-212	0.47**	0.41**
hsa-miR-23b	0.71*	0.62**
hsa-miR-106b	1.05	0.97
hsa-let-7d	0.73	0.66*
hsa-miR-181a	0.69	0.62*

*$p < 0.05$, **$p < 0.01$.

Localization of miR-23a and Sirt1 in the Frontal Cortex

Dual miR-23a *in situ* hybridization and sirt1 immunohistochemistry revealed nuclear miR-23a labeling in frontal cortex layer III neurons in tissue obtained from an 87 year-old female subject who died with a clinical diagnosis of aMCI (**Figure 3**). Sirt1 protein immunoreactivity was found primarily in the nucleus but also within the cytoplasm of the same neurons, providing evidence that miR-23a has direct access to its sirt1 target and that miR-directed sirt1 regulation in cortex is neuronal in origin.

miR-212 and miR-23a Down-regulation Increases Sirt1 Protein Expression in Human Neuronal Cells

To determine the extent to which concomitant down-regulation of miR-212/132 and miR-23a/b and up-regulation of sirt1 observed in aMCI frontal cortex represented a functionally significant relationship, we treated human hNT neuronotypic cells with specific inhibitors of these miRNAs and measured sirt1 protein expression. Interestingly, inhibition of miR-212, miR-132, miR-23a, or miR-23b individually had no effect on sirt1 expression, but we found that concurrent inhibition of miR-212 and miR-23a resulted in a significant ∼100% increase in sirt1 (**Figure 4**), whereas co-inhibition of miR-132 and miR-23a resulted in a ∼40% increase in sirt1 ($p < 0.05$, data not shown). By contrast, miR-23b co-inhibition with miR-212 or miR-132 had no effect on sirt1 expression (data not shown).

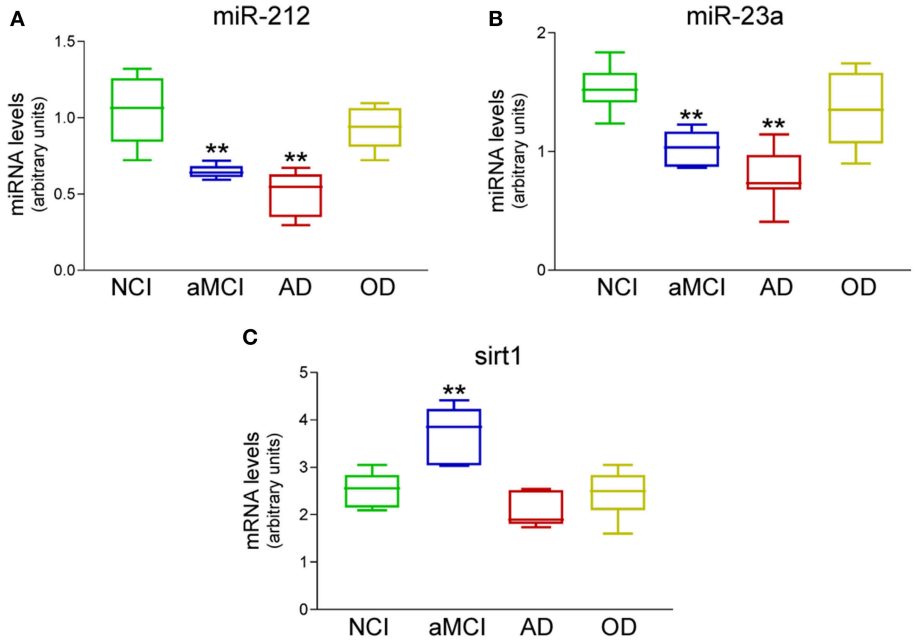

FIGURE 1 | Differential expression of miR-212, miR-23a, and sirt1 transcripts in the frontal cortex of aMCI subjects. qPCR analysis was performed on frozen frontal cortex tissue samples harvested from NCI ($n = 12$), aMCI ($n = 10$), mild AD ($n = 10$), and other dementia (OD, $n = 5$) neurologic control subjects. Box plots show that **(A)** miR-212 and **(B)** miR-23a were significantly down-regulated by ~50% in aMCI and by ~60% in AD. **(C)** Their predicted mRNA target sirt1 was up-regulated by ~40% in aMCI. miR expression levels were normalized to the human RNU48 control miRNA, whereas sirt1 mRNA was normalized to GAPDH for quantitative analysis. $^{**}p < 0.01$ via One-way ANOVA with Bonferroni correction for multiple comparisons.

TABLE 4 | qPCR analysis of select miRNAs and sirt1 mRNA in temporal cortex of NCI, aMCI, and AD subjects.

Probe set	aMCI fold Δ vs. NCI	AD fold Δ vs. NCI
hsa-miR-212	0.96	0.63*
hsa-miR-132	0.88	0.65*
hsa-miR-23a	0.92	0.87
hsa-miR-23b	1.03	0.98
hsa-miR-150	1.32	1.47**
has-miR-668	0.95	1.01
sirt 1 mRNA	1.12	0.94

*$p < 0.05$, **$p < 0.01$.

miR-23a and miR-212 Down-regulation Protects against $A\beta_{1-42}$ Induced Cell Death via Sirt1

To test whether miR-212 and miR-23a regulation of sirt1 results in neuronal protection, hNT cells were treated with inhibitors of either miRNA independently, or with inhibitors of both miRNAs combined, followed by challenge with $A\beta_{1-42}$, which has been shown to induce cell death in these neuronal cells (Counts and Mufson, 2010). Inhibition of both miRNAs was sufficient to reduce $A\beta_{1-42}$ induced cell death (**Figure 5**). This response was blocked by co-administration with the sirt1 inhibitor EX527 (**Figure 5**), supporting the concept that miRNA regulation of sirt1 expression is a viable neuroprotective pathway.

DISCUSSION

The discovery of miRNAs (Lagos-Quintana et al., 2001) introduced a new layer of complexity to gene regulation, but also afforded the opportunity to better understand the molecular underpinnings of cellular function and dysfunction. This concept has already been well-demonstrated in cancer research and the potential therapeutic value of targeting miRNAs has been gaining acceptance in that field (Ma and Weinberg, 2008). With respect to neurodegenerative disorders such as AD, the presence of miRNAs in the human brain and evidence for miRNA function in a wide variety of complex neuronal processes, including synaptic plasticity, suggests that miRNA regulation could have immense implications not only for disease pathogenesis but also for neuronal responses to progressive neurodegeneration (Kosik, 2006). Prior studies show that miRNAs could impact AD progression through several mechanisms. For instance, postmortem human tissue studies in well-characterized cohorts have shown decreased neocortical levels of miR-29a/b, miR-9, and miR-107 in AD compared to control subjects, which was associated with increased BACE1 mRNA expression and Aβ generation (Hébert et al., 2008; Wang et al., 2008; Che et al., 2014); in particular, miR-107 is downregulated very early in the disease process (Wang et al., 2008). By contrast, miR-15a is decreased in AD brain compared to healthy controls and is implicated in tau hyperphosphorylation *in vivo* (Hébert et al., 2010). Here, we show that mir-132/212 and miR-23a/b are selectively down-regulated in the frontal cortex

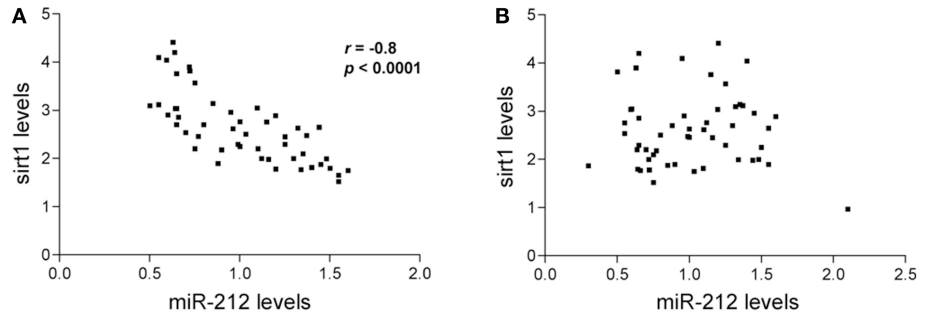

FIGURE 2 | Inverse relationship between miR-212 and sirt1 expression in the frontal cortex. Spearman rank correlations were performed to test for relationships between miR-212 and sirt1 expression. Scatterplots show that lower miR-212 transcript expression was associated with higher sirt1 transcript expression in **(A)** frontal cortex ($r = -0.81$, $p < 0.0001$), but not in **(B)** temporal cortex in the subjects examined.

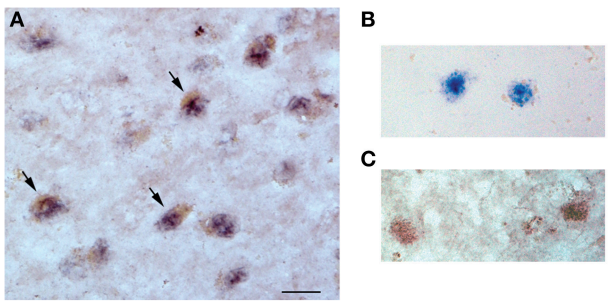

FIGURE 3 | miR-23a and sirt1 co-localize in the nucleus of frontal cortex layer III neurons. Dual *in situ* hybridization/immunohistochemistry of miR-23a RNA and sirt1 protein expression was performed in frontal cortex of an 87 year-old female subject who died with a clinical diagnosis of aMCI. **(A)** Photomicrograph shows layer III neurons co-labeled with miR-23a labeling (dark purple) and sirt1 (brown, arrows). Note that miR-23a appears localized to the nucleus, whereas sirt1 labeling is nuclear but also with immunoreactivity in the cytoplasm. **(B)** Dual miR-23a/sirt1 detection in the absence of sirt1 primary antibody (anti-rabbit IgG only). **(C)** Dual miR-23a/sirt1 detection with control scrambled miRNA probe. Scale bar = 30 μm.

in subjects clinically diagnosed with aMCI and that these alterations appear to be functionally linked to an up-regulation of sirt-1 and sirt-1 mediated protective responses. This novel finding adds to a growing literature on miRNA involvement in AD pathophysiology. However, rather than implicating another group of miRNAs in promoting neurodegeneration, our data support the concept that innate neuronal compensatory miRNA-mediated pathways are also activated in aMCI. A greater understanding of these and other miRNA pathways functioning during these putative prodromal stages of AD holds the promise that these pathways could be harnessed pharmacologically for drug development.

The miR-132/212 cluster has been implicated in several neuronal pathways, including dendritic elaboration (Magill et al., 2010) and learning and memory (Wang et al., 2013), and is downregulated in AD neocortex (Cogswell et al., 2008; Hébert et al., 2013; Lau et al., 2013; Wong et al., 2013). In particular, Wong and colleagues have previously shown that the miR-212/132 cluster is down-regulated in temporal cortex in AD, and that inhibition of miR-212 and/or miR-132 expression can induce apoptosis in primary neurons after 1 week in culture via activation of a foxo3a-mediated cell death pathway (Wong et al., 2013). In this regard, we replicated the finding that the miR-212/132 cluster is down-regulated in the temporal cortex in AD. However, we found no reductions in miR-212/132 in this region in aMCI; miR-212/132 was down-regulated in aMCI only in frontal cortex. In addition, in our hands experimental inhibition of miR-212 and/or miR-132 had no effect on cell survival of hNT neurons after 48 h in the absence of $A\beta_{1-42}$. However, co-inhibition of either miR-212 or miR-132 with miR-23a conferred neuroprotection against $A\beta_{1-42}$ in a sirt1-dependent manner. These discrepancies are likely explained by several factors. For instance, down-regulation of these miRNAs may have different functional implications in the AD temporal cortex, which displays a greater degree of degenerative changes than the aMCI frontal cortex. Notably, we found that sirt1 up-regulation in frontal cortex was confined to aMCI, suggesting that other AD-related pathways prevent sirt1 activation as the disease progresses. The down-regulation of miR-212/132 in aMCI frontal cortex may initially participate in protective compensatory mechanisms, but with sustained reductions these miRNAs may join a pathological cascade that promotes disease progression through foxo3a. In this regard, sirt1 activity has been shown to protect against foxo3a-mediated pro-apoptotic pathways by the deacetylation of this transcription factor (Brunet et al., 2004; Qin et al., 2008). Moreover, the differential roles for this cluster may depend on target binding partners, since miR-212/132 and miR-23a co-inhibition was required for sirt1 activation and neuroprotection. Hence, the activity of the miR-212/132 cluster may be context dependent during the progression of AD.

miR-23a/b has also been shown to be dysregulated in the AD brain (Cogswell et al., 2008; Lau et al., 2013), yet much less is known about this miRNA cluster in neuronal function. However, like miR-212/132, miR-23a levels have been inversely linked to apoptosis. In a recent report detailing mechanisms for neuronal cell death in a model of traumatic brain injury, *in vitro* studies revealed that miR-23a inhibition

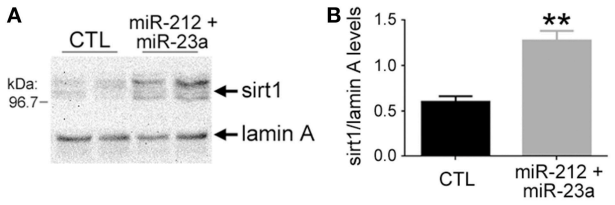

FIGURE 4 | Experimental down-regulation of miR-212 and miR-23a increases sirt1 protein expression. Human hNT neuronotypic cells were co-transfected with small inhibitors of miR-212 and 23a or scrambled negative controls (CTL). Given the co-localization of miR-23a and sirt1 in the nucleus, cells were harvested 36 h following transfection with the miRNA inhibitors or controls and nuclear fractions were immunoblotted for detection of sirt1. **(A)** A representative western shows that miR-212/23a co-repression resulted in an increase in sirt1 immunoreactivity. **(B)** Quantitative analysis revealed that sirt1 levels were significantly increased by ~100% in miR-212/23a inhibitor-transfected cells compared to CTL-transfected cells. $n = 6$/group; $^{**}p < 0.01$ via Student's unpaired test (two-tailed).

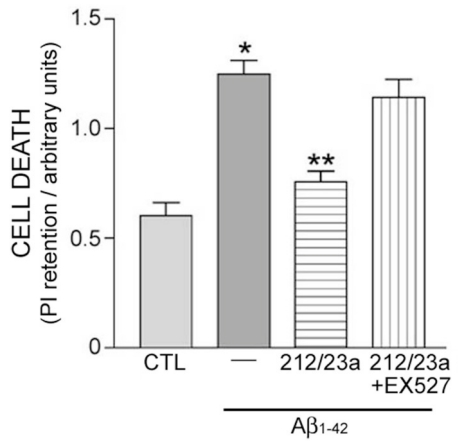

FIGURE 5 | Experimental down-regulation of miR-212 and miR-23a protects against $A\beta_{1-42}$ in a sirt1-dependent manner. Human hNT neuronotypic cells were co-transfected with small inhibitors of miR-212 and 23a or scrambled negative controls (CTL). Cells were then treated with 10 μM $A\beta_{1-42}$ for 48 h in the presence or absence of 100 nM EX527, a sirt1-specific inhibitor, followed by incubation with propidium iodide (PI) as an assay for cell death. Bar graph shows that $A\beta_{1-42}$ treatment resulted in significant cell death that was significantly reduced by miR-212/23a co-repression. This rescue effect was reversed by EX527, suggesting that the protective effects of miR-212/23a co-repression was mediated by sirt1. $n = 8$/group; $^{*}p < 0.05$ vs. CTL; $^{**}p < 0.05$ vs. $A\beta_{1-42}$ via One-way ANOVA with Bonferroni correction for multiple comparisons.

increased etoposide-induced cell death after 24 h in cortical neurons via caspase activation (Sabirzhanov et al., 2014). By contrast, we found that miR-23a inhibition alone had no effect on Aβ-induced cell death after 48 h, yet was neuroprotective in the presence of miR-212 by activating sirt1. Again, these discordances are not incompatible, but suggest that miR-23a has both pro-apoptotic and neuroprotective properties that depend on specific miRNA binding partners and whether these miRNAs are targeting pro-apoptotic factors such as caspases or, as presently shown, factors such as sirt1 that promote neuronal viability.

While the functional consequences of sirt1 up-regulation in this paradigm are unclear, sirt1 deacetylase activity plays an important role in regulating diverse cellular processes including aging, inflammation, and stress resistance (Imai et al., 2000; Brunet et al., 2004; North and Verdin, 2004; Herskovits and Guarente, 2014). In the adult brain, sirt1 can also modulate dendritic (Codocedo et al., 2012) and synaptic plasticity as well as memory formation (Gao et al., 2010; Michán et al., 2010). In addition to its importance during normal brain aging, sirt1 may also confer protective properties in neurodegenerative disorders such as AD (Qin et al., 2006, 2008; Min et al., 2010). Experimentally, sirt1 can reduce both Aβ-induced toxicity in neuronal cell lines (Conte et al., 2003; Han et al., 2004) and amyloid plaque formation in AD transgenic mice (Karuppagounder et al., 2009; Vingtdeux et al., 2010). Neuronal sirt1 expression has also been linked to non-amyloidogenic APP processing (Qin et al., 2006). Hence, we validated these concepts by showing that miR212/132 and miR-23a-mediated neuroprotection against Aβ is prevented by a sirt1-specific inhibitor. Sirt1 has also been shown to ameliorate tangle-like pathology in tau mutant mice (Kim et al., 2007; Min et al., 2010), possibly by tau deacetylation that allows ubiquitin ligases to target tau for degradation (Min et al., 2010; Cohen et al., 2011). Hence, there are several mechanisms by which miR-212/132 and miR-23a co-regulation of sirt1 expression may promote neuroprotection in the frontal cortex in aMCI. However, it is sirt1's role in the regulation and maintenance of dendritic growth (Codocedo et al., 2012) that could also play an important role in frontal cortex plasticity responses in MCI. In this regard, sirt1 knockout (KO) mice display reduced dendritic profiles relative to wild-type mice (Michán et al., 2010). Furthermore, overexpression of sirt1 can increase dendritic arborization, a phenotype that renders neurons resistant to dendritic dystrophy induced by Aβ (Codocedo et al., 2012). These data, taken together with multiple lines of evidence for a paradoxical up-regulation of synaptic elements within the frontal cortex during MCI (Counts et al., 2006; Bell et al., 2007; Williams et al., 2009; Bossers et al., 2010), suggest that sirt1 may mediate a synaptic remodeling response to mounting pathology during the progression of AD.

In summary, our data suggest that the transition from normal cognitive function in aging to a clinical diagnosis of aMCI may involve the suppression of brain microRNA networks such as miR212/132 and miR-23a. In the frontal cortex, this may result in the induction of cellular survival pathways including the stabilization of sirt1. Ultimately, as aMCI progresses to AD, the robust sirt1 expression is lost in frontal cortex, mimicking the situation seen in the temporal cortex. Recent microarray data suggest that the pattern of expression seen in sirt1 is also seen in other genes, particularly those involved in metabolic and synaptic machinery (Berchtold et al., 2013; Counts et al., 2013). Future research is necessary to confirm these findings in other AD cohorts, clarify whether the cumulative effect of these expression patterns is protective *in vivo* and by which sirt1-mediated mechanism, and determine whether augmentation of cell survival pathways such as sirt1

represents a viable strategy to delay neurodegeneration in prodromal AD. Linking these concepts to recent advances in miRNA knockdown and mimetic technology will usher in a new class of powerful *in vitro* and *in vivo* approaches to test the efficacy of miRNA-based therapies in preclinical AD models.

AUTHOR CONTRIBUTIONS

RW participated in the experimentation and manuscript preparation. EM participated in the case selection, data analysis, and manuscript preparation. SC participated in the study design, experimentation, data analysis, and manuscript preparation.

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MINI REVIEW
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EFhd2, a Protein Linked to Alzheimer's Disease and Other Neurological Disorders

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EFhd2 is a conserved calcium binding protein linked to different neurological disorders and types of cancer. Although, EFhd2 is more abundant in neurons, it is also found in other cell types. The physiological function of this novel protein is still unclear, but it has been shown *in vitro* to play a role in calcium signaling, apoptosis, actin cytoskeleton, and regulation of synapse formation. Recently, EFhd2 was shown to promote cell motility by modulating the activity of Rac1, Cdc42, and RhoA. Although, EFhd2's role in promoting cell invasion and metastasis is of great interest in cancer biology, this review focusses on the evidence that links EFhd2 to Alzheimer's disease (AD) and other neurological disorders. Altered expression of EFhd2 has been documented in AD, Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis, and schizophrenia, indicating that *Efhd2* gene expression is regulated in response to neuropathological processes. However, the specific role that EFhd2 plays in the pathophysiology of neurological disorders is still poorly understood. Recent studies demonstrated that EFhd2 has structural characteristics similar to amyloid proteins found in neurological disorders. Moreover, EFhd2 co-aggregates and interacts with known neuropathological proteins, such as tau, C9orf72, and Lrrk2. These results suggest that EFhd2 may play an important role in the pathophysiology of neurodegenerative diseases. Therefore, the understanding of EFhd2's role in health and disease could lead to decipher molecular mechanisms that become activated in response to neuronal stress and degeneration.

Keywords: EFhd2, tau, neurodegenerative disease, neurological disorders, Alzheimer's disease

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INTRODUCTION

Efhd2 gene codes for a 26.8 kDa highly conserved protein, from nematodes to human, located in chromosome 4 (4E1;4 74.75 cM) in mice and chromosome 1 (1p36.21) in humans. EFhd2 was first identified in a proteomics screen designed to discriminate CD8 from CD4 and CD19 lymphocytes. The abundance of this novel protein was found to be reduced in CD4 and C19 in comparison to CD8 lymphocytes (Vuadens et al., 2004; Dütting et al., 2011). In this original study, the novel protein was named Swiprosin 1, in reference to the Swiss-Prot database used for the tandem mass spectrometry data analysis (Vuadens et al., 2004). Subsequently, the name of this novel protein was changed to EF-hand domain family, member D2 (EFhd2) due to the presence of two EF-hand calcium binding motifs. Characterization of EFhd2 demonstrated that it

is an ubiquitous calcium binding protein, preferentially expressed in the central nervous system (Avramidou et al., 2007; Vega et al., 2008; Hagen et al., 2012; Ferrer-Acosta et al., 2013b). Further sequence analyses indicated that EFhd2 has a coiled-coil domain at the C-terminus, which is a conserved domain among fibrillar proteins and required for protein–protein interaction (Ferrer-Acosta et al., 2013a). At the N-terminus, EFhd2 has a distinctive polyalanine motif that varies in size (between 6 and 9 alanines) and it is only present in mammals (Dütting et al., 2011; Ferrer-Acosta et al., 2013a). The function of EFhd2's polyalanine motif is still unknown, but proteins containing polyalanine expansions have been shown to be linked to different neurological disorders (Albrecht and Mundlos, 2005). However, the physiological and pathological roles of EFhd2 are still poorly understood.

EFhd2 may function as a signaling or cytoskeleton regulatory protein. In WEHI231 cells, it was shown that EFhd2 is required for the regulation of the canonical NFkB pathway upon activation of the B-cell receptor (BCR; Avramidou et al., 2007; Hagen et al., 2012; Kim et al., 2013). ShRNA-mediated EFhd2 knockdown led to increase IkB phosphorylation, which is a prerequisite for translocation of NFkB to the nucleus, upon BCR activation (Avramidou et al., 2007; Kim et al., 2013). Based on this result, the authors suggested that EFhd2 may play a role as negative regulator of NFkB in the BCR signaling pathway (Avramidou et al., 2007). Alternatively, another study found EFhd2 at the plasma membrane, where it facilitates the assembly of the BCR and appears to work as a scaffold protein required for the function of Syk, SLP-65, and PLCγ2 during BCR-induced calcium flux (Kroczek et al., 2010). The functional interaction of EFhd2 with BCR signaling pathway and modulation of IkB phosphorylation indicates a potential regulatory role in cell survival or fate. Interestingly, EFhd2 was identified as a novel pro-caspase-9-interacting protein in H460 cells (Chęcińska et al., 2009). EFhd2 association with (inactive) pro-caspase-9 protein suggests that it may regulate the activation of apoptosis (Chęcińska et al., 2009). In contrast, other reports indicated that EFhd2 mediates actin bundling and regulates cell spreading and migration (Huh et al., 2013; Kwon et al., 2013). Moreover, recent studies indicated that EFhd2 was upregulated by epidermal growth factor signaling and mediates cell migration through the modulation of Rac1, Cdc42, and RhoA activity (Huh et al., 2015). Although, the direct role of EFhd2 in regulating cellular survival and apoptosis is still unclear, these results indicate that this novel protein plays an important role in modulating cellular responses elicited by different environmental cues, such as trophic factors or stimulating antibodies (Avramidou et al., 2007; Huh et al., 2015).

EFhd2 has also been associated with different pathological processes, from cancer to neurological disorders (**Table 1**). In cancer, EFhd2 was found to be overexpressed in the majority of carcinomas, colon cancer, and melanoma (Huh et al., 2015). The results indicated that EFhd2 may mediate invasion and metastasis of cancerous cells, suggesting it plays an important role in the biology of cancer. *EFhd2* gene expression and protein abundance have been also shown to be altered in AD and other neurological disorders. Thus, even though the role that EFhd2 plays in cancer biology is of great interest, this review focusses on EFhd2's role in the central nervous system, specifically its association with AD and other neurological disorders.

IS EFHD2 A MODULATOR OF FUNCTIONAL SYNAPSE FORMATION?

EFhd2 is highly expressed in neurons compared to other cell types of the central nervous system, where may play an important role in synapse formation (Reynolds et al., 2008; Vega et al., 2008; Ferrer-Acosta et al., 2013b; Borger et al., 2014). EFhd2 proteins were found in the cytosol and proximal to the membrane in neurons of most brain regions, including higher expression in the deeper layers of the cortex and hippocampus (Ferrer-Acosta et al., 2013b; Borger et al., 2014). EFhd2 co-localized with neurite markers such as tau, MAP2, synapsin, and PSD95, suggesting that its neuronal function could be associated with vesicle transport and synapse homeostasis (Borger et al., 2014; Purohit et al., 2014). Consistent with this putative function, a recent study showed *in vitro* that knockdown of EFhd2 increased synpasin 1a/b puncta labeling in neurites, suggesting that modulation of EFhd2 affects the development of functional synapses, but it had no effect on converting them to mature synapses as determined by the co-localization of synapsin and PSD95 (Borger et al., 2014). These results imply that EFhd2 may modulate the formation of new synapses, a process that it is relevant for different brain functions, such as learning and memory.

Characterization of an *Efhd2* knockout mouse provided further insights about its function in the central nervous system. Purohit et al. (2014) showed that deletion of *Efhd2* gene has no detectable effect on brain anatomy or function. Interestingly, they showed that vesicle transport velocity was enhanced in *Efhd2*$^{(-/-)}$ knockout primary hippocampal neurons and that EFhd2 protein inhibited kinesin mediated microtubule gliding *in vitro* (Purohit et al., 2014). The authors proposed that EFhd2 may interfere with the interaction between kinesin and microtubules (Purohit et al., 2014). In this regard, EFhd2 has been showed to co-purify with tubulin in the synaptosome fraction as well as to mediate actin bundling (Huh et al., 2013; Kwon et al., 2013; Purohit et al., 2014). Although, there is no evidence that *Efhd2* gene knockout affects brain function, these results suggest that EFhd2 may serve as a modulator of synapse formation by regulating the velocity of vesicle transport and cytoskeleton rearrangement.

EFHD2 IN ALZHEIMER'S DISEASE

EFhd2 protein abundance was found altered in AD, suggesting that *Efhd2* gene expression may be regulated in response to neurodegeneration (Vega et al., 2008; Borger et al., 2014; **Table 1**). Two different studies showed that the chromosome region encompassing the *Efhd2* gene locus is linked to (and a third study showed that it is associated with) late-onset Alzheimer's disease (LOAD; Hiltunen et al., 2001; Myers et al., 2002; Holmans et al., 2005). The linkage between *Efhd2* gene and LOAD is yet to be determined; however, we and others have shown that EFhd2 expression and protein abundance is altered in AD and

TABLE 1 | Identification of EFhd2 associated with neurological disorders.

Disorders	Disease	Results Summary	Method	References
Movement	Parkinson's disease (PD)	EFhd2 was found to be secreted from microglia after exposure to nitrated α-syn	SELDI-TOF ProteinChip	Reynolds et al., 2008
		EFhd2 protein abundance was shown to be reduced in midbrain (*substantia nigra*) of PINK1-KO mice	2D-DIGE and Tandem Mass Spectrometry	Diedrich et al., 2011
		EFhd2 was identified as Lrrk2 interacting protein, suggesting a potential role in actin polymerization	Quantitative immunoprecipitation combined with knockdown (QUICK); Tandem Mass Spectrometry	Meixner et al., 2011
		EFhd2 increased expression correlates with IFN-γ and SNCA switch to positive co-expression in *substantia nigra* in PD cases gathered from four different datasets; relationship between inflammation and PD	Microarray	Liscovitch and French, 2014
	Amyotrophic Lateral Sclerosis (ALS)	EFhd2 was one of the proteins uniquely identified in lipid raft isolated from mouse overexpressing G93A mutant SOD1	2D-gel and Tandem Mass Spectrometry	Zhai et al., 2009
		EFhd2 was identified as a C9orf72 poly-GA co-aggregating protein	Immunoprecipitation and Tandem Mass Spectrometry	May et al., 2014
	Huntington's disease	EFhd2 down-regulation preceded phenotype onset and it was one of only nine identified proteins with sustained altered expression after onset in a mouse model for Huntington's disease	2D-gel and Tandem Mass Spectrometry	Zabel et al., 2009
Dementia and psychiatric disorders	Suicide	EFhd2 was found down-regulated in prefrontal cortex and up-regulated in amygdala of individuals that committed suicide	2D-DIGE and Tandem Mass Spectrometry	Kékesi et al., 2012
	Schizophrenia	EFhd2 protein was found up-regulated in dorsolateral prefrontal cortex samples from schizophrenia patients	Quantitative Mass Spectrometry	Martins-de-Souza et al., 2009
		EFhd2 protein was found up-regulated in postmortem mediodorsal thalamus samples from schizophrenia patients	Quantitative Mass Spectrometry	Martins-de-Souza et al., 2010
	Alzheimer's disease (AD) and related dementias	EFhd2 was shown to co-immunoprecipitate with tau proteins in temporal cortex derived from AD and FTDP cases and in brain samples from a tauopathy mouse model (JNPL3)	Immunoprecipitation and Tandem Mass Spectrometry	Vega et al., 2008
		EFhd2 is found overexpressed in AD (APP23) and stroke (pMCAO) mouse models	Microarray	Tseveleki et al., 2010
		Increased alternative splicing of EFhd2 in frontal cortex of AD patients in comparison to normal aging	RNA-Seq	Twine et al., 2011
		EFhd2 was found associated with tau aggregates in the somatodendritic compartment and co-purified with tau filaments; EFhd2 protein abundance also found increased in AD cases	Immunoblotting, immune-gold electron microscopy, and histology	Ferrer-Acosta et al., 2013b
		EFhd2 protein levels were found reduced in frontal cortices from different types of tauopathies and other dementias	Immunoblotting	Borger et al., 2014

animal models that mimic the pathophysiology associated with AD (Vega et al., 2008; Tseveleki et al., 2010; Borger et al., 2014). Previously, we showed that EFhd2 protein is increased in the tauopathy mouse model JNPL3, which expresses the human P301L mutant tau protein (Vega et al., 2008; Ferrer-Acosta et al., 2013b). We showed that EFhd2 protein abundance increased as the accumulation of pathological tau and progression of the motor impairment phenotype augmented in JNPL3 mice (Vega et al., 2008). In an independent expression profiling study, using a different AD mouse model, APP23, EFhd2 was also found to be overexpressed (Tseveleki et al., 2010). It is important to mention that the APP23 AD mouse model overexpresses human Amyloid Precursor Protein with double Swedish mutation (K670M/N671L) and it has been shown that tau mediates the toxicity observed in this mouse model (Sturchler-Pierrat et al., 1997; Ittner et al., 2010). These results suggest that EFhd2 is up-regulated in response to pathological processes associated with tau-mediated neurodegeneration.

EFhd2 protein increased abundance in mouse models of neurodegeneration was validated in postmortem AD brain (Vega et al., 2008; Ferrer-Acosta et al., 2013b). Using quantitative immunoblotting, we demonstrated that EFhd2

protein abundance is increased in postmortem frontal cortices in AD cases (Ferrer-Acosta et al., 2013b). In contrast, Borger et al. (2014) showed that EFhd2 protein abundance is reduced in AD cases and other dementias, including frontotemporal lobar degeneration with TDP43 pathology. Moreover, they showed that EFhd2 mRNA was also significantly lower in AD, when compare to normal aging controls. The discrepancy between these two reports could be due to differences in postmortem brain sample selection, agonal stage, activated glial cells, and/or differential immune cell infiltration. For example, in our study, we included only AD postmortem brain with Braak stage IV or higher (Ferrer-Acosta et al., 2013b). In Borger et al. (2014), Braak stage is reported for only a few of the cases used. It is important to mention that in those reported cases with Braak stage VI, EFhd2 protein level is higher than in the other AD cases that the Braak stage was not indicated. Other technical reasons, such as anti-EFhd2 antibodies used, protein extraction method and differential subcellular localization, could contribute to the discrepancy. Interestingly, Twine et al. (2011) identified EFhd2 as one of the genes with increased alternative splicing in frontal cortices of AD patients. This is consistent with previous results that identified two protein bands corresponding to EFhd2, which might contribute to the detection of different EFhd2 isoforms depending on the antibody used (Avramidou et al., 2007; Vega et al., 2008). Nevertheless, what is consistent among these studies is that EFhd2 expression was found altered in AD. Further studies are required to determine the molecular mechanisms involved in the regulation of the *Efhd2* gene and protein under pathological or cellular stress conditions.

Further biochemical characterization of EFhd2 protein demonstrated that this novel protein has the molecular and structural features of amyloid proteins (Ferrer-Acosta et al., 2013b). *In vitro* studies showed that Thioflavin S (a dye that selectively binds to amyloid structures) binds recombinant EFhd2 protein, indicating that EFhd2 transition from a mostly helical and random coil structure to cross-beta-sheet (Ferrer-Acosta et al., 2013a,b). Amyloid proteins tend to form oligomers and filamentous structures. Electron microscopy analyses confirmed that EFhd2 forms filaments *in vitro* without the requirement of a nucleation factor (Congdon et al., 2008; Ferrer-Acosta et al., 2013b). Furthermore, the presence of calcium reduces EFhd2's ability to form filaments, and the coiled-coil domain was shown to be required for the formation of EFhd2 homodimers (Ferrer-Acosta et al., 2013b). These results indicated that formation of EFhd2 filaments could promote the association with pathological tau filaments. To test this hypothesis, immunohistological analyses of AD brain slices were performed. The results showed that EFhd2 co-localized with PHF1 (an antibody that recognized tau filaments) in the somatodendritic compartment, validating the association of EFhd2 with filamentous tau structures (Ferrer-Acosta et al., 2013b). Moreover, immune-gold electron microscopy showed that EFhd2 and tau, purified from AD brain, formed co-filaments (Ferrer-Acosta et al., 2013b). Moreover, *in vitro* protein–protein interaction assays demonstrated that EFhd2's coiled-coil domain is necessary for its association with tau proteins purified from brain extract derived from JNPL3 mice (Ferrer-Acosta et al., 2013b). Thus, it is plausible to hypothesize that formation of EFhd2 oligomers may serve as nucleation factor for tau oligomerization and, consequently, NFTs in AD and other tauopathies (**Figure 1**). Nevertheless, further studies are required to determine EFhd2's capability to enhance protein aggregation in tauopathy and other neurodegenerative disorders.

EFHD2 IS ASSOCIATED WITH PARKINSON'S DISEASE AND OTHER NEUROLOGICAL DISORDERS

EFhd2 has been also found associated with Parkinson's disease (PD). Liscovitch and French (2014) showed that EFhd2 expression is increased in substantia nigra in PD. In this study, they found that EFhd2 overexpression correlated with the positive co-expression of α-synuclein and IFN-γ, establishing a molecular relationship between PD and inflammation (Liscovitch and French, 2014; **Table 1**). In contrast, EFhd2 protein abundance was found reduced in the substantia nigra of a mouse model where PTEN-induced kinase 1 (PINK1) has been knockout (Diedrich et al., 2011). PINK1 is a mitochondrial protein, the loss-of-function mutation of which induces early-onset PD. These conflicting reports could be due to the difference between mechanisms associated with sporadic PD cases and those underlying the phenotype of the PINK1 knockout mice. However, *in vitro* studies identified EFhd2 as a protein secreted/released by microglia cells upon incubation with nitrated α-synuclein (Reynolds et al., 2008). This is the first study that shows expression of EFhd2 in microglia cells, but, more importantly, it also indicates that EFhd2, a cytosolic protein, could be released from cells in response to a pathological signal. Taken together, EFhd2 altered protein abundance in PD provides strong evidence that it is involved in molecular mechanisms associated with neurodegeneration.

EFhd2 associates with known proteins linked to the pathophysiology of PD (**Table 1**). In a proteomics approach, EFhd2 was identified associated with leucine-rich repeat kinase 2 (Lrrk2), the most common causative gene of inherited PD (Meixner et al., 2011). Interestingly, this study demonstrated that Lrrk2 decreased actin polymerization, promoting the accumulation of monomeric actin (G-actin; Meixner et al., 2011). Knockdown of Lrrk2 affects actin cytoskeleton and cellular morphology, indicating that Lrrk2 plays a direct role on actin (Meixner et al., 2011). In contrast, EFhd2 promotes actin bundling (Huh et al., 2013). Thus, these results suggest that Lrrk2 and EFhd2 may compete for the interaction with F-actin, as regulators of actin cytoskeleton dynamics. Moreover, Lrrk2 has been shown to phosphorylate tau and promote tau aggregation, as observed in postmortem brain tissue from PD cases with Lrrk2 mutations (Guerreiro et al., 2015). On the basis of their link to neurodegeneration, it is reasonable to speculate that there could be a pathological connection between Lrrk2 and tau through EFhd2.

EFhd2 altered protein abundance and its association with pathological proteins have also been found in other neurological disorders. EFhd2 protein was identified as a co-aggregating

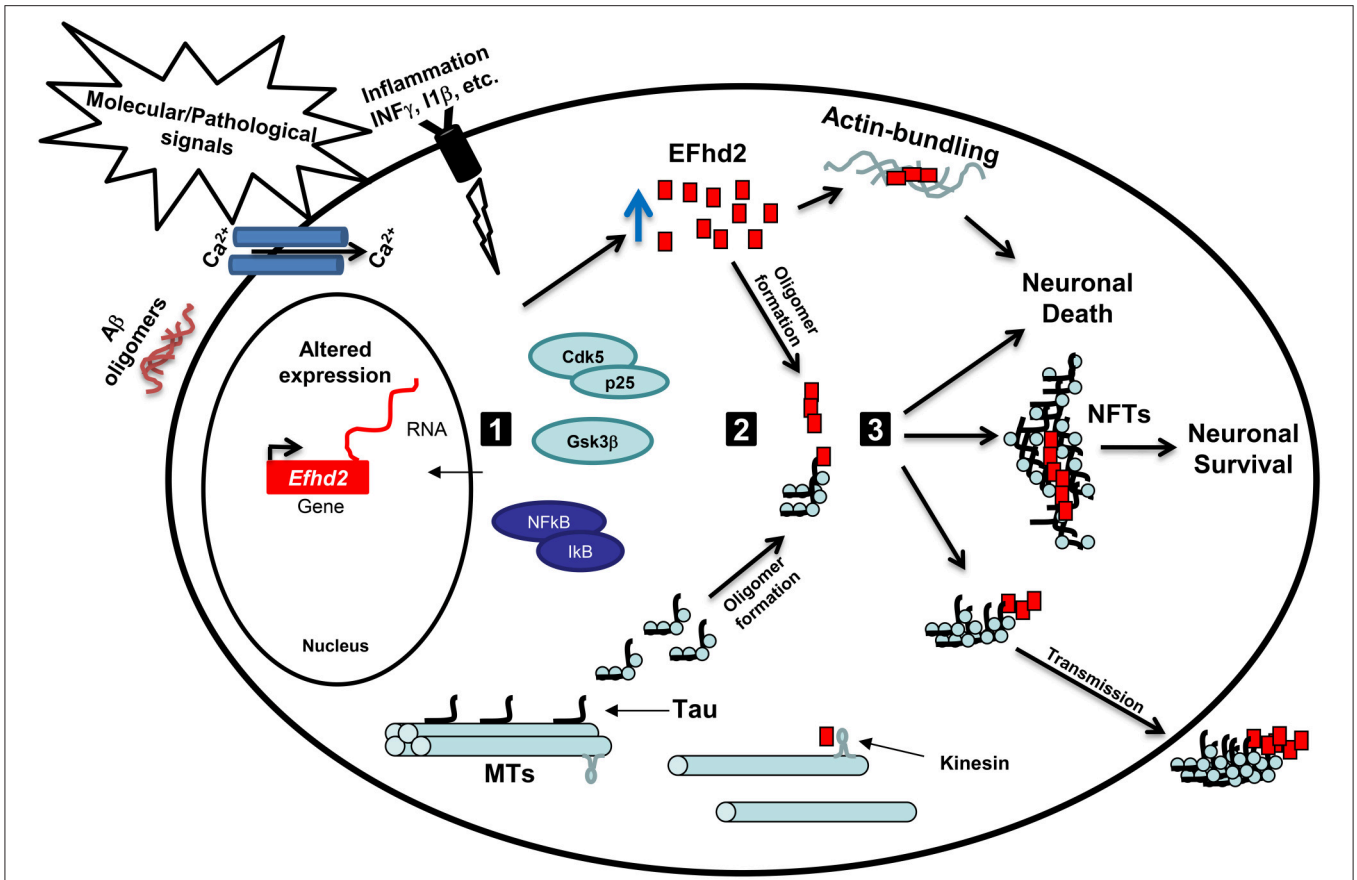

FIGURE 1 | Working hypothesis: EFhd2 role in neurodegeneration. (1) Activation: Pathological signals, such accumulation of Aβ oligomers, calcium influx, and neuroinflammation, lead to the activation of specific kinases, such as GSK3β and Cdk5/p25, and upregulation of gene expression. These kinases mediate the hyperphosphorylation of tau proteins and promote its release from the microtubules (MTs). Altered expression of *Efhd2* gene leads to increase abundance of EFhd2 protein. (2) Oligomerization: The accumulation of EFhd2 proteins and its self-oligomerization properties could serve as a nucleation factor for hyperphosphorylated tau proteins, enhancing the kinetics of the formation of tau oligomers and/or neurofibrillary tangles at the somatodendritic compartment. Additionally, the accumulation of EFhd2 proteins affects kinesin-mediated fast axonal transport and promotes actin-bundling. (3) Toxicity: The accumulation of EFhd2 and tau oligomers leads to neuronal death and its release to the interstitial fluid, where are up-taken by neighboring neurons. Alternatively, EFhd2 could accelerate the transition from tau oligomers to neurofibrillary tangles (NFTs) as a neuroprotective mechanism, reducing the capacity of toxic tau transmission. Further studies are required to define the pathological role of EFhd2 in AD and other neurological disorders.

protein with poly-GA C9orf72, a common pathogenic protein in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD; May et al., 2014). This result suggests that EFhd2 may be involved in the molecular mechanisms that lead to aggregation of pathological proteins, other than tau, in proteinopathies such as ALS. Consistently, EFhd2 was one of the uniquely identified proteins found in lipid rafts isolated from a mouse model that expresses the G93A SOD1 mutant; a mutation found in inherited ALS cases (Zhai et al., 2009). This result indicates that EFhd2 subcellular localization could also be altered in neurodegeneration.

In addition to its association with neurodegenerative diseases, *Efhd2* gene expression has also been found altered in psychiatric disorders. Two independent studies demonstrated that EFhd2 is up-regulated in schizophrenia (Martins-de-Souza et al., 2009, 2010). Postmortem analysis of dorsolateral prefrontal cortex and mediodorsal thalamus, two brain regions associated with the pathophysiology of schizophrenia, revealed a significant increase in EFhd2 protein level in comparison to normal control cases (Martins-de-Souza et al., 2009, 2010). In contrast, EFhd2 was found down-regulated in prefrontal cortex and up-regulated in amygdala of individuals that committed suicide (Kékesi et al., 2012). These results suggest that altered levels of EFhd2 are directly associated with brain pathology.

CONCLUSION

In summary, EFhd2 harbors similarities to known and well-studied neuropathological proteins. For instance, EFhd2 is a structural disorder protein mainly composed of random coils and alpha helices, forms filamentous structures, associates with vesicle trafficking and cytoskeleton rearrangement, co-aggregate, and co-purify with pathological proteins, its expression and protein abundance is altered in neurodegenerative diseases. Based on these similarities and the published results discussed

above, it is plausible to hypothesize that neuronal stress due to environmental cues (such as cytokines) or pathological protein aggregation induces changes in *Efhd2* gene expression (**Figure 1**). In this context, activation of signaling proteins, such as Cdk5 (Vázquez-Rosa et al., 2014), induces posttranslational modifications on EFhd2 that affect its calcium binding activity and promotes self-oligomerization (**Figure 1**). The accumulation of EFhd2 oligomers could serve as a nucleation factor of tau proteins facilitating its accumulation at the somatodendritic compartment, which also affects kinesin-mediated fast axonal transport (**Figure 1**; Kanaan et al., 2013). Consequently, the accumulation of EFhd2 proteins and tau aggregates would lead to activation of apoptosis (**Figure 1**). Interestingly, up-regulation of EFhd2 could also promote aberrant actin bundling, leading to the formation of Hirano bodies in AD (**Figure 1**, Sonoda et al., 2015).

Alternatively, EFhd2 altered expression in neurological disorders could be related to neuroprotection. The identification of EFhd2 co-localization with neurofibrillary tangles (NFTs) in the somatodendric compartment and its filament formation capability suggest that EFhd2 may affect the kinetic of tau filament formation, promoting the generation of stable ultrastructures (**Figure 1**). The generation of NFTs may sequester tau oligomers, preventing the spread of toxic tau to the interstitial fluid. Concomitantly, EFhd2's accumulation in NFTs induces a loss-of-function effect that promotes the generation of new functional synapses through increase vesicle transport velocity similar to that observed in *Efhd2* knockout mice. This may explain why NFT bearing neurons are still synaptically active and functionally integrated in neuronal circuits (Kuchibhotla et al., 2014). Nevertheless, further studies are crucial to determine the role that EFhd2 plays in the pathophysiology of neurological disorders.

AUTHOR CONTRIBUTIONS

IEV revised the literature and wrote the manuscript.

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METHODS
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A Rapid, Semi-Quantitative Assay to Screen for Modulators of Alpha-Synuclein Oligomerization *Ex vivo*

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Alpha synuclein (αsyn) aggregates are associated with the pathogenesis of Parkinson's disease and others related disorders. Although modulation of αsyn aggregation is an attractive therapeutic target, new powerful methodologies are desperately needed to facilitate *in vivo* screening of novel therapeutics. Here, we describe an *in vivo* rodent model with the unique ability to rapidly track αsyn-αsyn interactions and thus oligomerization using a bioluminescent protein complementation strategy that monitors spatial and temporal αsyn oligomerization *ex vivo*. We find that αsyn forms oligomers *in vivo* as early as 1 week after stereotactic AAV injection into rat substantia nigra. Strikingly, although abundant αsyn expression is also detected in *striatum* at 1 week, no αsyn oligomers are detected at this time point. By 4 weeks, oligomerization of αsyn is detected in both striatum and substantia nigra homogenates. Moreover, in a proof-of-principle experiment, the effect of a previously described Hsp90 inhibitor known to prevent αsyn oligomer formation, demonstrates the utility of this rapid and sensitive animal model to monitor αsyn oligomerization status in the rat brain.

Keywords: alpha-synuclein, oligomers, protein-fragment complementation, bioluminescence, viral vector model

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INTRODUCTION

Under pathological conditions alpha-synuclein (αsyn) can misfold and aggregate into insoluble deposits that accumulate in cells to toxic levels. The conversion of αsyn from its functional conformation into a misfolded and toxic conformation constitutes the basis of a group of diseases known as synucleinopathies which include Parkinson's disease (PD), multiple system atrophy, and dementia with Lewy bodies (Goedert and Spillantini, 1998; Kim et al., 2014). Several lines of evidence demonstrate a strong association between αsyn aggregation and neurodegeneration (Irizarry et al., 1998; Chartier-Harlin et al., 2004; Winner et al., 2011). Fibrillar forms of αsyn are the major component of glial cytoplasmic inclusions, Lewy bodies (LBs), and Lewy neurites, defined as intracytoplasmatic inclusions and considered the pathological hallmarks of synucleopathies (Forno, 1996; Spillantini et al., 1997; Halliday et al., 2011). Normally, αsyn is a soluble, presynaptic protein that may exist as a natively unfolded monomer or a functional tetramer (Bartels et al., 2011; Wang et al., 2011; Fauvet et al., 2012; Burré et al., 2013; Gurry et al., 2013; Selkoe et al., 2014; Dettmer et al., 2015a,b). The processes that lead to pathological aggregate formation occur through

the formation of several soluble oligomeric intermediates that mature into the insoluble amyloid fibrils found in LBs. It has been proposed that prefibrillar forms of αsyn are the disease-causative toxic species, while the insoluble fibrils might represent a protective pathway for surviving cells (Ross and Poirier, 2004; Paleologou et al., 2009; Winner et al., 2011; Kalia et al., 2013).

Although the mechanisms of αsyn-induced toxicity remain unclear, inhibition of oligomerization represents an attractive therapeutic approach. Such a strategy requires the availability of powerful cellular and rodent models. Current methods for detection of αsyn oligomers are challenging due to their dynamic nature and sensitivity to external conditions (Uversky, 2003; Drescher et al., 2012; Gurry et al., 2013) Thus, far, oligomer studies have used indirect methods and biochemical techniques that prohibit the study of αsyn oligomerization in a live cellular environment or in intact brain. More recently, protein fragment complementation assay (PCA) strategies that allow the detection of αsyn-αsyn interactions using fluorescence or bioluminescent reporters, have been widely developed and have been successfully applied to monitor αsyn oligomers not only in living cells (Outeiro et al., 2008; Putcha et al., 2010; Danzer et al., 2012) but also in rodent brain (Dimant et al., 2013, 2014; Aelvoet et al., 2014; McFarland et al., 2014).

Here, we generated and characterized a viral vector rodent model where humanized *Gaussia princeps* luciferase (hGluc) is used as a surrogate reporter of αsyn oligomerization *in vivo* in a fast, sensitive, and semi quantitative assay. We demonstrate that this rodent model can be utilized to track αsyn oligomerization *in vivo* and validate the potential use of the model by testing a novel Hsp90 inhibitor compound, known to reduce αsyn oligomerization *in vitro*, on our *in vivo* bioluminescence read out.

MATERIALS AND METHODS

Viral Vector Production

The viral vectors pAAV-CBA-synuclein-LUC1-WPRE (SL1) and pAAV-CBA-SYNUCLEIN-luc2-WPRE (SL2) were constructed by inserting the human SNCA cDNA (h αsyn) fused to either the N-terminus half of humanized *G. princeps* luciferase (hGluc) (Tannous et al., 2005) or the C-terminus half of hGluc, into the EcoRV and NheI sites of the pAAV-CBA-WPRE. Viral vector pAAV-CBA-*G. princeps* luciferase was constructed by inserting the full length of hGluc gene into the XhoI and NheI sites of pAAV-CBA-WPRE vector. Adeno-associated virus (AAV) serotype 2/8 was produced by plasmid transfection with helper plasmids in HEK293T cells. Forty-eight hours later, the cells were harvested and lysed in the presence of 0.5% sodium deoxycholate and 50μ/ml Benzonase (Sigma-Aldrich, St. Louis, MO) by freeze-thawing, and the virus was isolated using a discontinuous iodixanol gradient. The genomic titer of each virus was determined by quantitative PCR.

Surgical Procedure

Adult Female Sprague Dawley rats (225–250 g, Harlan, USA) were housed and treated in accordance with the NIH Guide for Care and Use of Laboratory animals. All procedures were approved and conducted in accordance with the Mayo Clinic Institutional Animal Care and Use committee. Rats were housed 3 per cage with ad-libitum access to food and water during a 12 h light/dark cycle. Surgery was conducted under 2% isoflurane anesthesia mixed with O2 and N2 using a stereotaxic frame and a 10 μl Hamilton syringe fitted with a 30 gauge needle. The scalp was exposed and a unilateral injection targeting the substantia nigra (SN) was performed at coordinates 5.2 mm posterior and 2 mm lateral to bregma, and 7.2 mm ventral relative to dura. AAV8 vectors were normalized by titer and volume, resulting in injection of an equal amount of genomes per copy per vector. Two microliter of a mixture of two viruses (SL1 8.10^{12} g/ml + SL2 8.10^{12} g/ml) were injected at a rate of 0.4 μl/min using a microinjection pump (Stoelting Co, Wood Dale, IL). Control animals were injected with one virus only (2 μl of AAV8-SL2 at 16.10^{12} genome/ml), or received one injection of 1 μl of SL1 (8.10^{12} g/ml) in the SN of the left hemisphere (ML: −2 mm) and one injection of 1 μl of SL2 (8.10^{12} g/ml) in the SN of the other hemisphere (ML: 2 mm). At the end of injection the needle remained in place for 5 min before being slowly retracted. Animals were then sutured with metal clips and monitored until fully recovered.

Tissue Processing

For histological analyses, animals were deeply anaesthetized at 1 and 4 weeks post-injection with pentobarbital and perfused transcardially with ice-cold 0.9% saline, followed by 4% paraformaldehyde (PFA). Brains were removed and post-fixed for 4 h in the same solution and were then transferred overnight to 25% sucrose solution for cryoprotection. The brains were cross-sectioned using a freezing stage sliding microtome (Leica SM2010R, Germany) at 40 μm in the coronal plane. Brains from a subset of animals from 1 to 4 weeks post injection were harvested fresh without fixation. The two hemispheres were separated and the striatum (STR) and midbrain from both sides were quickly dissected on ice and used directly for biochemical analysis.

Immunohistochemistry

Immunohistochemical analysis was performed on free-floating sections (40 μm) using primary antibodies against tyrosine hydroxylase (TH) (1:3000, MAB318, Millipore), and hαsyn (1:2000, 4B12, specific antibody to human form of αsyn, Covance). Sections were washed with phosphate buffer saline (PBS) before each incubation. After the initial wash, the sections were quenched for 20 min at RT with PBS solution, supplemented with 3% H_2O_2 (v/v) (Sigma-Aldrich), and 10% Methanol (v/v) (Pharmaco-Aaper) and were then blocked with 5% goat serum (Life technologies, Carlsbad, CA, USA) in T-PBS (0.25% Triton dissolved in PBS). The sections were incubated overnight at RT with one of the indicated antibodies, diluted in 2.5% goat serum and T-PBS. After washing, the brain slices were treated with 1:200 dilution of the appropriate biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) in 1% goat serum and T-PBS. Thereafter, the sections were rinsed and incubated with avidin-biotin-peroxidase complex (ABC peroxidase kit, Thermo Scientific). The staining was visualized by 3,3-diaminobenzidine (DAB; Sigma-Aldrich) used

as a chromagen and activated by 1% H_2O_2. The sections were then mounted on gelatin-coated slides, dehydrated with alcohol, cleared with xylene and coverslipped with DePex mounting medium (Sigma-Aldrich).

Stereology

Nigrostriatal cell loss was assessed using an unbiased stereology method using the optical fractionator principle as described previously (West et al., 1991). Lower magnification was used to trace the region of interest (SN in each hemisphere) at all levels in the rostro-caudal axis in 9–10 sections per animals. TH+ cells in the ventral tegmental area (VTA) were excluded. The counting was performed using the Stereo Investigator software (MBF bioscience). In addition, the counting frame size was adjusted so that ∼100–200 TH+ positive cells were counted in each side of the SN. The estimation of total neuron numbers was performed by using the optical fractionator formula and coefficient of error <0.1 was accepted (West, 1999).

Densitometry

Images of forebrain were captured using the Aperio slide scanner (Aperio, Vista, CA, USA). The optical density of striatal TH+ fibers was measured using ImageJ software (Version 1.46, National Institutes of Health) at four coronal levels according to the rat brain atlas of Paxinos and Watson (6th edition, 2007): AP: +1.2,+ 0.8, 0.0 and −0.4 mm relative to bregma. The measured values were corrected for non-specific background staining by subtracting values obtained from adjacent cortical areas. The data are expressed as a percentage of the corresponding area from the intact side.

Ex vivo Luciferase Complementation Assay

Fresh STR and SN were homogenized briefly in 200 µl cold PBS buffer using a Teflon pestle. One-hundred microliter of the homogenate was transferred to a 96 well plate (Costar, Corning, NY, USA) and the remainder was preserved for biochemical analysis. Luciferase activity from αsyn-αsyn interaction was measured in an automated plate reader (Victor 3 multilabel counter, Perkin Elmer, Waltham, MA, USA) at 480 nm following the injection of 100 µl of the substrate coelenterazine (40 µm, NanoLight tech, AZ, USA) with a signal integration of 2 s.

Human αsyn Elisa

Quantitative analysis of hαsyn concentration in rat tissue was performed using hαsyn specific ELISA (#KHBOO61, Life tech) according to the manufacturer's instructions. Absorbance at 450 nm is directly proportional to the concentration of hαsyn present in the original specimen. αSyn concentrations were normalized to the total amount of protein in the homogenate. Protein content was measured using Bicinchoninic acid assay (BCA) (Thermo Fisher Scientific, Waltham, MA, USA).

Western Blotting

Striatal and midbrain samples were suspended in RIPA buffer (Millipore, 20-188) containing protease inhibitor (Roche Diagnostics), homogenized on ice, and centrifuged at 13,000 × g for 10 min. Protein concentration of each lysate was determined by BCA. Twenty microgram proteins was loaded on a denaturing 4–12% Bis- Tris gradient gel (Nupage, Novex Gel, Life tech) and run according to the manufacturer's instructions. Subsequently, separated protein was transferred to polyvinylidene difluoride (PVDF) membrane, blocked in 5% non-fat milk in TBS-T, and immunoblotted for Hsp70 (1:5000, rabbit, ADI-SPA-812, Enzo). Anti-actin (1:10000, rabbit polyclonal, A2668, Sigma-Aldrich) was used as a loading control. All membranes were then incubated with a secondary antibody conjugated to HRP for 1 h at room temperature. Immunoreactivity was visualized using an ECL chemiluminescent detection Kit (Thermo Fisher Sci) and images were acquired with a CCD imaging system (LAS-4000, Fujifilm, Japan).

SNX-9114 Treatment *In vivo*

SNX-9114 (Pfizer, PF-04944733) was described previously (McFarland et al., 2014) and was dissolved in a 0.5% methylcellulose solution before use. SNX-9114 was administered at a dose of 2 mg/kg by oral gavage twice for 1 week in a volume of 1 mL. As a control, the same volume of vehicle (0.5% methylcellulose) was simultaneously administered in a separate group of rats (vehicle group). SNX-9114 administration started 2 days after the stereotactic viral injection.

Cell Cultures

H4 neuroglioma cells (ATCC, HTB-148, Manassas, USA) were maintained in Opti-MEM supplemented with 10% Fetal Bovine Serum (FBS). H4 cells were transfected with full length hGluc using Superfect transfection reagents (Qiagen, Chatsworth, CA, USA) with an equimolar ratio of plasmids according to the manufacturer's instructions.

A tetracycline-driven stable H4 cell line co-expressing hαsyn tagged with either the amino-terminal (SL1) or the carboxy-terminal fragments (SL2) of hGluc luciferase was generated and described previously (Moussaud et al., 2015). SL1SL2 cells were cultured in Opti-MEM supplemented with 10% FBS, 200 µg/ml geneticin, 200 µg/ml hygromycin B, and 1 µg/ml tetracycline to block the expression of the transgenes (SL1 and SL2). Cells were maintained at 37°C in a 95% air/5% CO_2 humidified incubator and all the cell cultures reagents were from Life technologies.

SL1SL2 stable cells were seeded into 6-well plates at the density of 1×10^5 cells/well and in a 96-well plate at the density of 3×10^4 cells/well in the absence of tetracycline. Twenty-four hours later, media was replaced with FBS-free media supplemented with SNX-9114 (25, 50, 100, or 200 nM) or with DMSO alone as control. After 24 h treatment, cells in the six well plates were lysed (150 mM NaCl, 5 mM Trisbase, 0.1% TritonX, cOmplete Mini, pH 7.4) and analyzed by western blotting using antibodies against: hαsyn (1:1000,mouse, 610787, Becton Dickinson, NJ, USA), Hsp70 (1:5000, rabbit, ADI-SPA-812, Enzo life), and actin (1:10000; rabbit polyclonal, A2668, Sigma-Aldrich), whereas cells in 96 well plates were used for luciferase activity measurement as described previously (Putcha et al., 2010).

Statistics

Data were analyzed using GraphPad Prism 6 (San Diego, CA) and are presented as mean ± standard error of the mean (S.E.M.).

Statistical significance was determined using a Student's *t*-test or one-way analysis of variance with Tukey's multiple comparison *post-hoc*. $P < 0.05$ was considered significant.

RESULTS

Ex vivo Monitoring of αsyn Oligomers using Bioluminescent PCA

The bioluminescent αsyn PCA strategy has been widely used by our lab and others (Outeiro et al., 2008; Putcha et al., 2010; Danzer et al., 2011; Moussaud et al., 2015) to investigate αsyn oligomerization in living cells. Briefly, two hαsyn proteins fused to N- or C-terminal halves of a reporter protein can reconstitute the enzymatic activity of the reporter when αsyn-αsyn interactions occur, thus providing a readout of αsyn-αsyn interactions and oligomerization. In this study, two AAV8 vectors expressing hαsyn fused with either the N-terminus or C-terminus of hGluc (referred hereafter as AAV-SL1 and AAV-SL2) were stereotactically co-injected unilaterally into the SN of adult rats to establish the *in vivo* bioluminescent PCA. Previous studies from our lab have characterized the oligomeric species formed in the bioluminescent complementation assay as a heterogeneous population of soluble dimers and higher order multimers (Danzer et al., 2011; Dimant et al., 2013). The term oligomer is used henceforth to represent the heterogenous population.

Delivery of AAV-SL1 and AAV-SL2 into the SN of the rat produces widespread overexpression of hαsyn along the nigrostriatal pathway as revealed by histological analysis (**Figures 1A,B**). Immunostaining with an antibody specific for hαsyn shows numerous cell bodies in the SN expressing the transgenes at 1 week and at 4 weeks while the non-injected side is devoid of hαsyn and is used as an internal control (**Figure 1A**). Transport of virally transduced αsyn is indicated by the extensive network of αsyn positive fibers at the striatal level (**Figure 1B**). Hαsyn is observed along the axons from neurons of the SN, terminating in the STR. Expression of hαsyn is detected from the most anterior to the most posterior extent of the STR. This event seems to happen rapidly after the viral injection since expression of the transgenes in the terminals is observed after 1 week. (**Figure 1B**, injected side). Moreover, these data demonstrate that the luciferase reporter does not affect the ability of hαsyn protein to be transported along the nigrostriatal pathway.

Because the substrate for hGluc, coelenterazine, does not permeate the blood brain barrier, *in vivo* luciferase signal was measured in homogenates *ex vivo* from the SN and STR overexpressing tagged hαsyn at 1 week ($n = 7$) and at 4 weeks ($n = 8$) post injection. In each group, all brains were processed at the same time and under the same conditions. During homogenization no detergent, no intense shaking, and no sonication are used. The tissue is briefly homogenized in PBS with a tissue grinder pestle and vortexed for 1 min. The contralateral non-injected sides (STR and SN) were processed in the same manner and served as internal controls for non-specific background signal. Interestingly, luciferase activity could be detected in the injected SN as early as 1 week post injection (**Figure 2A**), increasing dramatically by 13-fold at 4 weeks (**Figure 2A**). By contrast, in the STR, no luciferase activity is detected at 1 week post injection (**Figure 2B**) whereas, abundant luciferase activity is detected at 4 weeks (**Figure 2B**). The luciferase signal in tissue homogenate from injected animals indicates *in vivo* luciferase complementation, the surrogate marker of αsyn-αsyn interactions. As expected, in animals injected with only one half (AAV-SL2 only), no luciferase activity was detected after 4 weeks (**Supplementary Figures 1A,B**) demonstrating that fragmented luciferase does not give rise to aberrant luminescence signal. A second control incorporated into our experimental paradigm was a group of animals injected with one half of the complementation pair (AAV-SL1) in the left SN

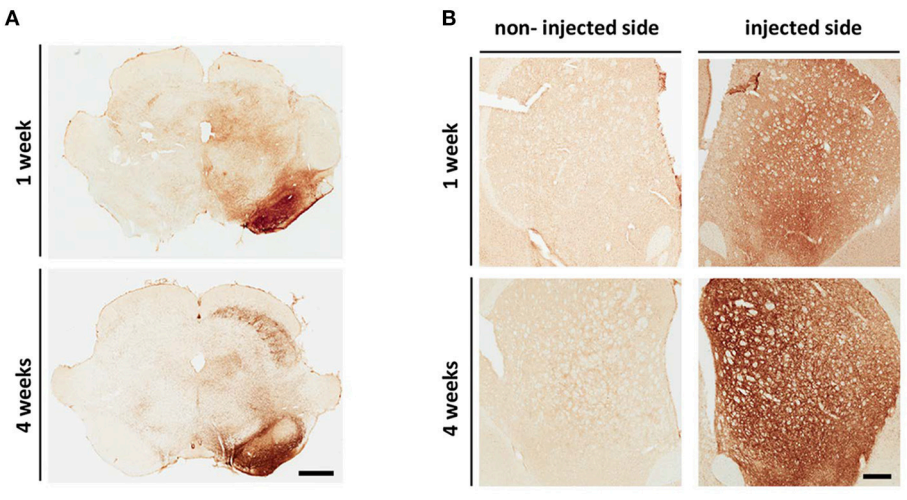

FIGURE 1 | AAV8 SL1SL2 transduction results in human αsyn over-expression along the nigrostriatal pathway. (A,B) Representative brightfield photomicrographs of SN **(A)** and STR **(B)** at 1 and 4 weeks. Coronal sections immunostained with an antibody specific for hαsyn (clone 4B12) had expression of the transgenes in the injected SN **(A)**. The expression was also detected at the striatal level **(B)** with a more intense staining at 4 weeks, scale bar A, 1 mm; B, 500 μm.

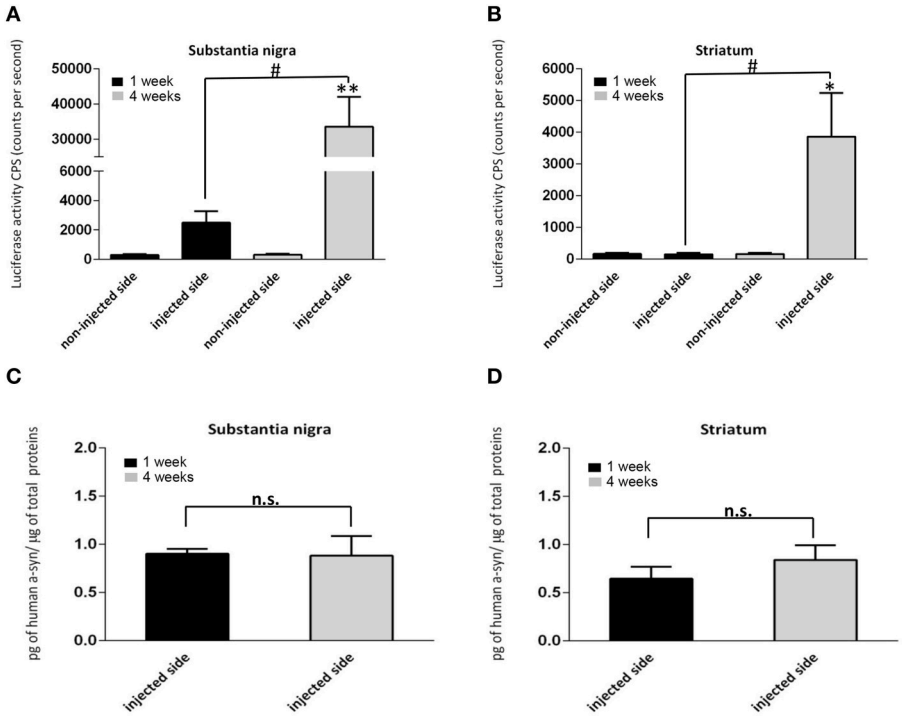

FIGURE 2 | Monitoring of αsyn oligomerization *ex vivo* using protein complementation assay. (A,B) αsyn oligomers were quantified via luciferase assay in brain homogenates at 1 and 4 weeks after stereotaxic injection of SL1 and SL2. Signal detected in the non-injected side was used as a baseline. **(A)** In the SN, αsyn oligomers were detected at 1 week, increasing significantly by 4 weeks [$n = 6$ per group, Student t-test, $T_{(10)} = 3.6$; $^{**}p < 0.001$]. **(B)** In the STR no luciferase activity was detected at 1 week, but were observed by 4 weeks [$n = 6$ per group, Student's T-test, $T_{(10)} = 2.4$; $^{*}p < 0.05$]. **(C,D)** represent the total expression of h-αsyn assessed by ELISA in the homogenates of the SN **(C)** and STR **(D)** at 1 and 4 weeks. The data are expressed as the amount of h-αsyn normalized to the total amount of proteins in the homogenates. Statistical analysis showed no significant differences across the time for each regions ($p > 0.05$, $n = 6$ per group). In **(A,B)** number sign indicates significant differences between the injected side at 1 week and 4 week ($^{\#}p < 0.05$). n.s, not significant.

and the other half (AAV-SL2) in the right SN. After 4 weeks, homogenates from left and right SN or STR were mixed together and assayed for luciferase activity. As expected, no luciferase activity was detected (**Supplementary Figure 1C**) demonstrating that αsyn-αsyn interactions occur *in vivo* and not *ex-vivo* during tissue processing.

Reconstitution of luciferase activity *in vivo* demonstrates the successful complementation of luciferase halves via the formation of αsyn oligomers. Importantly, our data show the presence of αsyn oligomers in the SN as early as 1 week post-delivery of AAV-SL1 and AAV-SL2 (**Figure 2A**). The total level of hαsyn in the SN of the animals was quantified by ELISA at 1 and 4 weeks (**Figure 2C**). No significant difference was detected in hαsyn levels 1 and 4 weeks (**Figure 2C**, 1 week $= 0.90 \pm 0.05$ 4 weeks $= 0.88 \pm 0.22$, $p > 0.05$). Interestingly, although equivalent levels of hαsyn was detected in SN at 1 and 4 weeks, the oligomeric profile was very different (**Figure 2A**). No hαsyn oligomers were detected in the STR at 1 week (**Figure 2B**) despite abundant expression of αsyn by ELISA quantification (**Figure 2D**). There are two ways these data can be interpreted; αsyn oligomers form in the SN and are then transported to the striatal terminals which takes longer than a week or that oligomers form at the terminals over a longer period of time (**Figure 2B**). Regardless, the data support the fact that subcellular location affects αsyn oligomerization kinetics.

SL1 and SL2 Overexpression Induce Progressive Nigral and Striatal Dopaminergic Neurodegeneration

Previous viral vector rodent models of αsyn overexpression have described a progressive nigral cell loss and striatal terminal neurodegeneration (Kirik et al., 2002; Klein et al., 2002). To confirm that our *in vivo* αsyn-αsyn bioluminescent complementation model exhibits similar characteristics, we immunostained coronal sections at the level of the SN and STR with an antibody against tyrosine hydroxylase (TH), and performed unbiased stereology and densitometry analysis. At 1 week, TH immunostaining and stereology of the SN indicated no loss of TH+ neurons in the injected side overexpressing SL1 and SL2 when compared to the non-injected side (**Figure 3A**). However, by 4 weeks a general reduction in the dendritic arborization in the SN of the animals was observed (**Figure 3A**) with stereological quantification confirming a $38.5 \pm 7.5\%$ dopaminergic cell loss in animals expressing SL1 and SL2 (**Figure 3B**). Also, comparison of nigral

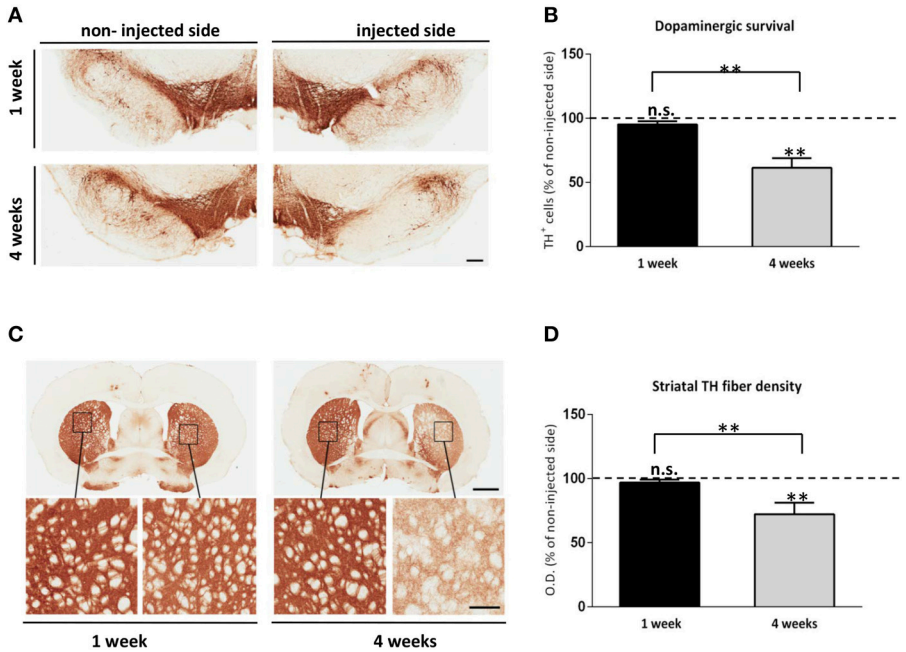

FIGURE 3 | Alpha synuclein associated neurotoxicity in the SL1SL2 rat model. (A) Representative TH immunohistochemical staining of nigral sections in animals injected with AAV8-SL1SL2 at 1 and 4 weeks. Scale bar represents 250 μm. **(B)** illustrates in a graph bar the estimation of total TH+ cells in the SN (expressed as % of non-injected side) using unbiased stereological counting at 1 and 4 weeks. 38.5 ± 7.5% significant dopaminergic cell loss was found at 4 weeks when compared to the non-injected side [Paired Students' *T*-test, $T_{(9)}$= 5.0, $^{**}p < 0.001$] and One-way ANOVA analysis followed by a Tuckey's for multiple testing revealed a 33.81 ± 7.9% significant difference between 1 and 4 weeks ($^{**}p < 0.001$). **(C)** Photomicrographs illustrating TH+ terminals in the STR in SL1SL2 animals. Scale bars represent 2 mm and 300 μm (zoomed-in pictures). **(D)** Illustrates in a bar graph the quantitative densitometry of the TH+ fibers measurements (expressed as percentage of non-injected side). The bars represent the average of four different rostro caudal levels of the STR measured for each animal. Statistical difference was found at 4 weeks in the injected side compared to the non-injected side [Paired Students' *T*-test, $T_{(9)} = 5.0$, $^{**}p<0.001$] and also between time point group ($^{**}p < 0.001$). $n = 6$, for 1 week group and $n = 8$, for 4 weeks group.

neurons at 1 and 4 weeks revealed a significant difference in the extent of cell loss between the groups. Dopaminergic cell death was further corroborated by the loss of fibers in the STR (**Figures 3C,D**). Densitometric analysis of TH stained sections showed a dense innervation throughout the STR of the non-injected side at all-time points. This was also true of the injected side at 1 week (**Figures 3C,D** OD black bar = 96.9 ± 2.2%). By contrast, at 4 weeks the injected side had a significant reduction of striatal TH+ fibers of 27.7 ± 7.4% relative to the contralateral side (**Figure 3D**). Thus, these data demonstrate a progressive cell and terminal loss through 4 weeks.

Modulation of αSyn Oligomerization in the Rodent Bioluminescent PCA Model

The fact that αsyn oligomers can be detected in the SN as soon as 1 week post virus delivery make this a very attractive model for rapid screening of modulators of αsyn oligomers. Therefore, we performed a proof-of-concept experiment whereby rats were treated with a previously described heat shock protein inhibitor, SNX-9114 (McFarland et al., 2014). *In vitro* αsyn-induced aggregation and toxicity is prevented by inhibition of Hsp90 (Klucken et al., 2004; McLean et al., 2004; Danzer et al., 2011; Jones et al., 2014). Hsp90 inhibition results in activation of heat shock factor 1 and by consequence an up-regulation of protective stress-induced proteins such as Hsp70. We recently showed a novel class of Hsp90 inhibitors that could significantly reduce αsyn oligomerization in a cellular model of αsyn aggregation (Putcha et al., 2010; McFarland et al., 2014).

Here we chose to use the Hsp90 inhibitor to validate our 1 week oligomers model. Because SNX-9114 has not been previously validated *in vitro,* we first evaluated its ability to reduce αsyn oligomer formation in our *in vitro* complementation assay. Stable cells expressing SL1 and SL2 were plated in a 96-well plate format and treated with 0.01% DMSO or increasing concentrations of the SNX-9114 (25, 50, 100, and 200 nM). Twenty-four hours later, cells were assayed for luciferase activity to determine the effect of the inhibitor on αsyn oligomerization as measured by reconstituted luciferase activity. A decrease in luciferase activity was first detected at 25 nM and 50 nM (**Figure 4A**, $p > 0.05$), however, at higher doses (100 and 200 nM) SNX-9114 significantly reduced αsyn oligomerization by 32.48 ± 2 and 35.1 ± 4.2% respectively compared to DMSO (**Figure 4A**, $p < 0.05$). Biochemical analysis of cells expressing SL1SL2 and treated with SNX-9114 confirmed the up-regulation of Hsp70 in a dose-dependent manner (**Figure 4B**) with little to no effect on steady state levels of αsyn except at the highest doses (**Figure 4B**).

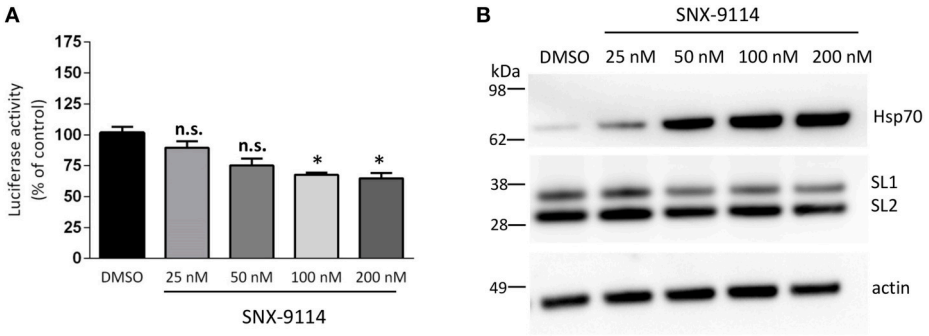

FIGURE 4 | Effect of SNX-9114 on αsyn oligomerization in cell culture. (A) A range of concentrations of SNX-9114 was added to SL1SL2 cells stably overexpressing hαsyn for 24 h. The extent of intracellular αsyn oligomers was evaluated by luciferase assay and was normalized to DMSO control. Oligomerization was significantly decreased with a 100 and 200 nM doses (*$p < 0.05$). **(B)** Induction of Hsp70 after treatment with SNX-9114 on SL1SL2 cells was monitored by western blot as well as the expression of h-αsyn. Actin was used as a loading control. Data are given as mean ± S.E.M., from three independent experiments. Statistical analysis: One-way ANOVA followed by Tuckey's multiple comparison test. n.s., not significant.

To determine if Hsp90 inhibition could reduce αsyn oligomer formation *in vivo* after 1 week, we stereotactically injected AAV-SL1 and AAV-SL2 into rat SN as described previously. Forty-eight hours after surgery we orally gavaged the rats with 2 mg/kg SNX-9114 or vehicle control. A second gavage of 2 mg/kg SNX-9114 or vehicle was performed 2 days later, and the rats were sacrificed at 7 days post-surgery (**Figure 5A**) A control group of rats transduced with AAV-hGluc were included to control for drug effects on the luciferase enzymatic reaction.

Western blot of homogenates prepared from the SN of injected rats confirmed the induction of Hsp70 in animals treated with SNX-9114 compared to animals treated with vehicle (**Figure 5B**). Furthermore, a luciferase activity assay on the same homogenates demonstrated a significant decrease in αsyn oligomers in the group of animals receiving the Hsp90 inhibitor (**Figure 5C**). No significant difference in hαsyn protein levels was detected between the drug and vehicle control groups when measured by ELISA (**Figure 5D**, $p > 0.05$). Of note, consistent with our *in vitro* experiments (**Figure 4B**), SNX-9114 did not directly affect the enzymatic luciferase signal *in vivo* (**Figure 5E**). These findings suggest that the decreased luciferase signal in the Hsp90 inhibitor treated group is indicative of less αsyn oligomer formation (**Figure 5C**).

DISCUSSION

Neuropathologic and genetic studies have provided strong evidence for the involvement of aggregated αsyn in the pathogenesis of synucleopathies (Tu et al., 1998; Dickson et al., 1999; Narhi et al., 1999; Masliah et al., 2000). Our present study proposes a rapid model for tracking αsyn oligomerization in selected brain regions of the rat brain using an *ex vivo* bioluminescent PCA.

Herein we demonstrate that, our split hGluc AAV-SL1 and SL2 vectors can be utilized to track αsyn oligomerization *in vivo*. As early as 1 week, we observe the expression of the transgenes along the nigrostriatal pathway and αsyn oligomers in the SN (**Figures 1**, **2**). At this time point no toxicity is detected in the SN and no loss of dopaminergic fibers at the striatal level (**Figure 3**). The expression of hαsyn and presence of oligomers in SN but no cell loss as early as 1 week post-transduction make this an attractive model with which to screen for novel oligomerization modulators. Because no significant difference was detected in total αsyn levels between SN and STR at 1 week, the absence of αsyn oligomers in STR suggests distinct dynamics of oligomer formation in striatum compared to SN or alludes to oligomer formation occurring in the soma and subsequent trafficking of oligomers to the terminals where additional oligomer formation is seeded. Differentiating between these two mechanisms will require further investigation.

By 4 weeks, abundant αsyn oligomers are detected in both SN and STR and cell loss is detected in the SN, with terminal loss detected in the STR. αSyn expression in the SN and STR did not increase even though there was a 13-fold increase in luciferase activity. These data could support a hypothesis whereby increased asyn oligomers correlate to neurotoxicity, however further studies will be required before this can be confirmed.

Given the essential role of αsyn oligomers in the pathogenesis of synucleopathies it is essential that we develop reliable methods for their detection. Oligomer-specific αsyn antibodies (Emadi et al., 2009; Fagerqvist et al., 2013; Maetzler et al., 2014) recognizing relevant pathology have been designed and are relevant for immunohistochemistry of αsyn brain pathology on human or transgenic mouse (Delenclos et al., 2015; Sengupta et al., 2015). They also hold potential as therapies and could be a relevant disease biomarkers. However, such antibodies cannot monitor the oligomeric state of αsyn in a live cell environment. PCA strategies using fluorescence or bioluminescence reporters have been developed and have proven to be a powerful approach to study protein aggregation. Recently, we generated a rodent model using YFP venus PCA to monitor αsyn oligomerization in live animals (Dimant et al., 2014). Using fluorescence as readout, we directly detected αsyn oligomers and monitored αsyn aggregation in cortical neurons of living mice using two photon microscopy. Recently, a mouse model expressing firefly luciferase

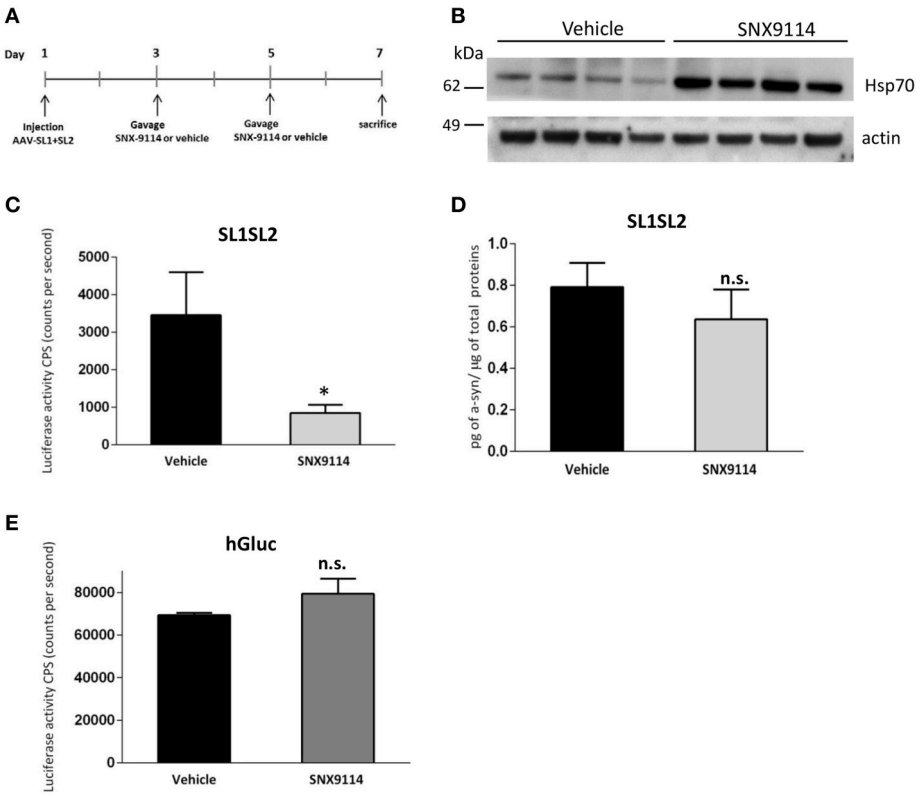

FIGURE 5 | Effect of SNX-9114 in SL1SL2 rat substantia nigra. (A) Illustration of the timeline experiment of SNX-9114 treatment *in vivo*. **(B)** Hsp70 induction in the SN was evaluated by western blotting and actin was used as a loading control. Western blot shows four representative animals for each group. **(C)** Level of oligomers in the SN of the vehicle group ($n = 6$) and the treated group ($n = 6$) was assessed by luciferase activity 1 week after viral vector delivery. A significant decrease of αsyn oligomerization was observed after 2 mg/kg SNX-9114 treatment [Student's T-test, $T_{(9)} = 2.46$; $^*p < 0.05$]. **(D)** Expression of hαsyn in injected SN homogenate was analyzed by ELISA and is expressed as amount of hαsyn normalized to the total amount of protein in the homogenates. **(E)** Control rats injected with hGluc and treated either with vehicle ($n = 2$) or SNX-9114 ($n = 2$). The luciferase activity was not affected by the drug treatment as $p > 0.05$. n.s, not significant.

tagged-αsyn *in vivo* was generated and bioluminescent imaging (IVIS) was used to non-invasively capture αsyn oligomerization in the mouse brain (Aelvoet et al., 2014). Both of these *in vivo* models have distinct advantages such as the ability to follow the response in the same animals over time, providing a kinetic readout of the oligomerization process. In our model we measure the oligomerization *ex-vivo*. Although, all brains were processed at the same time and under the same conditions, we cannot exclude the possibility that the extraction procedure could perturb αsyn aggregation state. On the other hand, even though in *vivo* imaging represents a good tool to monitor αsyn, these methods require expensive instrumentation, are time-consuming, and involve anesthesia and systemic substrate injections in the case of the firefly luciferase PCA. Lastly, the two photon microscopy is limited by photo absorption of the tissue and does not allow deep brain structure imaging.

The model presented herein has the advantage of having a rapid readout without expensive equipment. HGluc may also provide increased sensitivity as it is over 100-fold more sensitive than the commonly used luciferases, Firefly, and *Renilla reniformis* (Tannous et al., 2005; Remy and Michnick, 2006). Also Gluc is a smaller reporter (185 amino acid; Tannous et al., 2005) compared to the other forms of luciferase or the fluorescence reporter. Finally, hGluc PCA is a fully reversible interaction unlike the assay based on YFP venus fluorophore, thus facilitating the detection of the kinetics of protein complex assembly/disassembly *in vivo*. Better spatial and temporal resolution can be achieved compared with *in vivo* imaging and we believe that hGluc PCA may be more efficient and effective for some approaches. In our current experiment, oligomers were quantified *ex vivo* for several reasons. Brain lysate analysis provides a more accurate semi quantitative measure of aggregates and most importantly, allows a combination of different analyses on the same sample (e.g., ELISA, WB) for each brain. Because the PCA assay is effective in tissue directly placed in a micro titer plate, its utility to measure oligomers in *in vivo* brain slice preparations is also a possibility. Also, it is well established that αsyn oligomers can be transmitted from neuron(s) to neuron(s), thus inducing pathology along neuroanatomical pathways in the brain (El-Agnaf et al., 2006; Emmanouilidou et al., 2010). It remains to be determined if αsyn induces neurodegeneration via intracellular- or extracellular-associated mechanisms. Our AAV-model, coupled with *in vivo* microdialysis, could be a useful tool for the detection and analysis of αsyn oligomers in the interstitial

fluid (ISF) of living animals, allowing a real time monitoring of *in vivo* processes and opening possibilities for αsyn transmission studies. One disadvantage is that coelenterazine, the substrate of hGluc, does not cross the blood brain barrier and therefore excludes the use of our model for imaging in live animals. Despite this limitation, the use of hGluc PCA *in vivo* provides a rapid model to track αsyn oligomers depending on the desired readout.

To investigate the potential of our *in vivo* bioluminescent PCA to identify compounds that interfere with αsyn-αsyn interaction or modulate oligomerization, in a little as 1 week, we administered a cohort of AAV SL1 and SL2 transduced rats with 2 mg/kg SNX-9114, for one week. Hsp90 inhibition has been shown to modulate αsyn aggregation and toxicity (Auluck et al., 2002; Kalia et al., 2010; Luo et al., 2010; Putcha et al., 2010) and we have previously established that pharmacological pretreatment with geldanamycin, a naturally occurring Hsp90 inhibitor, protects against αsyn-induced toxicity and leads to degradation of high molecular-weight species (McLean et al., 2004) More recently we described the efficacy of novel brain permeable Hsp90 inhibitors to reduce the formation of αsyn oligomeric species in a dose dependent manner using our bioluminescent PCA system in H4 neuroglioma cells (Putcha et al., 2010). In line with these previous data, we show here that an Hsp90 inhibition can inhibit αsyn oligomerization in our AAV-SL1 and AAV-SL2 rodent model, and most importantly, we demonstrated that our luciferase assay is sensitive enough to detect these changes. In addition it is noteworthy to mention that the level of oligomers was assessed as early as 1 week after the AAV-injection, at a time when we do not detect cell death (**Figure 3B**) but we have an abundance of oligomers in the SN (**Figure 2A**). Therefore, the detected decrease in luciferase activity at 1 week in SN in animals receiving Hsp90 inhibitor, can be directly attributed to a decrease of αsyn oligomers.

While several strategies have been developed to monitor the modulation of αsyn aggregation *in vivo* and *in vitro*, the major advantage of the present approach over other techniques is the ability to quantify a change in αsyn oligomerization in a short period of time (1 week) with a fast, simple, and sensitive assay. In conclusion, our bioluminescent *in vivo* model represents a powerful new tool to study spatial and temporal changes in αsyn aggregation in response to new therapeutic agents that modulate αsyn oligomerization.

AUTHOR CONTRIBUTIONS

MD carried out the study design, animal procedures, histology, analysis, and drafting of the manuscript. TT performed immunohistochemistry, stereology analysis, densitometry analysis, and participated in all animal procedures. DJ, participated in the study design, animal procedures, and editing of the manuscript. SM and AB performed the *in vitro* assay. MY, assisted with animal procedures. WH provided the compound SNX-9114. PM participated in the experimental design, coordination, interpretation, drafting, and editing of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnins.2015.00511

Supplementary Figure 1 | Luciferase activity in control rats. (A) Animals were stereotactically injected with one viral vector (AAV8-SL2, $n = 2$). After 4 weeks, the luciferase activity was measured. No specific signal was detected in the injected side since the luciferase activity was similar to the non-injected side. In both regions, SN **(A)** and STR **(B,C)** To verify that the complementation occurs *in vivo* and not during homogenization process, animals were injected with AAV8-SL1 In the left SN and with AAV-SL2 in the right SN ($n = 2$). At 4 weeks homogenate of SN left and right or STR left and right, were mixed together respectively and luciferase activity was measured. No activity was detected in the mixture of SN or STR.

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HYPOTHESIS AND THEORY
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Is Alpha-Synuclein Loss-of-Function a Contributor to Parkinsonian Pathology? Evidence from Non-human Primates

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Accumulation of alpha-synuclein (α-syn) in Lewy bodies and neurites of midbrain dopamine neurons is diagnostic for Parkinson's disease (PD), leading to the proposal that PD is a toxic gain-of-function synucleinopathy. Here we discuss the alternative viewpoint that α-syn displacement from synapses by misfolding and aggregation results in a toxic loss-of-function. In support of this hypothesis we provide evidence from our pilot study demonstrating that knockdown of endogenous α-syn in dopamine neurons of non-human primates reproduces the pattern of nigrostriatal degeneration characteristic of PD.

Keywords: alpha-synuclein, Parkinson's disease, non-human primates, substantia nigra, dopamine

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ALPHA-SYNUCLEIN IN PARKINSON'S DISEASE

The best-validated participant in the molecular pathology of Parkinson's disease (PD) is alpha-synuclein (α-syn). Mutations and multiplications of the α-syn SNCA gene locus both produce inherited forms of PD (Polymeropoulos et al., 1997; Singleton et al., 2003; Ibanez et al., 2004). In addition, the presence of α-syn in Lewy bodies and neurites of midbrain dopamine (DA) neurons, the histological hallmark of PD, provides evidence for its association with idiopathic PD (Spillantini et al., 1997). While a pervasive viewpoint postulates that excess α-syn and consequent aggregation directly fuels neurotoxicity in a toxic gain-of-function event, it also is acknowledged that α-syn aggregation may endanger neurons by removing the protein from its normal cellular location and diminishing its function in a toxic loss-of-function event (Perez and Hastings, 2004; Cookson, 2006; Kanaan and Manfredsson, 2012). This issue remains a topic of debate.

Despite this ambiguity, approaches that may indiscriminately reduce α-syn in the central nervous system represent an active area of research as an approach for treating PD (e.g., AFFiRiS PD01, Prothena/Roche PRX002). In this Hypothesis and Theory article, we discuss evidence from rodent and non-human primate experiments suggesting that Parkinson's-like degeneration of the nigrostriatal DA system can be reproduced by eliminating endogenous α-syn from DA neurons vulnerable to degeneration in PD. These studies support the tentative conclusion that α-syn elimination therapies that do not distinguish between native and abnormal forms may compromise the viability of DA neurons and should proceed with caution.

KNOCKDOWN OF α-SYN IN THE ADULT RAT RESULTS IN DOSE-DEPENDENT NIGROSTRIATAL DEGENERATION

The lack of overt pathology in α-syn germ-line knockout animals (Abeliovich et al., 2000) and the commonly proposed toxic gain-of-function of the α-syn mutant versions (A53T, A30P, E46K; Kruger et al., 1998; Giasson et al., 2002; Klein et al., 2002; Zarranz et al., 2004) led many investigators, us among them, to pursue targeted knockdown of α-syn expression as a potential therapeutic avenue for PD. However, using recombinant adeno-associated virus (rAAV) expressing a short hairpin RNA (shRNA) to knock down α-syn in mature rat substantia nigra (SN) DA neurons we encountered a surprising result: DA neuron degeneration (Gorbatyuk et al., 2010). In our rodent studies, neurodegeneration could be rescued by co-expression of rat α-syn (rendered insensitive to the shRNA) demonstrating that neuronal loss was explicitly due to a toxic loss-of function of α-syn and not due to non-specific shRNA-mediated toxicity. Moreover, by utilizing several distinct shRNA sequences displaying varying efficiencies of endogenous rat α-syn mRNA knockdown we showed that the extent of neuronal loss was dependent on the level of remaining α-syn. The ability to rescue neurons by co-expression of rat α-syn and the fact that toxicity was proportional to the efficiencies of shRNAs demonstrate that neuronal loss was not due to "off-targeting" of other endogenous mRNAs or due to non-specific shRNA toxicity.

KNOCKDOWN OF α-SYN IN THE NON-HUMAN PRIMATE RESULTS IN DOSE-DEPENDENT NIGROSTRIATAL DEGENERATION: A CASE STUDY

To begin to further characterize the consequences of knockdown of endogenous α-syn and determine whether effects seen in rats were reproducible in a species more closely related to humans, we generated an α-syn shRNA specific for St.Kitts green monkeys (*Chlorocebus sabaeus*). We injected α-syn shRNA or scrambled shRNA, of two different titers, into the SN of individual monkeys ($N = 4$ total), waited 3 months, and examined DA neuron numbers, morphology, striatal innervation, and striatal DA content. α-Syn shRNA produced region-specific, titer-related, degeneration of SN tyrosine hydroxylase-positive (TH+) neurons and innervation of the striatum, reproducing the pattern of nigrostriatal degeneration observed in PD: SN degeneration was exaggerated in ventral tier neurons (vtSN; Gibb and Lees, 1991) with relative sparing of adjacent ventral tegmental area (VTA) DA neurons, and loss of TH+ fibers in the putamen (Pt) exceeded denervation of the caudate nucleus (Cd) (Kish et al., 1988; **Figure 1**). Stereologic quantification of TH+ neurons confirmed the qualitative observations: loss of TH+ neurons was greatest in vtSN with the VTA exhibiting relatively little neuron loss. However, the general pattern of TH+ neuron loss was not different in the High and Low titer α-syn shRNA conditions. These neurons also contain neuromelanin, and when counts of neuromelanin-only positive cells were added to the analysis, titer-related differences emerged. The Low titer shRNA condition was associated with the preservation of significantly more neuromelanin-only neurons, suggesting that while loss of TH+ phenotype was equivalent across titers, overt loss of neurons was greater in the High titer condition. The presence of TH-negative, neuromelanin-positive neurons is suggestive of ongoing pathology, an observation that also is seen in early PD. Importantly, co-localization of green fluorescent protein (GFP) as a marker of viral transduction within surviving midbrain DA neurons confirmed that dorsal SN and VTA neurons were transduced, but showed less degeneration (**Figure 1**). Titer-related differences in striatal DA depletion also were detected. Deficits in DA in the caudate nucleus and putamen were exaggerated in the α-syn shRNA High titer condition and a significant increase in the homovanillic acid (HVA)/DA ratio was only detected in the High titer subject. Increase in the HVA/DA ratio is known to be associated with a compensatory response to ongoing significant degeneration of DA neurons (Zigmond et al., 1990).

HOW DOES LOSS OF PRESYNAPTIC α-SYN CONTRIBUTE TO NEURODEGENERATION?

The scope of our one subject per treatment condition pilot study in non-human primates has clear limitations. Nevertheless, the results confirm our previous findings in the rat (Gorbatyuk et al., 2010; Kanaan and Manfredsson, 2012). At this stage our findings, while internally consistent among the treatment conditions, must be considered as generating a hypothesis, rather than being conclusive. While the idea that maintaining α-syn expression is critical for survival of populations of adult neurons has received relatively little investigation, the high prevalence and widespread distribution of α-syn in brain inarguably reflects an important function in synapses. While direct evidence for the loss-of-function hypothesis is in its early stages, many studies provide indirect support for the concept.

Alpha-synuclein exhibits a dramatic redistribution within the neuronal compartment as a function of aging: the best-validated primary risk factor for PD. A quantitative morphological postmortem analysis of α-syn immunoreactivity within SN DA neurons of humans reveals a distinct pattern of increased staining within neuronal soma with advancing chronological age (Chu and Kordower, 2007). Samples were from individuals with a mean age of 31 years (young group, $N = 6$), 55 years (middle-aged, $N = 4$) and 84 years (aged, $N = 8$). Alpha-synuclein immunoreactivity was rarely detected in the soma of SN neurons in young individuals and was restricted to its normal location in the neuropil. With advancing age, a progressive increase in somatic immunoreactivity was observed, culminating in a 639% increase in aged subjects. Quantitation of the intensity of somatic staining on a per neuron basis confirmed this pattern, illustrating a mean 57% increase in aged subjects as compared to young. It is noteworthy that none of these samples exhibited α-syn aggregates, suggesting that this intracellular redistribution of α-syn may be an ongoing aging-related event that precedes

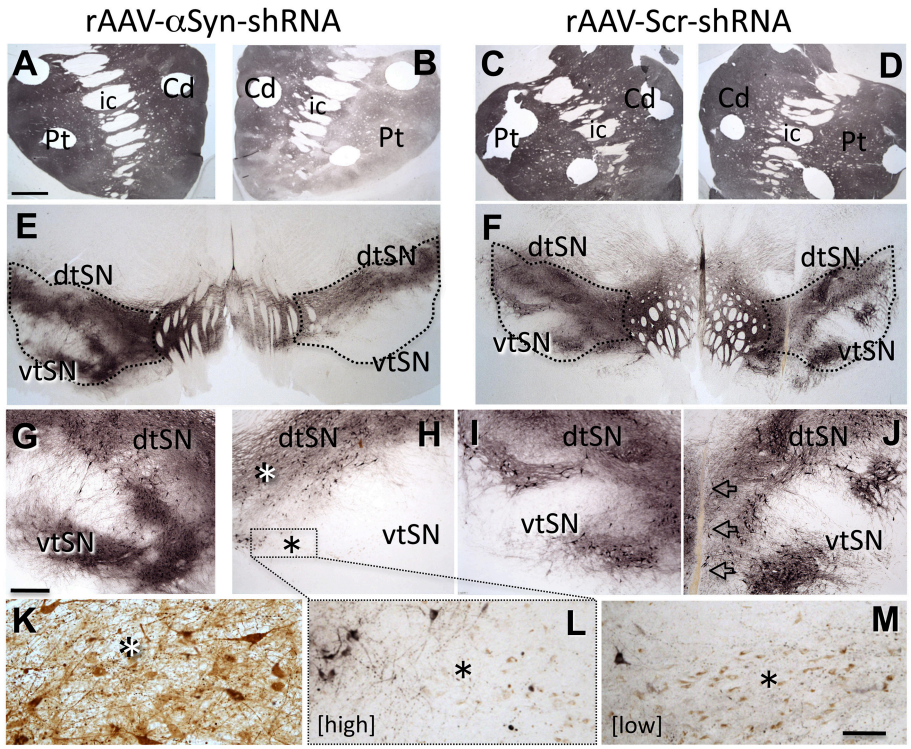

FIGURE 1 | Knock-down of endogenous α-syn in midbrain dopamine neurons reproduces a PD-like pattern of nigrostriatal degeneration. rAAV-α-syn-shRNA treatment produces exaggerated degeneration of ventral tier substantia nigra (vtSN) dopamine neurons and denervation of the putamen (Pt) that exceeds that in the caudate (Cd), reproducing the pattern of degeneration observed in Parkinson's disease **[A,B,E,G,H**, transduced hemisphere on right side of micrographs, staining for tyrosine hydroxylase (TH)]. This pattern is not observed with rAAV-scr-shRNA treatment **(C,D,F,I,J**, arrows in **(J)** indicate injection site). Dorsal tier substantia nigra (dtSN) neurons surviving α-syn-shRNA treatment (white asterisk in **H**) were transduced by the vector, but resistant to degeneration (**K**, brown stain for GFP vector tag). While rAAV- α-syn-shRNA of both High and Low titers produced equivalent loss of TH+ vtSN neurons, greater numbers of neuromelanin-positive neurons surviving in this region following Low titer treatment **(M)** indicates phenotype suppression with cell survival, and the low number of these cells in the High titer treatment **(L)** suggests greater overt cell loss [black asterisk in **(H)** indicates vtSN region shown in **(L)** and equivalent region in **(M)** with greater (Low shRNA titer **M**) and lesser (High titer in **L**) numbers of surviving neuromelanin neurons (brown pigment)]. Abbreviations: Cd, caudate nucleus; Pt, putamen; ic, internal capsule; dtSN, dorsal tier substantia nigra; vtSN, ventral tier substantia nigra; Calibration bar in **(A)** = 100 μm and applies to **(A–F)**; bar in **(G)** = 500 μm and applies to **(G–J)**; bar in **(M)** = 100 μm and applies to **(K–L)**.

processes of aggregation. The same pattern was observed in aging monkeys. Of importance to the loss-of-function hypothesis, no comparison to α-syn distribution/levels in striatum was performed, providing no opportunity to assess whether increased somatic levels are associated with decreased synaptic levels.

Oxidative stress is an established threat to DA neuron viability and can be significantly exacerbated by DA mishandling (i.e., increased cytosolic DA). Indeed, multiple lines of evidence suggest a regulatory role for synuclein in dopamine neurotransmission (Perez et al., 2002; Maries et al., 2003; Wersinger et al., 2003), consistent with the view that loss of presynaptic α-syn may contribute to increased oxidative stress via dysregulation of DA biosynthesis, release and metabolism. Studies in primary cultures of DA neurons demonstrate that in conditions of elevated oxidative stress, endogenous levels of α-syn increase and provide neuroprotection (Quilty et al., 2006; Musgrove et al., 2011). Similarly, transgenic mice over-expressing α-syn are resistant to paraquat-induced toxicity for DA neurons known to be associated with oxidative stress (Manning-Bog et al., 2003). Thus, endogenous α-syn likely provides an important DA neuron defense against oxidative damage.

Mishandling of synaptic DA and the toxic byproducts of its metabolism in the cytosol have a long history of association with the vulnerability of these neurons (e.g., Perez and Hastings, 2004; Segura-Aguilar et al., 2014). Studies of transgenic mice deficient in α-syn, while free of overt DA neuron pathology in adulthood, exhibit changes in DA neurotransmission, with some of these becoming exaggerated in aged animals. Adult, triple knock-out mice for all synuclein family proteins exhibit elevated evoked release of DA in striatum, enhanced turnover and reduced presynaptic DA stores (Anwar et al., 2011). Study of two α-syn deficient mouse lines found evidence for increased stimulated DA overflow in striatum, higher basal extracellular DA levels, decreased expression of the dopamine transporter (DAT) and reduced DA reuptake (Chadchankar et al., 2011). Aged (24–26 months old) α-syn null mice exhibit significant reduction of striatal DA, a decrease in TH+ fibers and decreased striatal levels

of TH and DAT (Al-Wandi et al., 2010). These data support the view that α-syn is a pivotal presynaptic regulator of DA neurotransmission and that disruption of this process may result in chronic accumulation of DA in the cytosol.

Perhaps the most direct evidence for an α-syn loss-of-function process related to aggregation comes from the mouse pre-formed fibril (PFF) model of synucleinopathy (Luk et al., 2012). In this model, intracerebral injection of pre-formed fibrils of α-syn seeds misfolding of endogenous α-syn, resulting in progressive accumulation of cytoplasmic aggregates of the protein. In a recent report (Osterberg et al., 2015) the formation of aggregates was tracked over time. The findings indicate that as inclusions mature into a compact form, detection of soluble α-syn shows a parallel decline.

IS TOXICITY DUE TO LOSS OF α-SYN RELEVANT TO PARKINSON'S DISEASE ETIOLOGY?

Direct assessment of the relevance of the α-syn loss-of-function hypothesis to PD is a challenge. At present, there is no imaging protocol specific for α-syn, although this is an active area of research. To the best of our knowledge, there has been no study of striatal synaptosome preparations comparing PD to controls and there may be significant technical limitations to this approach with postmortem tissue. In addition, since numerous neuronal circuits project to the striatum, it may be difficult to discern what effect is directly attributable to nigrostriatal projections. Studies of α-syn in body fluids as a biomarker for PD may hold clues, but often are difficult to interpret in the context of entry of α-syn into these compartments and the potential presence or impairment of mechanisms operating to maintain homeostasis. However, α-syn is enriched in brain and blood, and from the context of PD as a "whole body" syndrome it can be argued that a systemic change in α-syn that characterizes PD may be detectable in blood as a reflection of a general process that affects brain. Taken together, biomarker studies targeting α-syn present a set of mixed results (Malek et al., 2014 for review), documenting increases, decreases and no differences in PD subjects as compared to controls. Many of these conclusions likely are linked to differences in sample collection, preparation and the assay employed. One recent report takes a different approach to circumvent the complexities of accurately measuring α-syn protein in blood and measured α-syn transcripts. They analyzed blood samples from three large cohorts of PD patients and controls from prior and ongoing clinical trials individually. All three cohorts supported the finding of an approximate 20% reduction in α-syn transcripts even in newly diagnosed PD patients (Locascio et al., 2015). However, this finding could be interpreted as support of loss-of-function, gain-of-function, or be unrelated to processes operating in brain.

Abundant information from human genetic studies document a clear association between increases in α-syn gene-dosing, or mutations, that exacerbate aggregation and lead to PD. In line with our hypothesis, these all are processes that can directly increase the sequestration of α-syn away from the synapse via increased aggregation kinetics (e.g., enhanced molecular crowding). Nevertheless, one would expect that if α-syn is crucial for maintaining the viability of DA neurons, human genetic data on risk of PD also would implicate mutations or polymorphisms that result in decreased α-syn. Indeed, a recent report has described exactly that (Markopoulou et al., 2014). The dinucleotide repeat REP1 is positioned upstream of the SNCA transcription start site. Polymorphisms at this site are associated with either increased or decreased SNCA expression. Contrary to the investigator's hypothesis that polymorphisms associated with decreased expression of α-syn would be associated with decreased risk of PD, these low α-syn individuals exhibited increased risk of developing PD as well as worse motor and cognitive outcomes.

LOSS-OF-FUNCTION, GAIN-OF-FUNCTION OR A BIT OF BOTH?

Loss-of-function and gain-of-function hypotheses are not irreconcilable. The absence of an overt parkinsonian phenotype and DA neuron degeneration in germline α-syn knockout mice does not directly address the loss-of-function hypothesis. Whole genome expression analysis of SNCA(−/−) mice identified differential expression of 369 transcripts as compared to wild-type animals (Kuhn et al., 2007). This includes increased expression of transcripts of other synuclein family members, 14-3-3 proteins, TH and neurotrophic factors, decreased expression of pro-apoptotic transcripts, and changes in genes directly involved in vesicle function and neurotransmission. Thus, the lack of a severe phenotype in these animals is likely the result of complex germline compensation for α-syn function. While heritable forms of PD associated with duplication and triplication of the SNCA gene arguably support the toxic gain-of-function hypothesis, germline α-syn overexpression mice do not directly support this contention. The available mouse models exhibit little or no degeneration of SN DA neurons, although similar to α-syn knockout mice, show prominent changes in striatal DA neurotransmission (e.g., Chesselet and Richter, 2011; Crabtree and Zhang, 2012 for reviews). Nevertheless, if the α-syn transgene is induced in the mature animal, nigral degeneration does occur (Lin et al., 2012). Thus, it is plausible that germline compensation, similar to that seen in the knockout, occurs in overexpression transgenic models as well.

While germline transgenic models do little to inform the toxic gain-of-function/loss-of-function debate it is inarguable that too much α-syn and mutant α-syn can produce direct neurotoxicity. *In vitro* studies demonstrate that specific conformations of α-syn exert cytotoxic effects by permeabilizing vesicles, altering calcium flux, impairing mitochondrial function, and inducing apoptosis (Volles et al., 2001; Pieri et al., 2012; Luth et al., 2014; Pacheco et al., 2015). In addition, viral vector-mediated overexpression of α-syn in DA neurons uniformly produces significant, albeit variable in magnitude, neurodegeneration (e.g., Van der Perren et al., 2014 for review). However, in this case it is unclear what precipitates neurodegeneration. Nonetheless, in line with our hypothesis, and with data gained from the PFF model (i.e., Osterberg et al., 2015) it is plausible that loss of

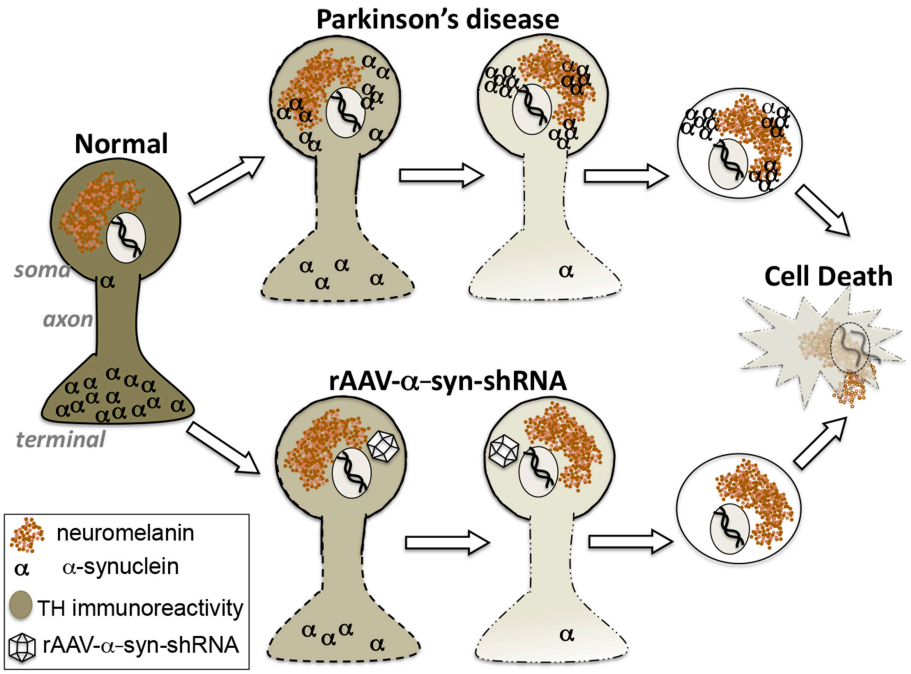

FIGURE 2 | Hypothesis: Alpha-synuclein loss-of-function as a contributor to parkinsonian pathology. Our findings suggest that the region-specific degeneration of ventral midbrain dopamine neurons characteristic of Parkinson's disease that is widely attributed to accumulation of toxic aggregates of alpha-synuclein can be accurately reproduced by knockdown of endogenous alpha-synuclein. These differing paths to degeneration converge upon displacement of alpha-synuclein from its natural location at synaptic terminals.

soluble endogenous α-syn to aggregation is one contributor to neurotoxicity. Thus, experimental support for both viewpoints suggests a "Goldilocks" biology commonly exhibited by many molecules: too much is bad, too little is bad, enough is just right (Kanaan and Manfredsson, 2012).

CONCLUSIONS

In this Hypothesis and Theory article we address the alpha-synuclein gain-of-function vs. loss-of-function debate as it applies to DA neuron pathology in PD. Our experimental findings in rats and non-human primates, combined with support for the concept from other reports in the literature, leads to the proposal that loss-of-function plays a significant role in this pathology. On its surface, the α-syn loss-of-function hypothesis seems counterintuitive since α-syn accumulation as aggregates, in the form of Lewy bodies, is a key neuropathologic feature of PD. However, while experimental observations largely have been interpreted as suggestive of a cytotoxic role for α-syn, it does not preclude the alternate interpretation that α-syn aggregation reflects processes that prevent α-syn from performing its normal biological functions. Our recent findings in non-human primates support the contention that α-syn loss-of-function in midbrain DA neurons accurately reproduces the pattern of nigrostriatal degeneration observed in PD, and that α-syn aggregation, acting as a "sink," is one possible driver of this process rather than a direct toxic culprit in cell loss (**Figure 2**). The findings suggest reconsideration of the relationship of α-syn to PD pathogenesis and caution in the implementation of α-syn clearance therapeutic strategies that do not distinguish between natural forms and pathological forms.

AUTHOR CONTRIBUTIONS

TC conception, design, acquisition analysis, data interpretation, drafting and revision, DR design, acquisition analysis, revision, KC acquisition analysis, revision, JL acquisition analysis, data interpretation, revision, FM conception, design, acquisition analysis, data interpretation, drafting and revision.

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Altered Expression Patterns of Inflammation-Associated and Trophic Molecules in Substantia Nigra and Striatum Brain Samples from Parkinson's Disease, Incidental Lewy Body Disease and Normal Control Cases

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Evidence of inflammation has been consistently associated with pathology in Parkinson's disease (PD)-affected brains, and has been suggested as a causative factor. Dopaminergic neurons in the substantia nigra (SN) pars compacta, whose loss results in the clinical symptoms associated with PD, are particularly susceptible to inflammatory damage and oxidative stress. Inflammation in the striatum, where SN dopaminergic neurons project, is also a feature of PD brains. It is not known whether inflammatory changes occur first in striatum or SN. Many animal models of PD have implicated certain inflammatory molecules with dopaminergic cell neuronal loss; however, there have been few studies to validate these findings by measuring the levels of these and other inflammatory factors in human PD brain samples. This study also included samples from incidental Lewy body disease (ILBD) cases, since ILBD is considered a non-symptomatic precursor to PD, with subjects having significant loss of tyrosine hydroxylase-producing neurons. We hypothesized that there may be a progressive change in key inflammatory factors in ILBD samples intermediate between neurologically normal and PD. To address this, we used a quantitative antibody-array platform (Raybiotech-Quantibody arrays) to measure the levels of 160 different inflammation-associated cytokines, chemokines, growth factors, and related molecules in extracts of SN and striatum from clinically and neuropathologically characterized PD, ILBD, and normal control cases. Patterns of changes in inflammation and related molecules were distinctly different between SN and striatum. Our results showed significantly different levels of interleukin (IL)-5, IL-15, monokine induced by gamma interferon, and IL-6 soluble receptor in SN between disease groups. A different panel of 13 proteins with significant changes in striatum, with IL-15 as the common feature, was identified. Although the ability to detect some proteins was limited by sensitivity, patterns of expression indicated involvement of certain

T-cell cytokines, vascular changes, and loss of certain growth factors, with disease progression. The results demonstrate the feasibility of profiling inflammatory molecules using diseased human brain samples, and have provided additional targets to validate in relation to PD pathology.

Keywords: inflammation, pathology, cytokines, antibody array, dopaminergic cell loss, Parkinson's disease, microglia, astrocytes

INTRODUCTION

Parkinson's disease (PD) is a chronic, progressive neurodegenerative disorder characterized by symptoms of tremor, bradykinesia, ataxia and rigidity, and is the main cause of movement disorders in the elderly. The current estimates of PD are one million cases in the U.S.A. with 60,000 cases being added each year (Parkinson's Disease Foundaton, 2015). This leads to significant morbidity and mortality in affected individuals along with the considerable cost of healthcare for PD patients. Sufferers of PD also have significant risk of progressing to dementia (Aarsland et al., 2005). There have been major strides in understanding the cause(s) of PD, but there is still lack of consensus of the sequence of events that lead to the loss of the dopaminergic neurons of the substantia nigra (SN) pars compacta and results in loss of dopaminergic neurotransmission in the striatum. Much research on disease mechanisms has focused on the properties of the presynaptic vesicle protein α-synuclein whose modifications can lead to the formation of aggregated and neurotoxic species (Vekrellis and Stefanis, 2012; Lawand et al., 2015; Osterberg et al., 2015; Sian-Hulsmann et al., 2015). Aggregated α-synuclein, particularly the phosphorylated form, is the major component of Lewy bodies, the major pathological feature of PD brains (Fujiwara et al., 2002). Most of the identified mutations in α-synuclein appear to increase the risk of PD by increasing its tendency to aggregate (Polymeropoulos, 1997; Baba et al., 1998; Conway et al., 1998; Ancolio et al., 2000; Ostrerova-Golts et al., 2000; Ghosh et al., 2013; Giráldez-Pérez et al., 2014).

Inflammation has also been a well-established feature of PD pathology (McGeer et al., 1988; Boka et al., 1994; Mogi et al., 1994; Hunot et al., 1999; Brochard et al., 2009), and some data have suggested it could be the primary pathological cause for SN neuronal cell death (Tansey et al., 2007, 2008; Vivekanantham et al., 2015). Inflammation in PD brains could be caused by different factors, but a number of studies have shown that aggregated forms of α-synuclein can activate microglia to produce toxic molecules that contribute to dopaminergic cell death (Zhang et al., 2005; Couch et al., 2011; Béraud et al., 2013; Acosta et al., 2015). Alpha-synuclein activation of microglia can be mediated through the Toll-like receptor (TLR)-2 (Codolo et al., 2013; Kim et al., 2013; Doorn et al., 2014; Daniele et al., 2015), though other microglial receptors have also been implicated, including TLR-4 (Stefanova et al., 2011; Fellner et al., 2013) and purinergic receptor P2X7 (Jiang et al., 2015). A recent immunohistochemical study of SN from control, ILBD and PD cases for the inflammation marker TLR-2 showed increased numbers of TLR-2-positive microglia in ILBD cases compared to PD cases (Doorn et al., 2014). This suggested that some inflammatory changes could be happening at early stages prior to development of symptoms of PD. By contrast, there was progressive increase from control to PD in numbers of CD68-positive amoeboid microglia/macrophages, a marker associated with phagocytosis, which correlated with an increase in α-synuclein deposits (Doorn et al., 2014). Independent of the presence of pathological α-synuclein, the human SN appears to be particularly sensitive to inflammation, possibly due to higher concentrations of iron, and neuromelanin, both of which can contribute to an environment of enhanced oxidative stress (Hirsch, 1993; Béraud et al., 2013; Taylor et al., 2013; Fischer and Maier, 2015). Purified neuromelanin can directly activate microglia to a proinflammatory state (Wilms et al., 2003; Zhang et al., 2013).

Animal models for PD have provided the most convincing evidence for how inflammation could be directly linked to SN neuronal cell loss. A widely used model involves the administration of the bacterial cell wall extract lipopolysaccharide (LPS) (Couch et al., 2011; Qin et al., 2013; Tanaka et al., 2013; Sharma and Nehru, 2015). Both direct injection of LPS into the SN or by intraperitoneal injection can lead to enhanced inflammation in the brain and selective loss of SN neurons. Administration of LPS results in increased production of free radicals and potentially toxic cytokines, including tumor necrosis factor-α (TNF-α) (McGuire et al., 2001; Pei et al., 2007; Tansey et al., 2007, 2008; Zhao et al., 2007; Gao et al., 2011; Tran et al., 2011; Montgomery and Bowers, 2012; Qin et al., 2013). Another feature of inflammation-induced models of PD is disruption of the blood-brain barrier, which enhances inflammation by permitting influx of components of cell-mediated immunity (Carvey et al., 2005b, 2006; Desai et al., 2007; Monahan et al., 2008).

The spread of abnormal forms of α-synuclein along neuroanatomical pathways is a significant pathological mechanism in humans and animal models of synucleinopathies (Beach et al., 2009; Luk et al., 2012; Masuda-Suzukake et al., 2014; Paumier et al., 2015). In animal models, instrastriatal injection of preformed α-synuclein fibrils resulted in neurodegeneration and inflammation in the SN; the reverse effect occurs with α-synuclein administered into the SN resulting in striatum pathology and inflammation (Koprich et al., 2010; Luk et al., 2012; Osterberg et al., 2015; Paumier et al., 2015). Direct injection of LPS into striatum or SN resulted in degeneration of nigrostriatal pathway neurons and motor impairments, along with microglial activation (Choi et al., 2009; Couch et al., 2011). Co-administration of α-synuclein and LPS can significantly enhance the generation of nigrostriatal pathology (Couch et al.,

2011; Gao et al., 2011). As it is still not known if spread of pathology or neuroinflammation occurs from striatum to SN or vice-versa, as part of this study, we sought to compare the changes in inflammation and trophic molecules between SN and striatum with disease progression. Recent mouse models of PD have focused on α-synuclein, either overexpression of normal or mutated α-synuclein under transgene control or overexpression by administration of α-synuclein viral transduction vectors (Watson et al., 2012; Béraud et al., 2013; Gardai et al., 2013; Harms et al., 2013). Animal models of PD can also be developed using the dopaminergic neurotoxin 1-methyl-4 phenyl-1,2,3,6-tetrahydropyridine (MPTP) to lesion the SN and striatum. This model can reproduce many PD features in non-human primates (Ohnuki et al., 2010). This study carried out gene expression profiling of SN and striatum tissue and showed significant downregulation of neuronal and dopaminergic genes in lesioned animals. With respect to inflammation-related genes, only upregulation of glial fibrillary acidic protein (GFAP), interleukin (IL)-11, and chemokines CXCL13 and CXCL4 were detected (Ohnuki et al., 2010).

Although there have been many immunohistological studies that used antibodies to activated microglia to demonstrate inflammation in PD brains, there have been few studies that biochemically measured levels of cytokines or other inflammation-associated molecules in human brain samples (Nagatsu et al., 2000a,b). A recent proteomics analysis comparing SN tissue from PD and control cases identified cytosolic non-specific dipeptidase 2 to be upregulated in PD tissue, but not inflammatory or growth factor molecules. This may have been due to lack of sensitivity of the methods employed to detect these low-abundance molecules (Licker et al., 2012). Such experiments are technically difficult as only low concentrations of key molecules are present in tissue. Cerebrospinal fluid (CSF), plasma and blood have also been used as surrogates to follow brain changes in PD (Rocha et al., 2014), but these results have generally been inconsistent between studies. Increased levels of IL-2, IL-6, and TNF-α have been detected in CSF samples of PD subjects compared to controls (Mogi et al., 1994, 1996). Screening of sera from control, PD, multiple system atrophy and corticobasal syndrome cases using a similar antibody array as used in this study, showed only platelet-derived growth factor (PDGF)-BB and prolactin having significant disease associated differences (Mahlknecht et al., 2012). Measurement of cytokines in CSF from PD and control cases identified significant changes in the cytokines/growth factors vascular endothelial growth factor (VEGF), placental growth factor (PIGF), soluble VEGF receptor (sVEGFR2), and angiopoietin2 (ANG2), associated with angiogenesis (Janelidze et al., 2015).

To address the problems of sensitivity, studies have used mRNA gene expression profiling of PD SN tissue, or laser-dissected SN dopaminergic neurons, to identify disease differences. These techniques have high sensitivity for detecting low-abundance gene expression, but there has been lack of consensus on the PD-associated differentially expressed genes between studies. However, these studies have identified multiple pathways affected in PD, with downregulation of genes associated with synaptic function, cytoskeletal function and neuroprotection and also ubiquitin-proteosome and mitochondrial function genes being features (Grünblatt et al., 2004; Hauser et al., 2005; Mandel et al., 2005; Duke et al., 2006; Elstner et al., 2009; Ohnuki et al., 2010; Gründemann et al., 2011; Botta-Orfila et al., 2012). A microarray study focusing on inflammatory gene expression in PD SN showed upregulation of the microglial purinergic receptor P2X7 (a receptor for ATP), colony stimulating factor-1 receptor (CSF1R), (a microglia growth factor receptor), and nitric oxide synthase 3 (a vascular marker) (Durrenberger et al., 2012).

With the development of high-sensitivity multiplex antibody arrays and other proteomic techniques, it is possible to profile large numbers of different biologically-active proteins in human brain tissue or other biological samples. We used this approach to examine the levels of 158 proteins in SN and striatum of control, ILBD and PD cases to determine if there were progressive changes in inflammation or related proteins. We particularly sought to determine if any of the cytokines identified in PD animal models could be validated in these human tissues. Our results demonstrated distinctly different patterns of inflammation and growth factor changes between SN and striatum with disease.

MATERIALS AND METHODS

Brain Tissue Samples

Brain tissue samples for this study were provided by the Banner Sun Health Research Institute Brain and Body Donation Program. The Brain and Body Donation Program operated with the approval of Western IRB (Puyallup, WA) under contract as the Institutional Review Board of Banner Research. A summary of the demographics of the cases is shown in **Table 1**. There were SN samples from 16 controls, 21 ILBD, and 18 PD cases; and striatum samples from 16 controls, 17 ILBD, and 16 PD cases. Tissue samples from both SN and striatum were not available for all cases—overlap of cases between brain regions was 92%. The selection of control, ILBD and PD cases used in this study was based on neuropathology diagnosis with reference to clinical records, with a diagnosis of Alzheimer's disease (AD) as the principal exclusion criteria. The degree of Lewy body (LB) pathology was assessed using a histological staging scheme in each of 10 brain regions (Beach et al., 2009). This involved obtaining a ranking score (0–4) using phosphorylated-alpha synuclein stained sections from each region. These numbers are summed to give Lewy body pathology scores of 0–40 for each brain. Histological ranking scores of plaques and tangles in five brain regions were used to assess how much age-associated AD-type pathology was present in each case (Beach et al., 2012; **Table 1**). This involved obtaining a ranking score (0–3) using Thioflavin S-stained tissue sections from entorhinal cortex, hippocampus, frontal, parietal, and temporal cortex. These numbers are summed to give plaque and tangle scores of 0–15 for each brain. Dementia was present in a number of the PD cases (**Table 1**); however, neuropathology and clinical records indicated this was not due to AD.

TABLE 1 | Demographic features of cases used in study.

Disease Type	*n*	Age (Mean + SD)	Sex M/F	Dementia (D/MCI/CN)	LB	Plaques (Mean + SD)	Tangles (Mean + SD)
SUBSTANTIA NIGRA							
Control	16	81.2 ± 11.4	9/7	0/16	0	1.4 ± 1.8	2.4 ± 1.6
ILBD	21	86.8 ± 6.8	17/4	0/3/18	1-3	3.5 ± 4.6	3.9 ± 2.2
PD (DD 12.9; 1–26)	18	81.3 ± 6.2	12/6	7/3/8	2b-4	3.2 + 3.6	3.9 ± 2.1
STRIATUM							
Control	16	81.4 ± 11.5	10/6	0/16	0	1.5 ± 1.9	2.5 ± 1.8
ILBD	17	85.2 ± 6.4	13/4	0/2/15	1–3	3.4 ± 3.8	3.2 ± 1.3
PD (DD 13.4; 1–26)	16	81.3 ± 6.2	12/6	7/2/7	2b–4	3.2 + 3.6	4.1 ± 2.1

ILBD, incidental Lewy body disease; PD, Parkinson's disease; n, number of cases; M/F, male/female; LB, Lewy body staging score; Plaques, Plaque score; Tangles, tangle score; D, demented; MCI, mild cognitive impairment; CN, cognitively normal; DD, disease duration/years—mean and range.

Tissue Preparation

Due to the limited amount of SN tissue available from each case, SN tissue was provided as 15 consecutive frozen (10 μm) SN sections (~6–10 mg) cut from each block with a cryostat and collected frozen. This approach to tissue sample preparation allowed the selection of matched samples containing approximately equivalent densities of SN pars compacta neuromelanin-containing neurons. Frozen striatum samples (20–30 mg) were dissected from putamen at the level of the lenticular nucleus.

Processing of Tissue Samples

Both series of tissue samples were extracted in six volumes (w/v) of a proprietary extraction buffer compatible with the Quantibody arrays (Raybiotech, Norcross, GA) supplemented with protease/phosphatase inhibitors (Thermo-Fisher/Pierce). Samples were briefly sonicated in extraction buffer and incubated on ice with constant shaking for 30 min. After centrifugation (18,000 g/30 min), the supernatants were collected for further analysis. Protein concentrations were determined using a micro BCA assay (Thermo-Fisher/Pierce). These same extracts were also used to prepare western blot samples.

Tissue Analyses

Preliminary analyses of protein extracts were carried out using western blot methods for tyrosine hydroxylase (TH), as a measure of degree of dopaminergic neuron cell loss; for IBA-1, an indicator of microglia abundance; for TLR-2 as a marker for inflammation; and for glial fibrillary acidic protein (GFAP), as an indicator of reactive astrocytosis. We employed our standard published western blot methods using antibodies to TH (Biolegend, Dedham, MA; rabbit polyclonal 1:2000 dilution); to IBA-1 (Wako Chemicals, Richmond, VA, 1:2000); to TLR-2 (Abcam, Cambridge, MA; rabbit monoclonal 1:2500 dilution); and to GFAP [BD Biosciences, Franklin Lakes, NJ; cocktail of three different monoclonal antibodies (1:2000)] (Walker et al., 2015a,b).

For the antibody array analysis, samples were processed as a service by Raybiotech (Norcross, GA). For SN, each sample protein concentration was adjusted to 500 μg/ml, and for striatum, each sample was adjusted to 1000 μg/ml. These were analyzed using 160 protein Quantibody arrays (catalog number QAH-CAA-3000) composed of antibody-coated glass slide arrays for detecting cytokines, chemokines, soluble receptor, and growth factors. **Table 2** shows the arrangement of the proteins on the four separate array slides. Each protein had a standard curve included with measurements from known dilutions of purified standard protein. Each slide array contained positive control samples that were used for normalization purposes. Measurements were based on fluorescent intensity of bound labeled antibodies to each spot, and calculated from the mean of four spots/antibody. Slides were measured, analyzed, and normalized to positive controls using Raybiotech software. Final results were expressed as pg of protein/ml extract.

Immunohistochemical Staining of SN and Striatum Tissue Sections

To characterize features of microglia activation in tissues being examined by antibody array analysis, 40 μm paraformaldehyde-fixed tissue sections of SN and striatum from control, ILBD and PD cases were stained using antibodies LN3 (Abcam, Cambridge, MA) and IBA-1 (Wako Chemicals, Richmond, VA). These are well-established markers for activated microglia (LN3) or pan microglia (IBA-1). Striatum sections were double-stained for TH (Biolegend—rabbit polyclonal, 1:2000) using a two-color method. Immunohistochemstry was carried out according to our published procedures (Walker et al., 2009, 2015b).

Data Analysis

The validity of the standard curve for each protein was confirmed by visual inspection of data. We found that standard curves for basic fibroblast growth factor and insulin growth factor binding protein-4 were unsatisfactory as the standard proteins did not produce a suitable dose-response curve; the results obtained for these proteins in tissue samples were excluded.

Data for each brain region samples were grouped as control, ILBD and PD and analyzed by One-way analysis of variance (ANOVA) using Graphpad Prism 5 software (Graphpad software, La Jolla, CA) without corrections for multiple comparisons. To

TABLE 2 | Quantibody array component proteins.

Array-CHE	Array-CYT	Array-REC	Array-GF
6Ckine	BLC	4-1BB	AR
Axl	Eotaxin	ALCAM	BDNF
BTC	Eotaxin-2	B7-1	bFGF
CCL28	G-CSF	BCMA	BMP-4
CTACK	GM-CSF	CD14	BMP-5
CXCL16	I-309	CD30	BMP-7
ENA-78	ICAM-1	CD40 L	b-NGF
Eotaxin-3	IFNγ	CEACAM-1	EGF
GCP-2	IL-1α	DR6	EGF-R
GRO	IL-1β	Dtk	EG-VEGF
HCC-1	IL-1ra	Endoglin	FGF-4
HCC-4	IL-2	ErbB3	FGF-7
IL-9	IL-4	E-Selectin	GDF-15
IL-17F	IL-5	Fas	GDNF
IL-18 BPa	IL-6	Flt-3L	GH
IL-28A	IL-6sR	GITR	HB-EGF
IL-29	IL-7	HVEM	HGF
IL-31	IL-8	ICAM-3	IGFBP-1
IP-10	IL-10	IL-1 R4	IGFBP-2
I-TAC	IL-11	IL-1 RI	IGFBP-3
LIF	IL-12p40	IL-2 Rg	IGFBP-4
LIGHT	IL-12p70	IL-10 Rb	IGFBP-6
Ltactin	IL-13	IL-17R	IGF-I
MCP-2	IL-15	IL-21R	Insulin
MCP-3	IL-16	LIMPII	MCF R
MCP-4	IL-17	Lipocalin-2	NGF R
MDC	MCP-1	L-Selectin	NT-3
MIF	MCSF	LYVE-1	NT-4
MIP-3a	MIG	MICA	OPG
MIP-3b	MIP-1a	MICB	PDGF-AA
MPIF-1	MIP-1b	NRG1-b1	PlGF
MSPa	MIP-1d	PDGF Rb	SCF
NAP-2	PDGF-BB	PECAM-1	SCF R
OPN	RANTES	RAGE	TGFa
PARC	TIMP-1	TIM-1	TGFb1
PF4	TIMP-2	TRAIL R3	TGFb3
SDF-1a	TNFa	Trappin-2	VEGF
TARC	TNFb	uPAR	VEGF R2
TECK	TNF RI	VCAM-1	VEGF R3
TSLP	TNF RII	XEDAR	VEGF-D

Abbreviations/Definitions: Supplemental Table 1.

determine whether age of patient or postmortem interval (PMI) were covariants for each measure, data from each protein was also analyzed by Analysis of Covariance (ANCOVA) with age, PMI, or age and PMI as covariants using Medcalc statistical software (Medcalc Software, Ostend, Belgium). Further analyses included correlation analyses, stepwise logistic regression analyses and receiver operating characteristic (ROC) curve analyses and were carried out using MedCalc. For each measure, $P < 0.05$ was considered statistically significant.

RESULTS

Characterization of Samples

The samples were selected based on consensus clinical and neuropathological criteria, but as shown in **Table 1**, there were different amounts of age-associated pathology in the samples. The control samples were free of LB pathology; while the ILBD and PD cases had varying degrees (**Table 1**). To support the clinical and neuropathological criteria used for case selection, samples were characterized for TH levels as an additional index of disease severity. There was significant variability between the samples in each disease group for TH, especially within the control groups (**Figure 1**). In SN, disease group differences in TH levels did not reach statistical significance by One-way ANOVA (**Figure 1A**), while in striatum, expected TH differences between each of the disease groups were shown (**Figure 1B**). Age and PMI were not significant covariant factors affecting TH levels in SN or striatum. Pathological variability within the disease groups was also highlighted by measures of gliosis and inflammation. GFAP levels showed no significant differences between disease groups for SN (**Figure 1C**) or striatum (**Figure 1D**) samples; however, GFAP levels in striatum were significantly affected by PMI ($P = 0.03$). There was significant negative correlation between TH and GFAP levels in striatum (Pearson $r = -0.399$, $P = 0.0071$; **Figure 1E**) suggesting increased gliosis as PD pathology progresses. Western blots measures of the microglial marker IBA-1 in SN (**Figure 1F**) and striatum (data not shown) did not show significant disease group differences; these measures were not affected by age or PMI.

Inflammatory Profiling of SN and Striatum

Initial data analysis of results from the Quantibody arrays consisted of individual ANOVA for each protein measure between the three disease groups for each of the brain regions. Results are shown in **Table 3**, which lists all *P*-values between groups for each protein. These results were not corrected for multiple comparisons. **Table 3** also identifies the proteins that gave negative values for each brain region. A negative designation was applied when fewer than 10 samples out of the total analyzed were above the limit of detection (LOD). For striatum samples, 57 of the 158 proteins measured were considered negative, while for SN, the same 57 were negative along with an additional 20 proteins (**Table 3**).

Protein Profile of SN

There were four proteins [IL-5, IL-15, monokine induced by gamma interferon (MIG) and IL-6 soluble receptor (IL6sR)] that gave $P < 0.05$ values between disease groups. The individual results for these proteins are shown as scatter plots in **Figure 2**. There were increases of IL-5, IL-15, and MIG, and a decrease of IL-6sR, in PD samples compared to controls or ILBD samples. Disease group differences did not become significantly different for any of these measures when corrected for age or PMI by ANCOVA; however, this analysis showed that IL-15 levels were significantly affected by PMI (ANCOVA—$P = 0.040$). In addition, when platelet derived growth factor (PDGF)-BB levels were corrected for age ($P = 0.031$—ANCOVA), a significant

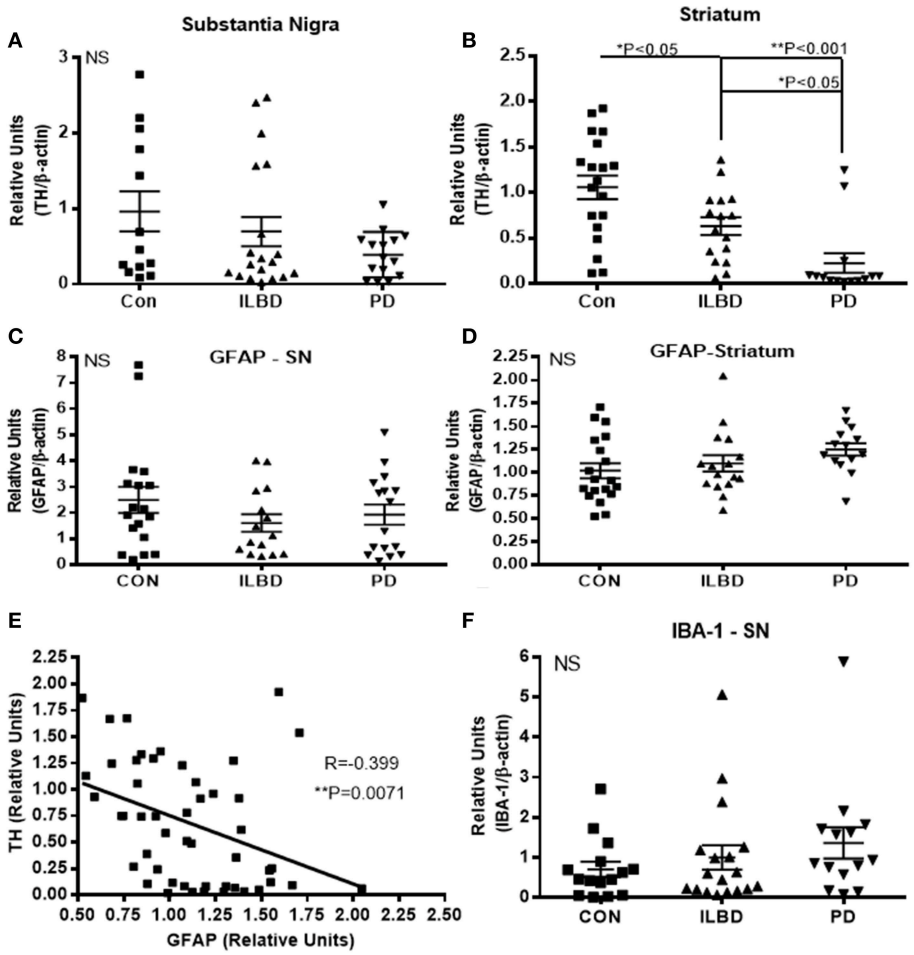

FIGURE 1 | Relative levels of tyrosine hydroxylase, glial fibrillary acidic protein or IBA-1 in substantia nigra. (A,B) Relative levels of tyrosine hydroxylase (TH) in control (Con), incidental Lewy body disease (ILBD), and Parkinson's disease (PD) samples of SN **(A)** or striatum **(B)** determined by western blot measures of TH with normalization for levels of β-actin. Statistical analysis by One-way analysis of variance (ANOVA) with Fisher LSD *post-hoc* test for between group differences. **(C,D)** Relative levels of glial fibrillary acidic protein (GFAP) in control (Con), incidental Lewy body disease (ILBD), and Parkinson's disease (PD) samples of SN **(C)** or striatum **(D)** determined by western blot measures of GFAP with normalization for levels of β-actin. Statistical analysis by One-way analysis of variance (ANOVA) showed no significance between disease groups. **(E)** Linear regression plot showing relation between striatum TH and striatum GFAP levels. Pearson correlation analysis showed significance between these measures ($R = -0.399$, $P = 0.0071$). **(F)** Relative levels of IBA-1 in control (Con), incidental Lewy body disease (ILBD), and Parkinson's disease (PD) samples of SN determined by western blot measures of with normalization for levels of β-actin. Statistical analysis by One-way analysis of variance (ANOVA) showed no significant difference between disease groups.

disease group difference was obtained ($P = 0.0323$ between ILBD and PD samples).

Stepwise logistic regression analysis of these four cytokines was carried out to determine if these measures had predictive value between disease groups. Comparing control to PD indicated that only IL-5 values had significant predictive value ($P = 0.003$) with ROC curve analysis for IL-5 with sensitivity of 85.7% and specificity of 62.5% with area under curve (AUC) of 0.81. Further stepwise logistic regression analysis between ILBD and PD values showed a combination of IL-15 and IL-6sR levels gave predictive value ($P = 0.0016$), with ROC AUC of 0.820, and sensitivity of 85.7% and specificity of 64.7%. These stepwise regression analysis models were not strengthened by inclusion of TH values. Results are summarized in **Table 4**. Multiple stepwise regression analyses for all measured proteins in SN showed no additional proteins or group of proteins could discriminate between control and ILBD cases, or between ILBD and PD cases (data not shown).

Protein Profile of Striatum

A panel of 13 proteins was shown to have significant differences in striatum samples between the disease groups by One-way ANOVA (**Table 3**)—CCL28 (C–C motif ligand 28 or mucosae-associated epithelial chemokine), HCC-1 (CCL-14), IL-18 binding protein a (IL-18BPa), PF4 (platelet factor 4), interferon-gamma (IFN-γ), IL-1 receptor antagonist (IL-1ra), IL-2, IL-15, PDGF-AA, PDGF-BB, TNF-α, Dtk (tyrosine-protein kinase receptor TYRO3), and ErbB3 (also HER3, human epidermal growth factor receptor 3). The individual scatter plot results for these proteins are shown in **Figure 3**. When correcting

TABLE 3 | Summary of One-way ANOVA results.

	Array			Array			Array			Array	
CHE	**SN**	**Striatum**	**CYTO**	**SN**	**Striatum**	**REC**	**SN**	**Striatumm**	**GF**	**SN**	**Striatum**
6Ckine	Neg	Neg	BLC	Neg	Neg	4-1BB	Neg	Neg	AR	0.76	0.756
Axl	0.179	0.643	Eota xin	Neg	Neg	ALCAM	0.836	0.527	BDNF	0.295	0.709
BTC	Neg	Neg	Eotax in-2	Neg	Neg	B7-1	Neg	Neg	*bFGF*	*Reject*	*Reject*
CCL28	0.503	**0.013**	G-CSF	Neg	Neg	BCMA	Neg	Neg	BMP-4	0.349	0.671
CTACK	Neg	Neg	GM-CSF	Neg	Neg	CD14	0.928	0.521	BMP-5	Neg	Neg
CXCL16	0.478	0.352	I-309	Neg	Neg	CD30	Neg	Neg	BMP-7	Neg	Neg
ENA-78	Neg	0.156	ICAM-1	0.621	0.83	CD40 L	Neg	Neg	b-NGF	Neg	0.867
Eotaxin-3	Neg	Neg	IFNg	0.832	**0.044**	CEACAM-1	0.941	0.655	EGF	0.406	0.79
GCP-2	Neg	Neg	IL-1α	Neg	Neg	DR6	Neg	0.967	EGF-R	0.77	0.483
GRO	0.575	0.289	IL-1β	Neg	Neg	Dtk	0.539	**0.031**	EG-VEGF	0.465	0.049
HCC-1	0.336	**0.045**	IL-1ra	0.671	**0.002**	Endoglin	0.935	0.299	FGF-4	Neg	Neg
HCC-4	Neg	Neg	IL-2	0.244	**0.028**	ErbB3	0.252	**0.004**	FGF-7	Neg	0.948
IL-9	Neg	Neg	IL-4	Neg	0.103	E-Selectin	0.496	0.582	GDF-15	0.218	0.401
IL-17F	Neg	Neg	IL-5	**0.035**	0.132	Fas	0.631	0.772	GDNF	0.766	0.394
IL-18 BPa	0.822	**0.015**	IL-6	0.219	0.067	Flt-3L	0.965	0.63	GH	0.663	0.397
IL-28A	Neg	Neg	IL-6sR	**0.022**	0.187	GITR	0.435	0.669	HB-EGF	Neg	0.83
IL-29	Neg	Neg	IL-7	0.631	0.146	HVEM	0.357	0.669	HGF	0.426	0.274
IL-31	Neg	Neg	IL-8	0.272	0.154	ICAM-3	0.103	0.073	IGFBP-1	0.553	0.192
IP-10	Neg	0.41	IL-10	Neg	Neg	IL-1 R4	Neg	0.277	IGFBP-2	0.731	0.316
I-TAC	Neg	0.91	IL-11	Neg	Neg	IL-1 RI	Neg	0.839	IGFBP-3	0.147	0.639
LIF	Neg	Neg	IL-12p40	Neg	Neg	IL-2 Rg	Neg	0.72	IGFBP-4	*Reject*	*Reject*
LIGHT	Neg	Neg	IL-12p70	Neg	Neg	IL-10 Rb	0.991	0.344	IGFBP-6	0.565	0.783
Ltactin	0.788	0.723	IL-13	0.231	0.091	IL-17R	0.345	0.336	IGF-I	0.097	0.391
MCP-2	0.524	0.482	IL-15	**0.033**	**0.009**	IL-21R	0.319	0.669	Insulin	Neg	Neg
MCP-3	0.887	0.753	IL-16	0.719	0.092	LIMPII	0.996	0.541	MCF R	0.389	0.32
MCP-4	Neg	Neg	IL-17	Neg	Neg	Lipoca lin-2	0.795	0.253	NGF R	0.183	0.309
MDC	Neg	Neg	MCP-1	0.081	0.295	L-Selectin	0.669	0.827	NT-3	Neg	Neg
MIF	0.883	0.477	MCSF	0.105	0.513	LYVE-1	0.781	0.988	NT-4	Neg	0.442
MIP-3a	Neg	0.544	MIG	**0.017**	0.389	MICA	0.809	0.422	OPG	Neg	0.363
MIP-3b	Neg	Neg	MIP-1a	Neg	Neg	MICB	Neg	Neg	PDGF-AA	0.14	**0.002**
MPIF-1	Neg	Neg	MIP-1b	Neg	Neg	NRG1-b1	Neg	Neg	PIGF	Neg	Neg
MSPa	Neg	Neg	MIP-1d	Neg	Neg	PDGF Rb	0.154	0.999	SCF	Neg	0.641
NAP-2	0.52	0.178	PDGF-BB	0.078	**0.003**	PECAM-1	0.394	0.184	SCF R	0.748	0.89
OPN	0.579	0.558	RANTES	0.65	0.109	RAGE	Neg	0.911	TGFa	Neg	Neg
PARC	Neg	Neg	TIMP-1	0.599	0.243	TIM-1	Neg	0.871	TGFb1	Neg	Neg
PF4	0.829	**0.02**	TIMP-2	0.184	0.08	TRAIL R3	0.754	0.839	TGFb3	Neg	Neg
SDF-1a	Neg	Neg	TNFa	0.144	**0.008**	Tra ppin-2	Neg	0.119	VEGF	Neg	0.999
TARC	Neg	Neg	TNFb	Neg	Neg	uPAR	0.078	0.533	VEGF R2	Neg	0.775
TECK	Neg	Neg	TNF RI	0.179	0.545	VCAM-1	0.432	0.21	VEGF R3	Neg	0.21
TSLP	Neg	Neg	TNF RII	0.508	0.517	XEDAR	Neg	Neg	VEGF-D	Neg	0.814

Results represent P-values from One-way analysis of variance for the comparison of values between Control, ILBD, and PD disease groups. Neg means the protein was deemed not to be present in sufficient number of the samples (we assessed this as <10 positive samples out of the 55 SN or 52 striatum). Measures with P values < 0.05 are highlighted in bold.

values for age, PMI, or age and PMI by ANCOVA, the disease group differences for these 13 proteins remained significant ($P < 0.05$). With these corrections, no additional proteins reached statistical significance.

Stepwise logistic regression analysis of these measures identified a combination of the values for ErbB3, IL-2, and PF4 as having predictive value between control and PD cases ($P = 0.0001$). Analysis by ROC with all three factors showed sensitivity of 86.67% and specificity of 87.5%, and an AUC of 0.917. As expected, TH values alone had significant predictive value for discriminating control from PD cases ($P = 0.0001$) with ROC analysis showing sensitivity of 80%, specificity of 85.71%, and AUC 0.914. Including TH values with the 13 proteins in step-wise regression analysis produced a different outcome; CCL28 in combination with TH had significant predictive value ($P < 0.0001$), with sensitivity of 80%, specificity of 78.7%, and AUC

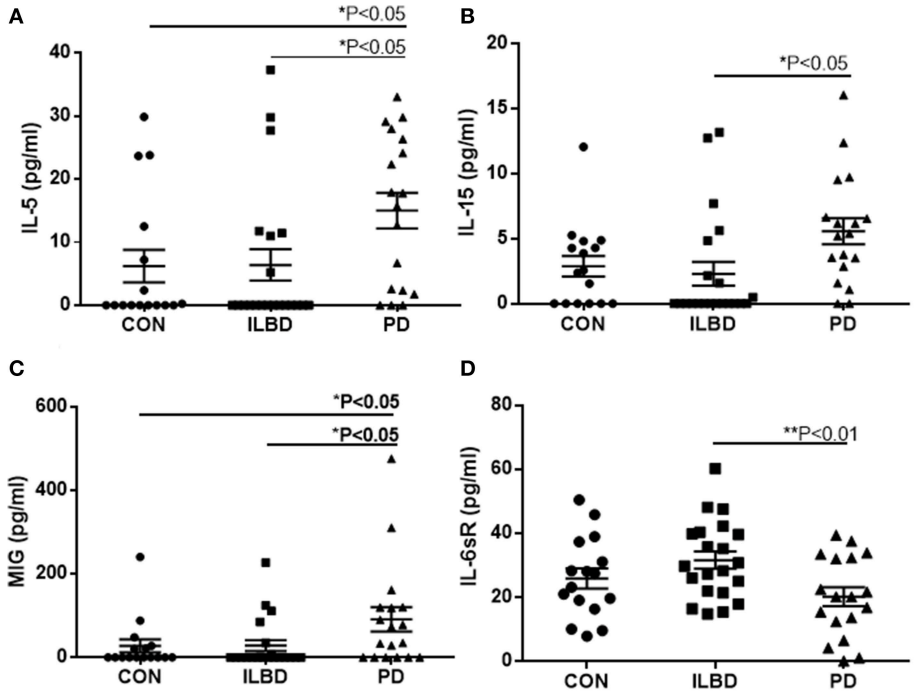

FIGURE 2 | Scatter plots showing distribution of results for proteins that showed disease group differences in substantia nigra samples. Disease group differences for interleukin-5 (IL-5) **(A)**, IL-15 **(B)**, monokine induced by gamma interferon (MIG) **(C)**, and IL-6 soluble receptor (IL-6sR) **(D)**. Statistical analysis by One-way analysis of variance (ANOVA) with Fisher LSD *post-hoc* test for between group differences.

TABLE 4 | Sensitivity and specificity for discriminating between disease groups.

Measures	Disease groups	% Sensitivity (95% CI)	% Specificity (95% CI)	Area under curve	*P*
(SN) IL-5	Control/PD	75	61.1	0.73	0.026
(SN) IL-15, IL-6sR	ILBD/PD	85.7	62.5	0.82	0.0016
(Str) ErbB3, IL-2, PF4	Control/PD	86.7	87.5	0.92	0.0001
(Str) TH	Control/PD	80	85.7	0.91	0.0001
(Str) TH, CCL28	Control/PD	80	78.7	0.94	<0.0001
(Str) PDGF-AA	ILBD/PD	72.2	62.5	0.72	0.015
(Str) TH, IL-15	ILBD/PD	82.3	85.7	0.90	0.0003
(Str) PDGF-AA	Control/ILBD	66.7	77.8	0.81	0.0014
(Str) TH	Control/ILBD	60	70.6	0.737	0.0157

of 0.938. Repeating the logistic regression analysis to determine if any of these measures gave predictive value between ILBD and PD cases identified PDGF-AA, with ROC analysis showing sensitivity of 72.2% and specificity of 62.5% and AUC of 0.722 ($P = 0.015$). Combining TH values with the 13 proteins showed IL-15 and TH values could discriminate between ILBD and PD ($P = 0.0003$), with sensitivity of 82.35 % and specificity of 85.71%, and AUC of 0.899. PDGF-AA levels alone gave discrimination between control and ILBD samples ($P = 0.0014$) with ROC analysis showing sensitivity of 66.67%, specificity of 77.78%, and AUC of 0.811. TH values alone discriminated between control and ILBD samples ($P = 0.0157$, sensitivity of 60%, specificity of 70.59%, and AUC of 0.737). Summary of these findings are shown in **Table 4**.

Correlation Analyses between Measures

Multiple correlation analyses were carried out for proteins that showed significant One-way ANOVA differences to indicate if levels of any of these correlated with each other. In SN, IL-5, IL-15, and MIG levels positively correlated with each other, while IL-6sR did not correlate with any measure (**Table 5**). This suggests that in samples showing inflammatory activation, a network of similar factors become upregulated. Significant correlations between some of the striatum proteins were identified (**Table 6**). Proinflammatory cytokines in this table showed significant positive correlations with each other, while there was also significant positive correlation between the PDGF-AA and PDGF-BB. The negative correlations between PDGF-BB, and IFN-γ or

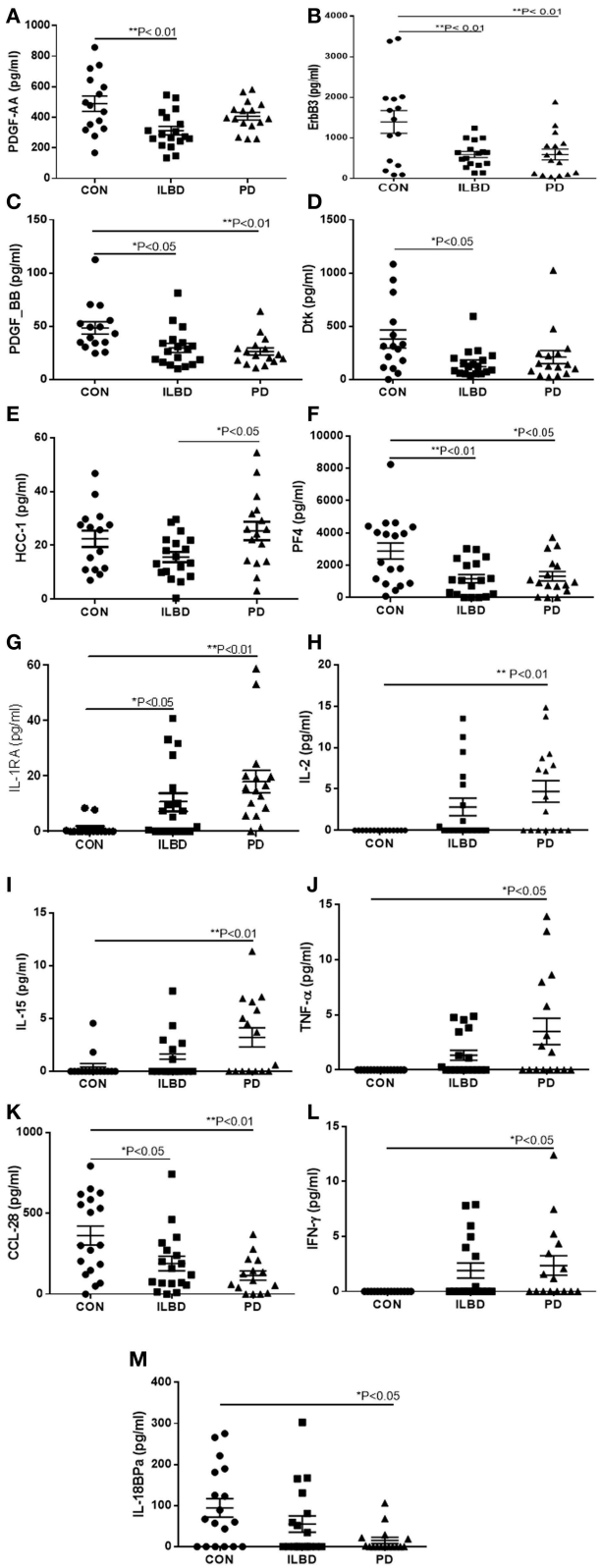

FIGURE 3 | Scatter plots showing distribution of results for proteins that showed disease group differences in striatum samples. Disease *(Continued)*

FIGURE 3 | Continued
group differences for platelet derived growth factor-AA (PDGF-AA) **(A)**, ErbB3 **(B)**, PDGF-BB **(C)**, and Dtk **(D)**. HCC-1, **(E)**, platelet factor-4 (PF4) **(F)**, IL-1 receptor antagonist (IL-1RA) **(G)**, IL-2 **(H)**, IL-15 **(I)**, TNF-α **(J)**, CCL-28 **(K)**, IFN-γ **(L)**, IL-18 binding protein (IL-18bpa) **(M)**. Statistical analysis by One-way analysis of variance (ANOVA) with Fisher LSD *post-hoc* test for between group differences.

TABLE 5 | Correlation between significant protein measures and with TH in substantia nigra.

	IL-15	IL5	MIG	IL-6SR
TH (R)	**0.315**	0.151	0.133	−0.075
(P)	**0.029**	0.306	0.367	0.61
IL-15		**0.737**	**0.488**	−0.191
		<0.0001	**0.0002**	0.166
IL-5			**0.656**	−0.254
			<0.0001	0.064
MIG				−0.177
				0.195

(R), Pearson correlation coefficient. (P), P values. Values highlighted in bold indicate statistically significant correlation between measures.

TNF-α suggested that the levels of this growth factor were affected by inflammation; this was not observed for PDGF-AA (**Table 6**).

Correlation Analysis of All Measured Array Features with TH

Multiple correlation analyses were carried out for all of the SN measures against TH levels. The significant correlations are shown in **Table 7**. Although CD14 and urokinase plasminogen activator receptor (uPAR), two classical markers of inflammation, did not show significant disease group differences, levels of the inflammation-associated markers negatively correlated with TH levels. The same analysis carried out for all striatal measures compared to striatum TH levels (**Table 7**) only showed significant positive correlations with activated leukocyte cell adhesion molecule (ALCAM-1), platelet-endothelial cell adhesion molecular (PECAM-1) and endoglin, all considered vascular adhesion proteins. The one common molecule from both brain regions was PECAM-1, which is involved in the integrity of the cerebral vasculature. To further assess inflammatory changes, we also measured levels of the astrocytic marker GFAP and the microglial markers IBA-1 and TLR-2 in samples by western blots. As had been shown (**Figure 1D**), the values between groups for GFAP in striatum were not statistically different (**Figure 1D**), but analysis of TH and GFAP across all disease groups did show significant negative correlation ($R = -0.399$, $P = 0.0071$; **Figure 1D**). Further separating these values into disease groups gave significant correlation between the control group GFAP values and TH ($R = -0.551$, $P = 0.0219$), but not between TH and GFAP levels for the ILBD or PD groups. There were no correlations between TH levels in SN and levels of IBA-1 or TLR-2 (data not shown).

TABLE 6 | Significant Correlation between key protein measures in striatum.

		Dtk	ErbB3			
CCL28	(R)	0.50	0.47			
	(P)	0.0002	0.0007			
		Dtk				
ErbB3	(R)	0.79				
	(P)	<0.0001				
		PDGF-AA	PF4			
HCC-1	(R)	0.46	0.37			
	(P)	0.0009	0.008			
		IL-2	IL-15	IFN-γ	PDGF-BB	TNF-α
IL-1ra	(R)	0.70	0.53	0.76	−0.385	0.566
	(P)	<0.0001	0.0001	<0.001	0.0063	<0.0001
		IL-15	IFN-γ	PDGF-BB	TNF-α	
IL-2	(R)	0.86	0.65	−0.45	0.80	
	(P)	<0.0001	<0.0001	0.0012	<0.0001	
		IFN-γ	PDGF-BB	TNF-α		
IL-15	(R)	0.56	0.42	0.74		
	(P)	0.0002	0.0026	<0.0001		
		PDGF-BB				
IFN-γ	(R)	−0.442				
	(P)	0.0015				
		PDGF-BB	PF4			
PDGF-AA	(R)	0.681	0.292			
	(P)	<0.0001	0.0418			
		PF4	IL-18 BPa	TNFa		
PDGF-BB	(R)	0.46	0.34	−0.35		
	(P)	0.0008	0.016	0.0128		
		TNFa				
IL-18BPa	(R)	−0.284				
	(P)	−0.048				

TABLE 7 | Significant Correlations between protein measures and TH.

Substantia nigra	TH	Striatum	TH
CD14 (R)	−0.306	ALCAM (R)	0.308
(P)	0.036	(P)	0.0375
CEACAM	0.461	Endoglin	0.401
	0.001		0.0057
EG-VEGF	0.674	PECAM-1	0.347
	< 0.0001		0.0183
EGFR	0.521	GFAP	−0.528
	0.0001		0.0002
GDNF	0.313		
	0.030		
IL-13	0.43		
	0.0023		
IL-15	0.315		
	0.029		
Lipocalin-2	−0.366		
	0.011		
LYVE-1	−0.366		
	0.010		
PECAM-1	0.371		
	0.0094		
SCF-R	0.731		
	< 0.0001		
TNF-α	0.411		
	0.0037		
uPAR	−0.299		
	0.039		

R, Pearson R correlation coefficient. See supplemental Table 1 for abbreviations for proteins.

TLR-2 Expression in SN

To follow up on the findings from a recent paper that examined tissue sections of SN from control, ILBD and PD cases and showed early increased expression of TLR-2 (Doorn et al., 2014), we carried out biochemical measurement of TLR-2 by western blot in our SN samples. Our analyses showed no significant differences between disease groups, but data obtained indicated TLR-2 levels had strong correlation with a number of the inflammatory markers measured (**Table 8**).

Microglial Activation Profiles in SN and Striatum

We stained tissue sections of SN and striatum from representative control, ILBD and PD cases to characterize activated microglia present in the disease groups. Staining was carried out using antibody LN3, which recognizes the MHCII protein HLA-DR, and antibody IBA-1, which recognizes all microglia. Results show increased immunoreactivity for HLA-DR in microglia associated with neuromelanin-containing neurons in the SN pars compacta, even in control cases (**Figures 4A,B**), with progressive change in microglial morphology in ILBD (**Figures 4C,D**), and PD cases (**Figures 4E,F**). The presence of smaller numbers of intensely-stained activated microglia in the control cases (**Figures 4A,B**) suggest ongoing inflammation even in the absence of disease. A noticeable feature was a difference within the PD groups, where some cases appeared to have a very strong inflammatory response in the vicinity of SN neurons (**Figures 4E,F**), while in others, there was a noticeable reduction in number of activated microglia (**Figures 4G,H**). Immunohistochemistry with antibody IBA-1 reveals greater numbers of microglia, but did not discriminate activated microglia in these sections (data not shown). For striatum, the sections were double-stained with antibody to TH (brown) to reveal the location of positive fibers. In control striatum, there were TH positive fibers throughout the structure, while in the PD cases, most of the TH fibers were lost. In **Figures 4J–L** particularly intense microglial response was observed in the vicinity of surviving TH fibers. Again, HLA-DR-positive microglia were also seen in control striatum

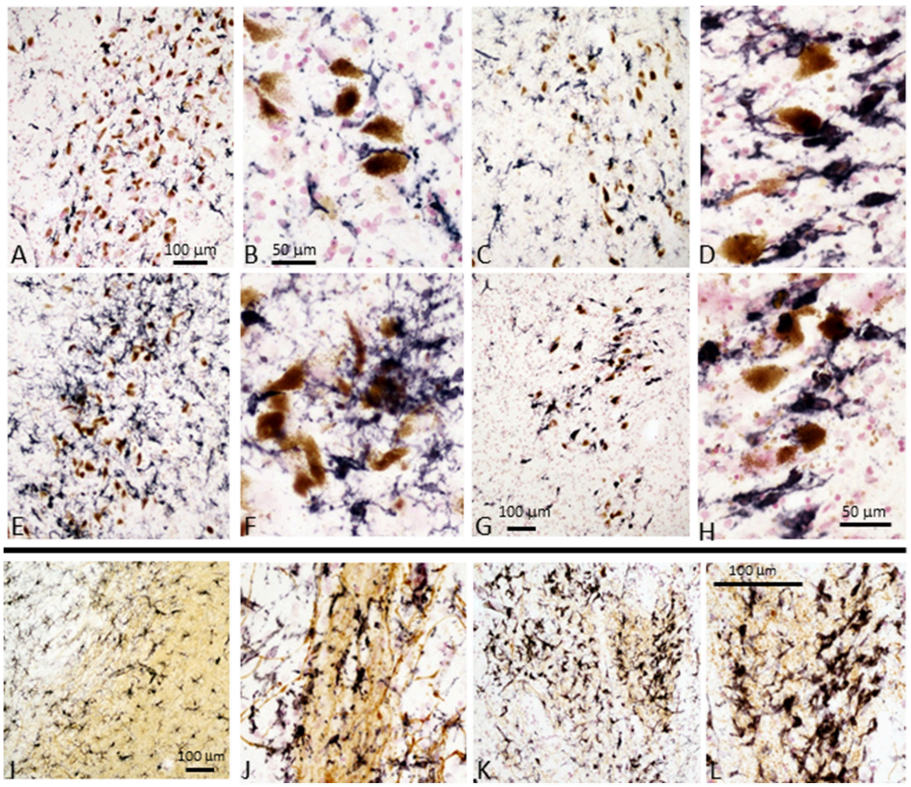

FIGURE 4 | Immunohistochemistry of microglia in tissue sections of SN and striatum. Substantia nigra: Immunohistochemical staining of sections from Control **(A,B)**, ILBD **(C,D)**, and PD **(E,F)** and PD **(G,H)** from SN tissue sections. **(A,C,E,G)** are lower magnification of SN tissue sections showing progressive increase in intensity of HLA-DR immunoreactive microglia (purple) from **(A–E)**, but PD case **(G)** showed reduced microglia intensity (burnt-out pathology) consistent with loss of SN neurons. Higher magnification images **(B,D,F,H)** illustrate the changes in morphology of microglia with progressive activation with reduction in burnt-out PD case. **(H)** shows microglia associated free neuromelanin. The preferential association of activated microglia with neuromelanin-containing dopaminergic neuron is apparent in all sections, even those from control cases. **Striatum**: Immunohistochemical staining of sections of Control **(I,J)** and PD **(K,L)** from striatum (putamen) stained with antibody LN3 to HLA-DR (purple) and tyrosine hydroxylase (brown). Control case **(I,J)** show association of microglia with tyrosine hydroxylase positive fibers. Stronger HLA-DR immunoreactivity **(K)** and activated morphology is apparent in association with remaining TH positive fibers in PD case **(K,L)**. Representative staining of sections from cases included in the antibody array series and are representative of each group.

sections (**Figures 4I,J**), though with a less-activated morphology compared to those in PD cases (**Figures 4K,L**).

Does the Presence of AD-Type Pathology in Brains Affect Inflammatory Protein Levels?

One limitation of human disease-focused studies can be the presence of mixed pathologies within the same tissue samples. For this study, the possible effects of age-associated AD-type pathology, namely plaques and tangles, on the inflammatory environment might be a confound factor. Although the primary diagnoses criteria used for case selection was the presence or absence of PD, along with the absence of AD, age-associated pathology was a feature of many of the cases used (**Table 1**). As the routine neuropathology diagnosis of each donor brains included an assessment of the overall burden of AD-type pathology by assessing the plaque and tangle scores of each brain, we used the above scores to identify any correlations of individual SN and striatum array measures with plaque and tangle scores using Spearman rank correlation analysis (**Table 9**). Proteins Dtk, ErBb3, IL-1ra, and IL-5 that had shown significant disease group differences in the antibody arrays for striatum showed significant correlations with tangle scores (**Table 9**, bold).

DISCUSSION

This work is the first description of large-scale unbiased multiplex quantitative protein profiling of tissue from human SN and striatum to elucidate changes in inflammatory and related molecules that might be involved in PD pathology, though this platform was used to screen sera from patients with Parkinsonian syndromes for inflammatory protein changes (Mahlknecht et al., 2012). We measured the levels of 158 different proteins in SN and striatum samples from control, ILBD, and PD cases. The study was based on the hypothesis that inflammatory molecules that become upregulated in ILBD cases might be more likely involved in disease mechanisms. ILBD seems to represent a precursor stage to PD; these subjects have no diagnosed movement disorders or dementia, but certain amounts of LB pathology, loss of dopaminergic neurons and reduced levels of TH (Beach et al.,

TABLE 8 | Significant correlations between key protein measures and TLR-2 in substantia nigra.

	TLR-2
IBA-1 (R)	0.565
(P)	0.0003
CD14	0.434
	0.0045
CXCL16	0.38
	0.0142
HCC-1	0.568
	0.0001
HGF	0.422
	0.0059
IL-2	0.377
	0.0152
IL-5	0.372
	0.0166
MCP-1	0.531
	0.0004
TNF-RII	0.643
	< 0.0001
TRAIL-R3	0.362
	0.0201

R, Pearson R correlation coefficient. See Supplemental Table 1 for abbreviations for proteins.

TABLE 9 | Correlations between Alzheimer's-type pathology and protein measures.

Plaques			Tangles		
Protein	***R***	***P***	**Protein**	***R***	***P***
SUBSTANTIA NIGRA					
ALCAM	−0.335	0.012	Dtk	−0.276	0.041
CCL28	0.441	0.0008	PDGF-AA	−0.303	0.024
CD14	−0.381	0.004			
Endoglin	−0.416	0.002			
ErbB3	−0.323	0.016			
Flt-3L	−0.324	0.016			
IL-18Bpa	0.361	0.007			
PDGF-AA	−0.271	0.045			
PECAM-1	−0.343	0.010			
PF4	0.277	0.041			
uPAR	−0.299	0.027			
STRIATUM					
L-tactin	−0.352	0.013	AR	0.346	0.015
MCP-2	−0.34	0.017	**Dtk**	**−0.462**	**0.0008**
MIP-3a	−0.415	0.003	**ErbB3**	**−0.397**	**0.005**
OPN	−0.311	0.029	FGF-7	0.299	0.037
Trappin-2	−0.308	0.031	**IL-1ra**	**0.29**	**0.043**
uPAR	−0.317	0.026	**IL-5**	**0.295**	**0.039**
			IP-10	0.36	0.011
			NT-4	0.458	0.0009

Plaques, Histological plaque score 0-15; Tangles, Histological tangle score 0-15; R, Spearman rank correlation. See Supplemental Table 1 for abbreviations for proteins. Results in bold identify proteins that showed significant differences between control, ILBD, and PD groups.

2008; Caviness et al., 2011; Iacono et al., 2015). In agreement with our earlier studies, we showed this group of tissue samples had intermediate TH values between control and PD cases in striatum (Beach et al., 2008, 2009).

The major findings from this study were the identification of candidate inflammatory or growth factor molecules that showed significant disease group differences. We identified four proteins in SN, and 13 proteins in striatum that had One-way ANOVA differences, and *post-hoc* differences between disease groups with $P < 0.05$, though these statistical analysis did not take account for multiple comparisons. The other feature of the study was the identification of a different inflammatory signature for SN compared to striatum. The only molecule that changed significantly for both brain regions was IL-5. Independent validation of these findings are now required using sensitive methodologies in a new group of tissue samples.

An inherent limitation to the approach using human tissues was the highly variable nature of disease and inflammatory changes within each group. This large variability for many measures reduced the potential of detecting disease group differences. As demonstrated in **Figure 1**, there was considerable variability in the values for TH as the fundamental biochemical measure of disease severity. This could also be seen in most individual measures (see scatter plots in **Figures 2**, **3** as examples) particularly within the control groups. Related to this was sensitivity of detection for certain proteins. It was observed that a number of samples within each group had values below the LOD of the assays. Although this was observed for IL-5, IL-15, and MIG in SN, and IL-2, TNF-α, IL1-RA, IL-15, and IFN-γ in striatum (**Figures 2**, **3**), a clear increase in the number of PD samples having higher positive values was apparent, confirming the changes in these cytokines in the diseased samples.

The analyses of protein levels showed a higher percentage of proteins being below the LOD in SN compared to striatum. Of the 158 array proteins being measured, 101 of these proteins could be detected and measurable in striatum, while 81 were measurable in SN. The difference in protein profiles between SN and striatum did not appear to be due to different sensitivity between tissues. All of the proteins that showed significant differences between disease groups in striatum samples could be detected in SN, while the reverse situation was present for significant SN proteins in striatum (**Table 3**). This feature provided support for the observation of differences in inflammatory features between SN and striatum.

Fundamental to this study was also a demonstration of differences in appearance of microglia in disease-affected tissues. Immunohistochemical staining of selected tissue sections of SN and striatum for the presence of morphologically activated microglia using antibody LN3 identified a progressive change in appearance of microglia associated with increasing pathology. This was particularly apparent in SN; however, two patterns of activated microglia could be seen in PD cases. Some cases had large numbers of highly-activated microglia potentially indicative of an active pathological process; while others showed

a reduced number of microglia with many having intracellular neuromelanin potentially indicative of "burnt-out" pathology (**Figures 4G,H** compared to **Figures 4E,F**). Similar patterns were apparent in striatum in association with TH positive fibers. One noticeable feature was the presence of considerable numbers of activated microglia in SN and striatum from control cases that had absence of LB pathology.

There were two patterns of changes in proteins between disease groups. For those proteins that had reduced levels with progression to PD, this could indicate loss of "protective" functions as a disease feature. For those proteins that showed increased levels with progression to PD, this indicated a possible role in enhancing inflammatory pathology. In SN, there were increases in PD for cytokines IL-5, IL-15, and MIG, but decrease in levels of IL-6sR. In striatum, there were decreases in PD compared to control for PDGF-AA, PDGF-BB, ErbB3, Dtk, PF4 and HCC-1, IL-18BPa and CCL28, but increases in PD for cytokines IL-2, IFN-γ, TNF-α, IL-1Ra, and IL-15. The factors downregulated are either inflammatory antagonists or growth factors, while the factors upregulated are inflammation-associated cytokines. With the exception of IL-2, TNF-α, and IFN-γ, the other factors we identified have not been associated with PD in previous animal or human studies.

Of the highlighted proteins, some features as they relate to possible brain function will be discussed. IL-5 has been described as a Th2-cell cytokine whose expression is upregulated in inflammatory-associated conditions associated with parasitic infections, similar to IL-4 or IL-13, with secretion of IL-5 by astrocytes and microglia having been described (Sawada et al., 1993). IL-5 can also be a colony stimulating factor for macrophages and microglia (Ringheim, 1995; Liva and de Vellis, 2001). By comparison, IL-15 expression in diseased brain has been more widely studied than IL-5. Both neurotoxic and proinflammatory consequences of IL-15 have been observed in brain. IL-15 receptors are present on neurons, and treatment of neurons *in vivo* with IL-15 resulted in reduced neurite outgrowth and reduction in expression of microtubule-associated brain (MAP)-2 (Huang et al., 2009). IL-15 can enhance the cytotoxic potential of CD4+ T cells in multiple sclerosis (Broux et al., 2015). IL-15 also has an effect at the blood-brain barrier resulting in weakening of tight junctions and increased permeability (Stone et al., 2011). Increased expression of MIG by microglia in brain has been demonstrated in response to IFN-γ and also IL-9 (Carter et al., 2007; Ellis et al., 2010; Ding et al., 2015). MIG activates cellular signaling through the receptor CXCR3, which is shared with CXCL10 (interferon gamma induced protein 10–IP-10). Compared to MIG, IP10 was not consistently detected in SN samples. Interpreting the decrease in levels of IL-6sR in PD compared to ILBD samples is complex as IL-6sR functions can have both agonist and antagonist properties. IL-6 interaction with IL-6sR interaction enhances signaling on cells expressing the other IL-6 receptor gp130, while blocking signaling on cells expressing both IL-6R and gp130. Microglia express IL-6R as well as gp130 so changes in IL-6sR levels might block rather than enhance activation of microglia. However, other studies have shown that IL-6 signaling in brain is predominantly enhanced by IL-6sR (Burton et al., 2011, 2013; Campbell et al., 2014).

The different pattern of changes for proteins were observed in striatum compared to SN was suggestive of a decline in growth factors along with enhanced levels of different inflammatory proteins were ongoing. Both PDGF-AA and PDGF-BB levels showed disease differences in striatum. It has been known that PDGF can be neurotrophic for transplanted dopaminergic neurons and induces neurogenesis (Smits et al., 1993; Mohapel et al., 2005). Clinical trials using intraventricular infusion of PDGF-BB in PD patients as a therapeutic treatment to support dopaminergic neurons are ongoing (Paul et al., 2015). In **Table 5**, the significant negative correlations of PDGF-BB levels with those of inflammatory markers IL-1RA, IL-2, IL-15, IFN-γ, and TNF-α suggested that levels were negatively affected by degree of inflammatory activation. The correlation between PDGF-BB and TH levels in striatum was close to significance ($P = 0.052$). CCL-28 is expressed by epithelial cells and has chemotactic properties for certain immune cells. Increased expression in periphery has been associated with inflammation, however, in brain, it has only been identified in neurons rather than glial cells (Liu et al., 2012). In a model of epilepsy, downregulated expression of CCL28 was associated with loss of neurons (Liu et al., 2012). Dtk (receptor tyrosine kinase TYRO3) is also predominantly expressed by neurons (Prieto et al., 2007; Zhao et al., 2012). Its function in the PD brain is unclear, but overexpression of Dtk in an AD mouse model resulted in reduced production of amyloid beta (Aβ) peptide (Zheng et al., 2012). Interleukin-18 binding protein α (IL-18BPa) is high-affinity antagonist for the proinflammatory cytokine IL-18 (interferon gamma inducing factor). Both factors normally occur in balance, but a decrease in IL-18BPa can be accompanied with increased IL-18 (Dinarello et al., 2013). This factor has not been studied in human brain Platelet factor 4 (PF4), also known as CXCL4, is a chemokine involved in wound repair and inflammation. It has anti-angiogenic properties, and is an antagonist for the basic fibroblast growth factor receptor (Sulpice et al., 2002; Chadderton and Stringer, 2003). ErbB3 is an epidermal growth factor-related receptor tyrosine kinase with neuregulin as a ligand. ErbB3 is widely expressed in brain in gray matter, white matter, neurons, astrocytes, endothelial cells, and oligodendrocytes (Ozaki et al., 1998; Steiner et al., 1999; Calaora et al., 2001; Lok et al., 2009; Sharif et al., 2009). Examination of human PD striatum samples for EGF and ErbB receptors 1-4 demonstrated different results from this study (Iwakura et al., 2005). A decrease of EGF in PD samples was reported, along with a decrease in ErbB1 and ErbB2, but not ErbB3 (Iwakura et al., 2005). We did not identify differences in EGF levels in striatum or SN samples in this study. HCC-1 (CCL-14) has not been studied in brain, but has been characterized as a marker for alternatively activated/anti-inflammatory macrophages (Jaguin et al., 2013). In the striatum samples, there was a significant increase between ILBD and PD samples.

Five of the inflammatory cytokines associated with disease differences were progressively upregulated in striatum between control, ILBD, and PD samples. IFN-γ is a potent proinflammatory cytokine primarily produced by T lymphocytes and natural killer cells rather than by brain resident cells. IFN-γ has been shown to have a central role in mediating dopaminergic cell loss or deterioration of the nigrostriatal pathway in PD

animal models (Mount et al., 2007; Brochard et al., 2009; Chakrabarty et al., 2011; Barcia et al., 2012; Mangano et al., 2012). By contrast, IL-1RA is protective and blocks the binding of the cytokines IL-1α or IL-1β to the IL-1 receptor. Its increased levels are suggestive of inflammation due to elevated levels of IL-1α or IL-1β. This cannot be confirmed in this study as IL-1α and IL-1β were not detectable in most SN or striatum samples. IL-1RA has significant neuroprotective properties in situations with brain injury with increased expression by neurons in AD and Pick's disease brains (Yasuhara et al., 1997; Sanderson et al., 1999; Yang et al., 1999; Vecil et al., 2000; Masada et al., 2003). IL-2 is also a T-lymphocyte produced cytokine that is involved in cellular immune responses mediated by T-lymphocytes. Increased IL-2 levels were reported in an earlier study in PD striatum (Mogi et al., 1996). TNF-α is been the cytokine most widely implicated in the pathogenesis of PD. The majority of studies have focused on TNF-α in various animal models not in human brain (Nagatsu et al., 2000a; Gayle et al., 2002; Carvey et al., 2005a; Zhao et al., 2007; De Lella Ezcurra et al., 2010; Chertoff et al., 2011; Daniele et al., 2015). Sensitivity of detection of this cytokine was an issue due to the number of samples with negative values included in the analysis. However, comparing TNF-α levels in SN control samples with control striatum samples illustrate difference between these two brain regions. In SN, half of the control samples had detectable levels of TNF-α, while the striatal control samples were all below the level of detection. As can be seen, the distribution of values in SN between control and PD were not significantly different ($P = 0.144$), while in striatum there was an increasing number of positive samples in the ILBD and PD groups ($P = 0.008$). Although the values were low, the amount of TNF-α detected in SN was overall higher than in striatum. Mean values in PD SN samples were 10.1 pg/ml compared to 3.5 pg/ml in striatum PD samples.

Although interpreting the implications of these findings for identifying therapeutic targets needs to be done with caution, interactions of different factors and also the presence of other pathologies needs to be considered. Correlation of SN measures with TH levels identified one panel of 13 proteins, while a panel of four proteins correlated with TH levels in striatum. One common feature was the number of vascular markers—this suggested that changes in vascular integrity could be a feature of PD pathogenesis. Pathology-based studies have shown increased vascular degeneration in PD affected brain tissue, as well as aberrant angiogenesis that also can promote vascular degeneration (Desai et al., 2009; Guan et al., 2013). The possible confounding effect of AD-type pathology also needs to be considered when interpreting findings. It has been recently described that many subjects diagnosed clinically with PD show evidence of additional neuropathology upon postmortem examination (Dugger et al., 2014). As demonstrated in **Table 9**, there were significant correlations between a number of these inflammatory proteins with plaque and tangle pathology, even though the AD pathology measures used in our analyses did not directly assess plaques and tangles in SN and striatum but were used as an overall index of brain load of AD-type pathology.

In summary, we had defined different inflammatory profiles for SN and striatum using an antibody array assay that measured 158 different biologically active proteins in tissue from control, ILBD, and PD cases. The study has identified candidate molecules whose role(s) in accelerating PD pathology can be validated in further analyses. One of the problems highlighted in this study is the heterogeneity in disease or aging changes in these carefully selected cases, as well as the presence of other neuropathologies that can affect measures. Variability is inevitable in human samples, but potentially masked some of the disease group differences. The very large range of values found for each of the factors suggests other causes beside PD pathology might be affecting levels of molecules in tissue. The use of correlation analyses along with assessment of disease group differences provided interesting leads to follow up. The strength of this application is the quality of the postmortem tissue used. These were collected and stored under defined conditions with short postmortem delays so the stability of these measured molecules should be assured (Beach et al., 2015). Further studies though will require methodology with higher sensitivity to be able to measure the low levels of key cytokines in all tissue samples.

AUTHOR CONTRIBUTIONS

DW planned the study, coordinated sample processing and analysis, performed data analysis, manuscript writing, and coordinating with funding agent. LL assisted in these processes. GS and LS selected and dissected the tissue samples used in the study and provided neuropathology consultations. JC and CA provided clinical assessments premortem on many of the recruited subjects in the study. TB provided neuropathology diagnosis, assisted in sample selection and coordinated tissue collection.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnins.2015.00507

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REVIEW
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Potential application of lithium in Parkinson's and other neurodegenerative diseases

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Lithium, the long-standing hallmark treatment for bipolar disorder, has recently been identified as a potential neuroprotective agent in neurodegeneration. Here we focus on introducing numerous *in vitro* and *in vivo* studies that have shown lithium treatment to be efficacious in reducing oxidative stress and inflammation, increasing autophagy, inhibiting apoptosis, and decreasing the accumulation of α-synulcein, with an emphasis on Parkinson's disease. A number of biological pathways have been shown to be involved in causing these neuroprotective effects. The inhibition of GSK-3β has been the mechanism most studied; however, other modes of action include the regulation of apoptotic proteins and glutamate excitotoxicity as well as down-regulation of calpain. This review provides a framework of the neuroprotective effects of lithium in neurodegenerative diseases and the putative mechanisms by which lithium provides the protection. Lithium-only treatment may not be a suitable therapeutic option for neurodegenerative diseases due to inconsistent efficacy and potential side-effects, however, the use of low dose lithium in combination with other potential or existing therapeutic compounds may be a promising approach to reduce symptoms and disease progression in neurodegenerative diseases.

Keywords: neuroprotection, calpain, GSK-3β, Bcl-2, and autophagy

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INTRODUCTION

Lithium, introduced in 1949, is the most commonly used drug for the treatment of bipolar disorder, a chronic mental illness characterized by manic and depressive cycles. The efficacy of lithium in treating acute mania is long-established, and it is the standard against which other medications for bipolar disorder are measured (Young and Hammond, 2007). Meta-analysis of 14 randomized control samples showed that lithium, when used prophylactically, primarily reduced manic relapses, although its efficacy in reducing depressive relapses was significantly lower

Abbreviations: 3-NPA, 3-nitropropionic acid; 6-OHDA, 6-hydroxydopamine; AD, Alzheimer's disease; AIMs, abnormal involuntary movements; ALS, amyotrophic lateral sclerosis; AP-1, activator protein-1; APP, amyloid precursor protein; Akt, protein kinase B; BAD, Bcl2-associated agonist of cell death; BAK, Bcl2-antagonist/killer; Bax, Bcl-2–associated X protein; Bcl, B-cell lymphoma; BDNF, brain derived neurotrophic factor; CAT, catalase; Cdk, cyclin dependent kinase; DOPAC, dihydroxyphenyl acetic acid; ERK, extracellular signal-regulated kinase; FOXO, forkhead box class O; GSH, glutathione peroxidase; GSK, glycogen synthase kinase; HD, Huntington's disease; IMPase, inositol monophosphatase; IP3, inositol 1,4,5-trisphosphate; JNK, Jun N-terminal kinase; MCI, mild cognitive impairment; MDM, murine double minute; MEF, myocyte enhancement factor; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NPCs, neural progenitor cells; NT-3, neurotrophin 3; NT-4, neurotrophin 4; PD, Parkinson's disease; SNpc, substantia nigra pars compacta; SOD, superoxide dismutase; Tcf, T-cell factor; TH, tyrosine hydroxylase; TrkA, receptor tyrosine kinase A; TrkB, receptor tyrosine kinase B; TrkC, receptor tyrosine kinase C; VPA, valproic acid.

(Smith et al., 2007). Additional meta-analyses showed that lithium treatment reduced the number of suicides and suicide attempts in individuals with mood disorders (Cipriani et al., 2005; Baldessarini et al., 2006).

A growing body of evidence suggests that the benefits of lithium extend beyond mood stabilization. Lithium treatment has been shown to provide neuroprotection against neurological insults including excitotoxicity, ischemic damage, and traumatic brain injury (Basselin et al., 2006; Zhu et al., 2010). In addition, lithium has been shown to contribute to remyelination and axonal regeneration (Makoukji et al., 2012). In particular, lithium treatment has been associated with neuroprotection against neurodegenerative conditions such as Parkinson's, Alzheimer's, and Huntington's diseases as well as Amyotrophic Lateral Sclerosis (ALS). This review focuses on the effects of lithium on Parkinson's disease and some of the presumed mechanisms by which lithium provides its protective properties.

While studies suggest lithium can be an efficacious treatment for mood disorders and neurodegenerative conditions, there are several reports about lithium-induced neurotoxicity that, at its worst, is irreversible. High lithium doses are generally required for inducing neurotoxicity, however, it can occur at therapeutic dosages as well (Donaldson and Cuningham, 1983). The signs exhibited by the effected patients were mostly extrapyramidal in nature (Johnels et al., 1976; Kane et al., 1978; Ghadirian and Lehmann, 1980). There have also been reports of patients with cerebellar signs and Creutzfeldt-Jakob disease-like syndrome induced by lithium treatment (Smith and Kocen, 1988; Finelli, 1992). Research has shown, however, a correlation between lower doses of lithium and lower side-effects (Abou-Saleh and Coppen, 1989).

Parkinson's Disease (PD) is a basal ganglia disease, which associates with clinical motor symptoms such as bradykinesia, akinesia, resting tremor, muscular rigidity and postural instability, and non-motor symptoms of sleep disturbance, constipation, dysarthria, dysphonia, dysphagia, sialorrhoea, urinary incontinence and, "at the last, constant sleepiness with slight delirium" (Parkinson, 2002). Pathologically, PD is characterized by intracytoplasmic Lewy body inclusions and degeneration of dopaminergic neurons primarily in the substantia nigra pars compacta (SNc). Like other neurodegenerative diseases, the etiology of PD is largely unknown. Less than 10% of cases are probably caused by genetic mutations, most notably, in the gene encoding the presynaptic alpha-synuclein protein (Polymeropoulos et al., 1997). Some studies have demonstrated that prolonged occupational exposure to certain chemicals, particularly pesticides and heavy metals, such as Fe, Mn, Zn and Cu, is associated with an elevated risk of PD (Betarbet et al., 2000; Fukushima et al., 2013; Stelmashook et al., 2014).

LITHIUM EFFECTS IN PARKINSON'S DISEASE

Diverse lithium studies in Parkinson's disease have been executed and included both *in vitro* and *in vivo* experiments. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and 6-OHDA (6-hydroxydopamine) have been used to generate PD animal models and have been shown to activate the pro-apoptotic cysteine protease, caspase-3. In human neuroblastoma SH-SY5Y cells treated with MPP+ and in cultured rat cerebellar granule neurons treated with 6-OHDA, lithium inhibited the caspase-3 activation and prevented cell death (King et al., 2001; Chen et al., 2004). Endoplasmic reticulum (ER) stress, in combination with abnormal protein degradation has been implicated in the pathophysiology of PD as well as other neurodegenerative diseases (Lindholm et al., 2006). Lithium, via GSK inhibition, has been shown to protect cells from ER stress-induced lipid accumulation (Kim et al., 2005). In addition, chronic low-dose lithium exposure on SH-SY5Y cells demonstrated its neuroprotective effects, possibly mediated by regulating stress gene expression, stimulating glycolysis, accumulating extracellular pyruvate, and inducing resistance to oxidative stress due to nuclear factor erythroid 2-related factor 2 (NRF)-2 activation and miR-34a inhibition (Nciri et al., 2013; Alural et al., 2015). Similarly, lithium was found to be protective against oxidative stress in rat dopaminergic N27 cells which over-express A53T alpha-synuclein. In the brains of alpha-synuclein A53T over-expressing transgenic mice, lithium prevents/degrades paraquat/maneb-induced alpha-synuclein protein aggregation (Kim et al., 2011). Chronic lithium treatment *in vivo* and *in vitro* in the absence of either neurotoxin showed significant increases in tyrosine hydroxylase in the frontal cortex, hippocampus, and striatum of rats and mice as well as in human neuroblastoma SH-SY5Y and rat dopaminergic N27 cells (Chen et al., 1998; Lieu et al., 2014; Lazzara et al., 2015).

The combination of low-dose lithium and L-Dopa/Carbidopa (Sinemet®) in MPTP-lesioned mice has been shown to reduce MPTP-induced abnormal involuntary movements (AIMs) (Lazzara et al., 2015), while in another study using the MPTP-induced PD mouse model, combined administration of lithium and valproate improved motor function and increased the number of dopaminergic neurons in the substantia nigra, compared to controls. There was also a decrease in the concentration of the dopamine metabolite, dihydroxyphenyl acetic acid (DOPAC), in both the MPTP-treated and control groups; however, DOPAC loss was less severe in mice receiving the combined lithium-valproate treatment (Li et al., 2013).

Lithium has been considered as a potential apoptotic inhibitor. Studies in PD animal models have demonstrated that lithium can prevent MPTP-induced dopamine depletion and stimulate the up-regulation of B-cell lymphoma 2 (Bcl-2) and the down-regulation of Bcl-2–associated X protein (Bax) in the striatum (Youdim and Arraf, 2004). Over-expression of Bax hastens apoptotic cell death, which can be reduced by lithium's anti-apoptotic effect (Oltval et al., 1993). Bcl-2, on the other hand, is a proto-oncogene that is one of the key regulators of apoptosis. Over-expression of Bcl-2 provides protection against dopaminergic neurotoxins, by which lithium may reduce apoptosis (Offen et al., 1998).

PUTATIVE MODES OF ACTION OF LITHIUM

Numerous *in vitro* and *in vivo* studies over the last two decades have demonstrated that lithium provides neuroprotection, and as such, has the potential to become a therapeutic agent in the treatment of neurodegenerative diseases. Mechanisms of action of lithium include activating neurotrophic and neuroprotective cellular cascades, reducing oxidative stress, and decreasing apoptosis and inflammation. It has also been shown to enhance neurotrophic factors, reduce excitotoxicity, and provide mitochondrial stability (see **Figure 1**).

Inhibition of GSK-3

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase that is involved in a number of intracellular signaling pathways. It exists in two isoforms, α and β. Dysfunction of the protein plays a role in the pathogenesis of both sporadic and familial forms of Alzheimer's disease. Up-regulation of GSK-3 activity leads to phosphorylation of the amyloid precursor protein (APP) and the protein tau, both of which are associated with the pathological processes that lead to the hallmarks of Alzheimer's disease (AD), amyloid-β plaques and neurofibrillary tangles (Hanger and Noble, 2011; Avila et al., 2012). Interestingly, in the MPTP mouse model, α-synuclein contributes to tau hyper-phosphorylation, an effect that is mediated by the activation of GSK-3β. This robust activation of GSK-3β was also observed in SH-SY5Y cells co-transfected with hDAT, mesencephalic neurons, striata of transgenic mice overexpressing α-synuclein, and in the postmortem striata of PD patients (Duka et al., 2009).

A number of researchers have demonstrated that lithium at concentrations of 1–2 mM inhibits GSK-3 (Bauer et al., 2003; Rowe and Chuang, 2004; Gould and Manji, 2005; Rowe et al., 2007). Lithium has been shown to reduce GSK-3 activity in two ways—both directly and indirectly—by increasing the inhibitory phosphorylation of GSK-3. The direct path by which GSK-3 is inhibited by lithium is via direct competition for a magnesium-binding site with GSK-3β (Jope, 1999). The indirect regulation of GSK-3 activity by lithium is through the activation of protein kinase B (also known as Akt). It is thought that the activation of Akt may provide additional neuroprotective effects downstream via modulation of forkhead box class O (FOXO), Bcl-2-associated death protein (Bad) (a pro-apoptotic protein of the Bcl-2 family), and murine double minute (MDM; Beaulieu et al., 2004, 2007; Avila and Hernández, 2007; Alural et al.,

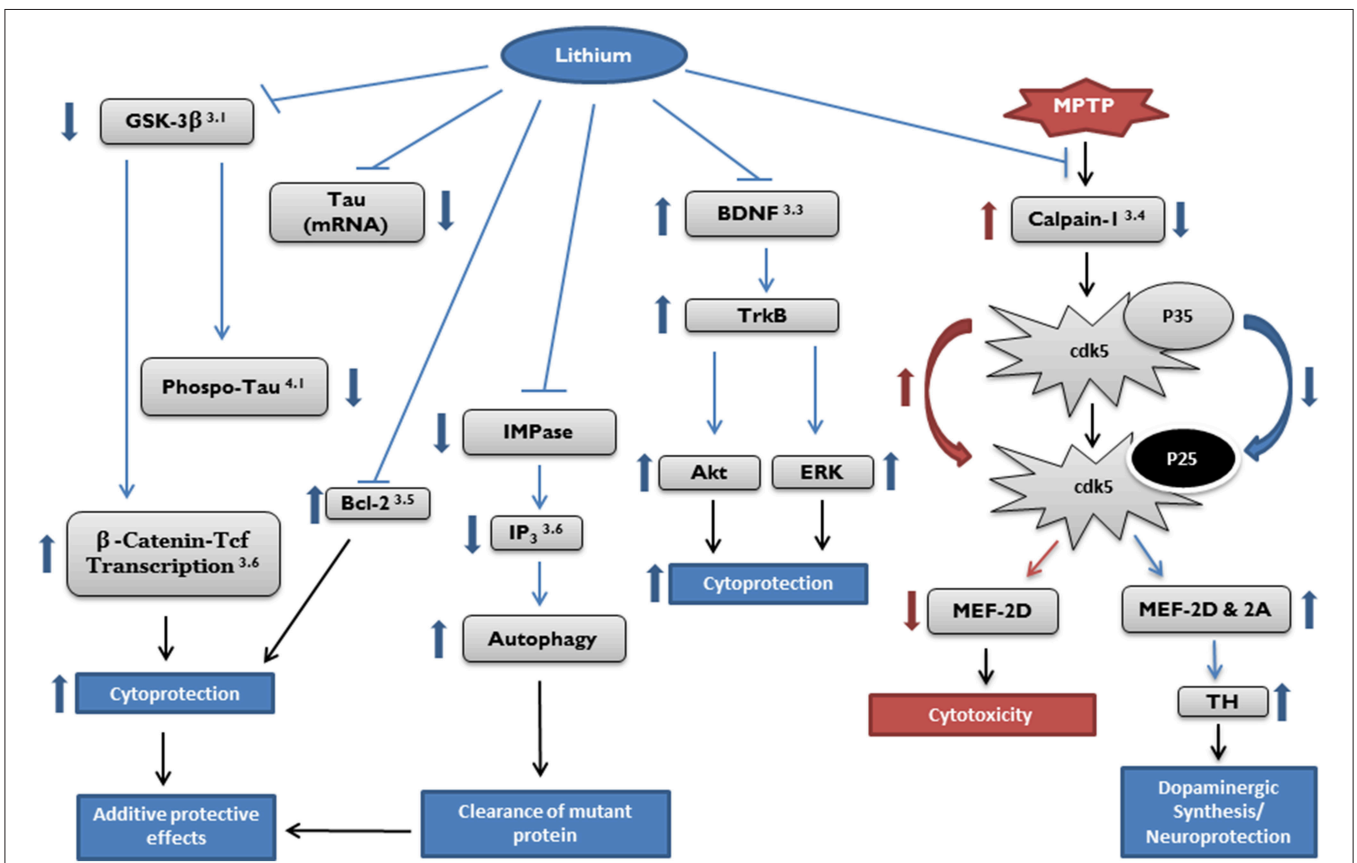

FIGURE 1 | Potential targets of cytoprotective effects by lithium. Target pathways of lithium in neurodegenerative diseases described in the article are marked by section number (right top). Akt, protein kinase B; Bcl, B-cell lymphoma; BDNF, brain derived neurotrophic factor; Cdk, cyclin dependent kinase; ERK, extracellular signal-regulated kinase; GSK, glycogen synthase kinase; IMPase, inositol monophosphatase; IP_3, inositol 1,4,5-trisphosphate; MEF, myocyte enhancement factor; Tcf, T-cell factor; TH, tyrosine hydroxylase; TrkB, receptor tyrosine kinase B.

2015). Further, this inhibition of GSK-3 by lithium correlates with reduced tauopathy and degeneration *in vivo* (Hong et al., 1997; Noble et al., 2005). However, the lithium-induced GSK-3β regulation may be an acute down-regulation since chronic lithium exposure does not appear to have an impact on the level of GSK-3β expression nor its activity (Kim et al., 2011; Nciri et al., 2015).

Inhibition of Oxidative Stress

Oxidative stress is believed to be one of the underlying causes of cellular dysfunction and death in PD. In PD patients, the cells in the SNpc exhibit increased levels of oxidative stress-induced damage in lipids, proteins, and DNA and decreased levels of glutathione (GSH) (Bosco et al., 2006; Nakabeppu et al., 2007; Zeevalk et al., 2008). The primary markers of oxidative stress include thiobarbituric acid reactive substances and regulation of several enzymes—superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Wang et al., 2003). When stress conditions are increased, SOD levels increase as well, leading to an elevated SOD/CAT ratio. An increase in oxidative stress is often linked to an increase in the cellular hydrogen peroxide concentration inducing lipid peroxidation in membranes, proteins and genes (Gsell et al., 1995).

In several *in vitro* studies, lithium administration was found to inhibit hydrogen peroxide-induced cell death as well as obstruct lipid peroxidation and protein oxidation in cortical cells (Shao et al., 2005; de Vasconcellos et al., 2006; Cui et al., 2007; Frey et al., 2007; Machado-Vieira et al., 2007; Kim et al., 2011). In addition, the ability of lithium to act as an anti-oxidant was ascribed to an increase in GSH levels in neurons, rat dopaminergic N27, and human SH-SY5Y neuroblastoma cells (de Vasconcellos et al., 2006; Kim et al., 2011).

Activation of Brain Derived Neurotrophic Factor/Receptor Tyrosine Kinase B

Brain derived neurotrophic factor (BDNF) helps to regulate neuronal and synaptic development and support the survival and plasticity of existing neurons (McAllister et al., 1999). The receptor tyrosine kinase B (TrkB) is activated by and facilitates the effects of the neurotrophins: BDNF, neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). These effects include neuronal differentiation and survival (Yoshii and Constantine-Paton, 2010).

Chronic lithium treatment was shown to significantly increase BDNF expression in the hippocampus as well as temporal and frontal cortices of rat brain; however, it was not accompanied by an increase in TrkB levels (Fukumoto et al., 2001). In a study which used primary cultures of rat cortical neurons, results with wild type and BDNF heterozygous and homozygous knock-outs showed that lithium application activates the BDNF/TrkB signaling pathway and protects neurons from glutamate excitotoxicity (**Figure 1**; Hashimoto et al., 2002). In two separate *in vitro* studies utilizing neural progenitor cells (NPCs), it has been suggested that lithium up-regulates BDNF production as evidenced by maximal cellular proliferation and neuronal differentiation (Su et al., 2007, 2009). It has been demonstrated that lithium increases mRNA of NGF, but not TrkA in rats, and inhibits TrkA-mediated signaling in PC12 cell cultures. In addition, NT-3 mRNA was decreased, but the mRNA of its receptor TrkC was increased by lithium (Burstein et al., 1985; Mudò et al., 1996). Although the cell proliferation effect by lithium in brain can be controversial, lithium-induced hippocampal neurogenesis in adult rodents has been reported by several groups (Fiorentini et al., 2010; O'Leary et al., 2012).

Inhibition of the Calpain—Cdk5 Pathway

In the MPTP-induced PD mouse model, Smith et al. demonstrated a mechanism for dopaminergic neuronal loss which involves the downstream pathway of calpain-1 (2006). This mechanism includes modulation of the transcription factor myocyte enhancer factor 2 (MEF2) via cyclin dependent kinase 5 (Cdk5). A co-activator of Cdk5, p35, is converted to p25, a pathogenic form, by calpain-mediated cleavage of p35. Cdk5, bound to p25, becomes pro-apoptotic and leads to phosphorylation of MEF2 at Ser444, an inactivating site. The inactivation of MEF2 plays a critical role in dopaminergic cell loss (Smith et al., 2006). There has been no evidence that lithium inhibits calpain activity directly (Sasaki et al., 2006); however, it has been shown that lithium treatment robustly inhibits NMDA-receptor mediated calcium influx (Nonaka et al., 1998). As calpain activation is tied to calcium influx, it may be reasonable to speculate that lithium down-regulates calpain via this mechanism. We recently reported that lithium suppresses MPTP-induced calpain-1 expression and activity, which is very likely up-stream of MEF2 and tyrosine hydroxylase (TH) in the mouse brain. The efficacy of lithium for PD is, in part, derived from increased dopamine synthesis through TH-upregulation (Lazzara et al., 2015). The calpain-mediated Cdk5 pathway can be a known target for AD pathology as well. For example, the loss of regulation of Cdk5 has also been implicated in the formation of the pathological characteristics and the neurodegeneration associated with AD. An *in vivo* study using mice has revealed that the neurotoxic Cdk5 activator, p25, resulted in increased inflammation, deposition of amyloid and phosphorylated tau, and neuronal death. (Cruz and Tsai, 2004). The use of Cdk5 inhibitory peptide has been shown to reduce the effects of increased activation of Cdk5/p25 in mice, exhibiting decreased neuroinflammation, brain atrophy and cognitive decline (Sundaram et al., 2013). Lithium treatment in cultured cerebellar granule neurons prevents the increase of Cdk5/p35 fragmentation to Cdk5/p25 induced by colchicine (Jordà et al., 2005). In addition, treatment of cultured primary hippocampal neurons and rat striatum with lithium down-regulated calpain activity, Cdk5 activation, and cellular death induced by 3-nitropropionic acid (3-NPA) (Crespo-Biel et al., 2009).

It has been established that GSK3β is a key mediator of tau hyper-phosphorylation, and that lithium treatment inhibits GSK3β and consequently, tau hyperphosphorylation. Plattner et al. has demonstrated a connection between GSK-3β and Cdk5, showing that Cdk5 acts as a modulator of tau hyper-phosphorylation via the inhibitory regulation of GSK-3 (Plattner et al., 2006).

Regulation of Apoptotic Proteins and Glutamate Excitotoxicity

Lithium has also been shown to influence levels of pro-apoptotic proteins. Bax, also known as Bcl-2–associated X protein, is a regulator that promotes apoptosis by binding to and antagonizing the Bcl-2 protein. The tumor suppressor protein, p53, targets both Bcl-2 and Bax and promotes growth arrest and cell death in response to cell damage (Basu and Haldar, 1998). In addition to being a major anti-apoptotic protein, Bcl-2 has been shown to induce regeneration of axons after injury (Huang et al., 2003).

A number of *in vitro* and *in vivo* experiments have demonstrated the neuroprotective effects of lithium attributed to increased Bcl-2 levels. Lithium treatment of cultured cerebellar granule cells stimulated an increase of mRNA and protein levels of Bcl-2; the Bcl-2/Bax protein level ratio increased by 5-fold after treatment for 5–7 days (Chen and Chuang, 1999). Lithium-induced increases in Bcl-2 expression were shown to cause neurogenesis in the hippocampus and entorhinal cortex in adult rodents as evidenced by an increase of axon diameters and improved neurite growth in the CA3 area of the hippocampus and an increase of myelination in the entorhinal cortex (Chen et al., 2000). In the MPTP-induced mouse PD model, a diet which included a high dose of lithium almost completely prevented the depletion of striatal dopamine and tyrosine hydroxylase, and the expected increase in dopamine turnover was prevented. Lithium was credited with providing neuroprotection by stimulating anti-apoptotic activity—increasing Bcl-2 level and reducing Bax (Youdim and Arraf, 2004). Phosphorylation of Bcl-2 at serine 70 is required for its complete anti-apoptotic function (Ruvolo et al., 2001), and Chen et al. have shown that lithium blocks induced apoptosis in mouse T hybridoma cells treated with ceramide and etoposide (2006). In this model, lithium inhibited Bcl-2 dephosphorylation and caspase-2 activation via reduction of protein phosphatase-2A activity (Chen et al., 2006). Changes in the expression of Bcl-2 and other pro-apoptotic genes have also been detected in human subjects taking lithium. The peripheral blood of patients with bipolar disorder was studied to observe changes in the gene expression profiles over time following treatment with lithium. This analysis identified the apoptotic pathway as the most affected by lithium, as after 1 month, those patients who responded positively to lithium treatment showed up-regulation of Bcl-2, while several pro-apoptotic genes, e.g.,: Bcl-2-antagonist/killer 1 (BAK1) and Bcl-2-associated agonist of cell death (BAD), were down-regulated (Lowthert et al., 2012).

Glutamate-induced excitotoxicity has been implicated in various neurodegenerative diseases including Huntington's disease, AD, and ALS, as well as in stroke, trauma and spinal cord injury (Friedlander, 2003; Lau and Tymianski, 2010). A number of studies have also associated glutamate-mediated excitotoxicity in the pathogenesis of PD. PARK2 is the E3 ubiquitin ligase parkin-encoding gene; its mutations cause PD. Mutations to the PARK2 gene can lead to an abnormally small parkin protein that is non-functional and is rapidly degraded. Parkin has also been shown to be involved in the function and stability of glutamatergic synapses. Further, the parkin mutations linked to PD trigger a proliferation of glutamatergic synapses with concomitant susceptibility to excitotoxicity (Helton et al., 2008).

Glutamate excitotoxicity has been shown to be associated with the up-regulation of Bax and p53, both of which are pro-apoptotic proteins, and the down-regulation of Bcl-2 (Chen and Chuang, 1999). The apoptosis attributed to glutamate was shown to be preceded by an increase in activator protein-1 (AP-1) caused by activation of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAP kinase) and phosphorylation of c-Jun (Ser63) and p53 (Ser15) (Chen et al., 2003). In a study using cultured rat cerebellar granule cells, treatment with lithium prevented these signaling events and ameliorated the increase in apoptosis (Chi-Tso and Chuang, 2011).

Other Proposed Pathways

There are a number of additional mechanisms by which lithium has been shown to act intracellularly. These include modification of cyclic adenosine monophosphate (cAMP)-mediated signal transduction (Jope, 1999; Gould et al., 2002; Einat et al., 2003); reduction in the arachidonic acid (AA) cascade (Chang et al., 1996, 2001; Chang and Jones, 1998; Rintala et al., 1999; Rapoport and Bosetti, 2002); negative regulation of the Smad3/4-transcription factor and protein levels of plasminogen activator inhibitor-1 (PAI-1); and induction of neurogenesis (Chen et al., 2000; Hashimoto et al., 2003). In addition, lithium has been shown to induce the survival pathway, MEK/ERK (Liang et al., 2008); increase levels of transcription factor β-catenin (Stambolic et al., 1996; Gould et al., 2004); and regulate autophagy via inositol inhibition and reduction of inositol 1, 4, 5-trisphosphate (IP3) levels (Sarkar et al., 2005; Sarkar and Rubinsztein, 2006, 2008; Fornai et al., 2008a; Klionsky et al., 2012).

LITHIUM EFFECTS IN OTHER NEURODEGENERATIVE DISEASES

Alzheimer's Disease

Abnormal levels of GSK-3 are associated with pathogenesis and neuronal death in individuals with AD (Bhat et al., 2004), and lithium has been shown to inhibit the GSK-3-related toxicity (Stambolic et al., 1996). In 1997, researchers demonstrated that lithium reduces phosphorylated tau *in vitro* and *in vivo* by inhibition of GSK-3 (Hong et al., 1997; Muñoz-Montaño et al., 1997). Lithium also prevented tau hyper-phosphorylation, thereby blocking its neurotoxicity and associated cell death (Alvarez et al., 1999). Further, lithium treatment resulted in a significant reduction of GSK-3 activity with concomitant decreases in the AD-associated tau phosphorylation, insoluble, aggregated tau accumulation, and axonal degeneration (Noble et al., 2005).

Various studies with AD animal models have shown that lithium can also provide beneficial effects. For example, aged double transgenic mice (AβPPSwe/PS1A246E) that display amyloid deposits were treated with lithium and showed attenuated γ-cleavage of amyloid precursor protein (APP) followed by reduction in amyloid-β plaque formation. These

animals also showed improvement in spatial learning and memory abilities in addition to reduced autophagy activation (Zhang et al., 2010).

The promising studies showing GSK-3 inhibition by lithium have prompted many researchers to regard lithium as a potential therapeutic agent for the prevention and treatment of Alzheimer's disease. However, most clinical trials have provided ambiguous or inconsistent results. A case-control study using data from the General Practice Research Database in the UK showed that patients who received lithium treatment had a higher risk of diagnosis of dementia, which was believed to correlate to higher doses of the drug (Dunn et al., 2005). In a placebo-controlled, randomized, single-blind, multicenter study, lithium was given to 71 AD patients over a period of 10 weeks. CSF and plasma biomarkers (total tau, phosphorylated tau, Aβ42 and GSK-3) were monitored during that time period, and there was no significant difference in the biomarkers or in cognitive performance as compared to patients receiving a placebo (Hampel et al., 2009). However, in a 2007 study that compared elderly, bipolar patients (who are at a higher risk for dementia) who had received chronic lithium treatment, with bipolar patients who had not received lithium, it was shown that the lithium-treated patients had lower prevalence of dementia than the untreated group. In fact, the prevalence of the treated group was equivalent to the general, age-comparable population. The non-lithium-treated patients had an incidence of dementia that was six times greater (5% on lithium vs. 33% no lithium; Nunes et al., 2007). Although this study remains to be validated, it suggests lithium as a potential therapeutic for AD.

A recent, more promising, lithium study differed from previous ones in two ways: it was long-term (2 years) and involved patients who had mild cognitive impairment (MCI), not AD. The researchers measured the CSF biomarkers: Aβ42, phosphorylated tau and total tau, and assessed cognitive performance and drug safety. There was a significantly lower concentration of phosphorylated tau in the CSF of lithium-treated patients compared to those receiving a placebo, but there were no differences in the levels of Aβ42 and total tau. The lithium-treated group also had fewer conversions from mild cognitive impairment to AD, but this number was statistically insignificant. Nonetheless, the investigators believe their results show that lithium may slow the disease progression from cognitive impairment to dementia (Forlenza et al., 2011).

Huntington's Disease

In the 1970s, there were several clinical trials of lithium therapy for patients with HD. These trials included only small numbers of patients (9, 6, and 6) for very short time periods (6–12 weeks). In each case, the findings indicated that there were no improvements in involuntary movements, hyperkinesia, motor skills, or in the ability to perform everyday tasks, leading to the conclusion that lithium did not appear to be of therapeutic value in HD (Aminoff and Marshall, 1974; Leonard et al., 1975; Vestergaard et al., 1977). Since the 1970s, there have not been any reported human clinical trials utilizing lithium for treatment of HD; however, there has been a recent off-label use of lithium in a case study involving three individuals. Each of the patients received low doses of lithium and showed no further progression of chorea over periods of 2–4 years (Danivas et al., 2013).

Other studies showed that lithium treatment suppresses striatal lesions, reduces neurodegeneration, and stimulates cell proliferation in an excitotoxic rat model of HD. It has also been shown to reduce poly(Q) toxicity in cell models of HD; significantly improve motor performance (albeit with no improvement in longevity) in an HD mouse model; and protect against poly(Q)-mediated toxicity in a Drosophila model of HD (Wei et al., 2001; Carmichael et al., 2002; Wood and Morton, 2003; Senatorov et al., 2004; Berger et al., 2005). Two different studies looked at the results of combining lithium with a second mood-stabilizing drug to improve the efficacy of the treatment. Sarkar et al. showed that the treatment of an HD fly model with lithium in concert with rapamycin offered synergistic protection against neurodegeneration compared with either agent alone (Sarkar et al., 2008). Furthermore, there was better motor deficit improvement in two different mouse models of HD co-treated with lithium and valproate compared with monotherapy (Chiu et al., 2011).

Amyotrophic Lateral Sclerosis

The protective effects of lithium in ALS were detailed in two papers (Fornai et al., 2008a,b). These include the normalization of the structure of altered mitochondria found in motor neurons as well as the removal of intracellular aggregates from motor neurons via increased autophagy; a stimulatory effect on mitochondrial biogenesis; inhibition of astroglial growth and proliferation; and neuronal differentiation. In a parallel study using human ALS patients and the G93A mouse (an ALS genetic animal model), researchers found that lithium provided significant neuroprotection. In the human trial, which lasted 15 months, the progression of the disease was significantly reduced in the lithium-treated group compared to the control group treated with riluzole for the same time period, while the mutant mice exhibited delayed onset of the disease and a longer life span (Fornai et al., 2008b). In another study with G93A mice in the same year, lithium in concert with valproic acid (VPA) was reported to delay the onset of ALS symptoms, increase life span, and the mice exhibited fewer neurological deficits as compared to mice treated with lithium or VPA alone (Feng et al., 2008). These results led the authors to suggest that lithium offers some promise as a treatment for human patients affected by ALS. In contrast, a subsequent multi-center consortium study reported that lithium treatment was not universally effective in ameliorating its symptoms in human patients (Chiò et al., 2010).

Based on the results from the Fornai et al. studies, a large 13-month phase II screening trial of lithium carbonate in ALS was undertaken in 2011. In contrast to earlier results, the researchers concluded that lithium carbonate does not slow the rate of decline of function in patients with ALS, as compared to a control group, and the lithium-treated patients showed no differences in quality of life and were more prone to adverse events (Miller et al., 2011). Around the same time, another randomized, double-blind, placebo-controlled trial showed similar negative results, and in fact, the trial was terminated prematurely because of futility (Aggarwal et al., 2010). These trials have demonstrated some

safety concerns and the lack of promising therapeutic benefits from lithium treatment in ALS patients.

The inconsistent efficacy of lithium in treating ALS may be due to the fact that ALS is a complex disease with a constellation of cellular and molecular pathways involved in its pathophysiology. There has been a small subset of patients that demonstrated some improvement; however, it may be that lithium does not selectively target the pathways that would provide benefits to a majority of the cases. Another factor to consider is that there may be an optimal dose of lithium, which can be unique to each individual's disease stage, required to alleviate symptoms and prevent the pathology. However, most clinical trials were likely performed without identifying the optimal doses for patients. While some patients may show improvement with a standard dose, that dose in others may not impact on their pathophysiology or may create undesirable side-effects.

CONCLUSIONS

Numerous *in vitro* and *in vivo* studies have shown that lithium provides potential therapeutic value in the prevention and/or treatment of neurodegenerative conditions. Multiple different biological mechanisms have been shown to contribute to these protective effects including the up-regulation of neuroprotective proteins including Bcl-2 and its actions on regulation of apoptosis and cellular resilience, such as GSK-3. Further clinical and experimental studies with lithium are needed to determine if the cellular and molecular biological properties of the drug can be a part of a therapeutic strategy for Parkinson's and other neurodegenerative diseases. Lithium-only treatment may not be a suitable therapeutic for neurodegenerative diseases due to inconsistent efficacy and potential side-effects, however, the use of low dose of lithium in combination with other potential or existing therapeutic compounds may be a promising prophylactic approach to reduce symptoms and disease progression in neurodegenerative diseases.

AUTHOR CONTRIBUTIONS

CL wrote the article. YK provided critical revisions and final approval and had overall responsibility for the article.

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