MOLECULAR DIAGNOSTICS IN THE DETECTION OF NEURODEGENERATIVE DISORDERS

EDITED BY: Megha Agrawal and William C. Cho

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MOLECULAR DIAGNOSTICS IN THE DETECTION OF NEURODEGENERATIVE DISORDERS

Topic Editors:
Megha Agrawal, University of Illinois at Chicago, USA
William C. Cho, Queen Elizabeth Hospital, Hong Kong

Molecular Diagnostics in the Detection of Neurodegenerative Disorders.
Image by Megha Agrawal

Neurodegeneration is characterized by the progressive loss of neural tissue that result in various neurodegeneration-initiated cerebral failures and complex diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease. All these medical conditions are accompanied by the disruption of blood-brain barrier (BBB). The BBB is an interface, separating the brain from the circulatory system and protecting the central nervous system from potentially harmful chemicals

while regulating transport of essential molecules and maintaining a stable environment. Owing to the inability of the neurons to regenerate on their own after neurodegeneration or severe damage to the neural tissue, neurodegenerative disorders do not have natural cures on their own. Neuroregeneration is a viable way to curb neurodegeneration. One of the current approaches is stem cell-based therapy that has been shown to be potentially helpful for the application of neuronal cell replacement for neuroregeneration.

It is vital that the neurodegenerative disorder being detected at an early stage as it can provide a chance for treatment that may be helpful to prevent further progression of the fatal disease. Thus, research has focused on developing effective non-invasive diagnostic methods for early detection of these disorders. Molecular diagnostics can provide a powerful method to detect and diagnose various neurological disorders. Such diagnosis can enhance early detection, provide subsequent medical counsel based on medical pathway, as well as to gain better insight of neurogenesis and hopefully eventual cure of the neurodegenerative diseases.

With research reports, reviews, mini-reviews and commentary, this research topic covers a wide range of areas in neurodegeneration research, including diagnosis and prognosis; regulating central nervous system; biomarkers and brain injury induced neurobehavioral outcomes among other timely reports on neurodegeneration.

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EDITORIAL
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Editorial: Molecular Diagnostics in the Detection of Neurodegenerative Disorders

Megha Agrawal[1]* *and William C. Cho*[2]

[1] Department of Bioengineering, University of Illinois at Chicago, Chicago, IL, USA, [2] Department of Clinical Oncology, Queen Elizabeth Hospital, Kowloon, Hong Kong

Keywords: Alzheimer's disease, biomarkers, neurons, Parkinson's disease, stem cells

Editorial on the Research Topic

Molecular Diagnostics in the Detection of Neurodegenerative Disorders

Neurodegenerative disorders encompass a range of medical conditions which primarily affect the neurons in the human brains. Such conditions result in the disorders of the central nervous system which eventually lead to the progressive loss of neural tissues including death of neurons (Agrawal and Biswas, 2015). An impressive volume of research has been conducted over the last few decades to advance our understanding of this fatal disease. The challenge to treat neurodegenerative disorders lies in the inability of the neurons to regenerate on their own once they start functioning abnormally after the neural deterioration or once a severe damage occurs to a neural tissue. However, stem cell therapy has been proven to be potentially useful in neuroregeneration or even neuronal cell replacement (Chung et al., 2002; Rachakonda et al., 2004). One of the most important missions of diagnosis and prognosis of neurodegeneration is the ability of early detection of the onset of neurodegeneration. An early diagnosis of the disease is critical as it provides a chance for an early treatment that may be helpful to prevent further progression of the deadly neurodegeneration and its aftermaths that takes millions of lives every year globally (Miller and O'Callaghan, 2015).

With an aim to provide a discussion platform to neurologists, neuroscientists and pathologists for sharing the latest findings and knowledge on neurodegeneration and the molecular diagnostics to detect and combat neurodegeneration, we have launched this special research topic on molecular diagnostics in the detection of neurodegenerative disorders. We anticipate that molecular diagnostics will play an imperative role in near future for providing an effective diagnostic solution to the complex problem of neurodegenerative diseases. Based on the available research data, we firmly believe that molecular diagnostics can be effective to detect and diagnose various neurological diseases such as Amyotrophic lateral sclerosis, Huntington's, Alzheimer's, and Parkinson's disease, at an early stage (Gasser et al., 2001a,b, 2003; Agrawal and Biswas, 2015). Molecular diagnostics in neurodegenerative disorder is an emergent area of study, research and development. It represents a multidisciplinary research field that offers plenty of opportunities for collaboration between neurologists, psychologists, biologist and biomaterials scientists and other trained personnel with the necessary experience in managing the diseases. We expect that further developments in various molecular diagnostics will pave the way for the early detection of neurodegeneration and effective treatment.

This e-book showcases important and significant reports that cover a wide range of areas in neurodegeneration research and treatment. These include diagnosis and prognosis; role of neuroactive drugs in regulating central nervous system; advances in novel biomarkers; brain injury induced neurobehavioral outcomes and also connectivity between spinal muscular atrophy and loss of α-motors neurons among other reports. One of the reports investigates the origin and

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Edited by:
Sanjeev Kumar Srivastava, Mitchell Cancer Institute, USA

Reviewed by:
Sanjeev Kumar Srivastava, Mitchell Cancer Institute, USA
Rajeev Kumar, Affiliated Hospital of Guizhou Medical University, China

***Correspondence:**
Megha Agrawal
meghaagra@gmail.com; agrawalm@uic.edu

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potential function of corpora amylacea (CA) which are found in large numbers in the central nervous system of the patients with neurodegenerative diseases. Immunohistochemistry analyses were employed to reveal fungal proteins present in CA from patients diagnosed with Alzheimer's disease (Pisa et al.). An insight into the prospective roles of haptoglobin (Hp) (an endogenous hemoglobin-binding protein) in traumatic brain injury and other acute brain injuries is discussed in another report which portrays to be helpful in understanding the inconsistency in outcomes of clinical studies regarding the importance of Hp phenotypes in such brain injuries (Glushakov et al.). This study is a step forward to develop and progress with new therapeutics in the prevention of cerebral hemorrhage which is a common feature of traumatic brain injury and its associated chronic disabilities (Glushakov et al.). Furthermore, important and significant biomarkers for neurodegeneration have been investigated and studied for their sensitivity and specificity in this e-book that sheds new lights in treatment of irreversible cognitive deficit and dementia in elderly population. Biomarkers represent important molecular diagnostic tools and thus the development of novel biomarkers could bring significant breakthroughs in an early diagnosis of neurodegeneration (Sfera et al.).

In a particular review, the application of stem cells and induced pluripotent stem cells in combating neurodegeneration is discussed that addresses the issues of diagnosis, modeling, and therapeutic transplantation strategies (Singh et al.). In another review, adropin is discussed as a biomarker for the diagnosis of central nervous system disorders and is considered as a potential therapeutic candidate in central nervous system injuries (Shahjouei et al.). It is believed that hyper excitability in neuronal network possibly contributes to the cognitive deficits in Alzheimer's disease and this is addressed in a research report that sheds light on the mechanism of aberrant neuronal networks in Alzheimer's disease (Wang et al.). To this end, researchers conducted investigations on the excitability in cultured pyramidal neurons from APP/PS1 mice using patch clamp recording techniques. This provides an important insight into the pathogenesis of Alzheimer's disease that could be helpful in developing new therapeutic avenues in the future (Wang et al.). This e-book covers another area of neurodegeneration: spinal muscular atrophy, which is an early-onset, autosomal recessive neurodegenerative disease that is characterized by the loss of spinal α-motor neurons leading to infant deaths (Butchbach). The author highlights why a better understanding of the underlying mechanism causing spinal muscular atrophy and the accurate measurements thereof are crucial to control such neurodegenerative disease in infants (Butchbach). The molecular mechanisms of C9orf72 gene causing amyotrophic lateral sclerosis, frontotemporal dementias and atypical parkinsonian syndromes are also discussed (Munteanu and Lynch).

In concluding our thoughts and deliberations, we do hope that this discussion forum in the form of an edited e-book will advance our further understanding to have an enhanced insight of neurogenesis and eventual cure for neurodegenerative diseases. Future research directions might involve multifunctional biomolecular diagnostic markers and technology platforms that would significantly enhance and augment the accuracy, specificity and sensitivity that would drive an early diagnosis and prognosis of various neurodegenerative disorders.

AUTHOR CONTRIBUTIONS

Both authors have made substantial intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Age-Dependent Effects of Haptoglobin Deletion in Neurobehavioral and Anatomical Outcomes Following Traumatic Brain Injury

Alexander V. Glushakov[1]*, *Rodrigo A. Arias*[1], *Emanuela Tolosano*[2] and *Sylvain Doré*[1,3]*

[1] Department of Anesthesiology, Center for Translational Research in Neurodegenerative Disease, University of Florida College of Medicine, Gainesville, FL, USA, [2] Departments of Molecular Biotechnology and Health Sciences, University of Torino, Torino, Italy, [3] Departments of Anesthesiology, Neurology, Psychiatry, Psychology, Pharmaceutics and Neuroscience, University of Florida College of Medicine, Gainesville, FL, USA

Cerebral hemorrhages are common features of traumatic brain injury (TBI) and their presence is associated with chronic disabilities. Recent clinical and experimental evidence suggests that haptoglobin (Hp), an endogenous hemoglobin-binding protein most abundant in blood plasma, is involved in the intrinsic molecular defensive mechanism, though its role in TBI is poorly understood. The aim of this study was to investigate the effects of Hp deletion on the anatomical and behavioral outcomes in the controlled cortical impact model using wildtype (WT) C57BL/6 mice and genetically modified mice lacking the Hp gene ($Hp^{-/-}$) in two age cohorts [2–4 mo-old (young adult) and 7–8 mo-old (older adult)]. The data obtained suggest age-dependent significant effects on behavioral and anatomical TBI outcomes and recovery from injury. Moreover, in the adult cohort, neurological deficits in $Hp^{-/-}$ mice at 24 h were significantly improved compared to WT, whereas there were no significant differences in brain pathology between these genotypes. In contrast, in the older adult cohort, $Hp^{-/-}$ mice had significantly larger lesion volumes compared to WT, but neurological deficits were not significantly different. Immunohistochemistry for ionized calcium-binding adapter molecule 1 (Iba1) and glial fibrillary acidic protein (GFAP) revealed significant differences in microglial and astrocytic reactivity between $Hp^{-/-}$ and WT in selected brain regions of the adult but not the older adult-aged cohort. In conclusion, the data obtained in the study provide clarification on the age-dependent aspects of the intrinsic defensive mechanisms involving Hp that might be involved in complex pathways differentially affecting acute brain trauma outcomes.

Keywords: controlled cortical impact, Iba1, GFAP, gliosis, hemoglobin, hemorrhage, trauma

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Edited by:
William Cho,
Queen Elizabeth Hospital, China

Reviewed by:
Pedro José Carlos Rondot Radío,
University of Buenos Aires, Argentina
Anna Elizabeth King,
University of Tasmania, Australia
Vinita Ganesh Chittoor,
Oregon Health & Science University, USA

***Correspondence:**
Alexander V. Glushakov
glushakov@bellsouth.net
Sylvain Doré
sdore@ufl.edu

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INTRODUCTION

Traumatic brain injury (TBI) is one of the major causes of morbidity and mortality in the United States and worldwide with no current effective treatment. It is widely recognized that chronic neurological and psychological disabilities following TBI largely result from potentially preventable or treatable secondary pathophysiological cascades, so-called

secondary injuries, initiated by the acute brain trauma. However, despite extensive research and promising results obtained in preclinical studies, numerous clinical trials failed primarily due to the heterogeneity and complexity of the TBI and mechanisms underlying acute vs. chronic anatomical pathologies and neurological deficits. Secondary injuries resulting from acute TBI, such as mechanical brain damage and intracranial hemorrhages, are triggered by complex coexisting pathways involving excitotoxicity, oxidative damage, and neuroinflammatory cascades (McIntosh et al., 1996; Diaz-Arrastia et al., 2000; Saatman et al., 2008). On the other hand, the presence of acute subarachnoid or, to a lesser degree, parenchymal hemorrhages following TBI is highly associated with brain-injury severity and chronic cognitive and physical disabilities; and it is believed that the mechanisms are mainly initiated by the toxicity of hemoglobin and its heme-containing breakdown products released into the brain tissues from erythrocytes during hemolysis of cerebral hematomas (Xi et al., 1998; Bhasin et al., 2002), causing oxidative damage to macromolecules such as lipids, proteins, and nucleic acids (Nakamura et al., 2005, 2006), as well as activation caspase proteolysis (Regan and Panter, 1993; Wang et al., 2002), resulting in disruption of the blood-brain barrier (Xi et al., 1998; Bhasin et al., 2002; Keep et al., 2008) and neuronal death (Koeppen et al., 1995; Aronowski and Hall, 2005; Xi et al., 2006).

One of the critical mechanisms involved in deactivation of cell-free hemoglobin in the mammalian body during the hemorrhagic event is formation of highly stable complexes of hemoglobin with haptoglobin (Hp)—an endogenous hemoglobin-binding protein present in blood plasma and almost absent within the brain itself (Wada et al., 1970; Philippidis et al., 2004; Schaer et al., 2006)—and subsequent clearance of the Hp-hemoglobin complexes primarily by tissue macrophages and circulating monocytes, and likely by other cell types such as astrocytes and microglial cells that are mediated via CD163 (Liu and Sturner, 1988; D'Armiento et al., 1997; Ascenzi et al., 2005; Schaer et al., 2005, 2007; Zhang et al., 2012). However, at this point, the contribution of Hp to acute brain injury is still not fully understood (D'Armiento et al., 1997).

The changes in Hp expression have been shown in various disorders and diseases associated with inflammation (Carter and Worwood, 2007). It is well recognized that under physiological conditions the plasma pool of Hp is relatively high and its levels further increase up to 10-fold in different types of injuries associated with hemorrhages as a part of the acute phase-2 response (Petersen et al., 2004). Early studies have shown that serum Hp concentrations are increased in patients with severe head injuries and that the serum Hp level could potentially be a predictive biomarker of the hemorrhagic brain-lesion severity (Auer and Petek, 1978). In both physiological and pathophysiological conditions, Hp is synthesized mainly by hepatocytes and then released to the peripheral circulation (Bowman and Kurosky, 1982; Hoj et al., 1984; Yang et al., 2013). However, some studies suggest that Hp may have very limited expression within brain-cell types. The Hp mRNAs were found in the human retinal pigment epithelial cell line and post-mortem neural retina (Chen et al., 1998). Expression of Hp has also been demonstrated in human glioblastoma tissue but not in normal brain samples (Kumar et al., 2010). Few groups have documented that Hp is present within the brain of rodents following brain injuries (Lee et al., 2002; Zhao et al., 2009, 2011). Mouse studies from Dr. J. Aronowski and colleagues suggested that Hp is expressed and secreted by brain oligodendrocytes after experimental intracerebral hemorrhage and that brain-derived Hp plays a significant role in the protection of brain cells after injury (Zhao et al., 2009, 2011) in addition, a rat study by Dr. Kim and colleagues suggested Hp expression in the hippocampus following brain ischemia (Lee et al., 2002). Increased Hp immunoreactivity and upregulation of Hp mRNA in reactive astrocytes have been shown in an experimental ischemia model, suggesting *de novo* Hp synthesis in the brain (Lee et al., 2002). On the other hand, a human study of subarachnoid hemorrhage suggested an influx of Hp from peripheral circulation into cerebrospinal fluid, and that the intrathecal Hp-scavenging system could have limited capacity (Galea et al., 2012). Previously, Hp levels in cerebrospinal fluid have been proposed as a biomarker of blood-brain barrier disruption (Chamoun et al., 2001). It has also been demonstrated that, following TBI, the increase in brain Hp levels occurs due to extravasation of Hp, as well as other types of plasma proteins, into brain parenchyma resulting from the blood-brain barrier breakdown associated with intracranial hemorrhage and subsequent uptake of the plasma proteins by reactive astrocytes (Liu and Sturner, 1988).

Current interest is driven by recent studies suggesting that Hp phenotypes, primarily associated with different affinities to bind free hemoglobin and affinity of the hemoglobin-Hp complex to its receptors, may be associated with differential outcomes in subarachnoid hemorrhages (Chaichana et al., 2007, 2010; Leclerc et al., 2015) and that Hp may play an important role in the development of secondary injuries, particularly delayed arterial vasospasm and brain ischemia (Nonaka et al., 1979; Borsody et al., 2006). Of interest, post-traumatic cerebral vasospasm is a common complication of TBI, with incidences ranging from 2 to 63% mainly depending on the severity of injury and the method of diagnostics (Macpherson and Graham, 1978; Taneda et al., 1996; Mattioli et al., 2003). Although there is a strong association of cerebral vasospasm with traumatic subarachnoid hemorrhage (Macpherson and Graham, 1978; Gomez et al., 1991; Steiger et al., 1994; Kordestani et al., 1997; Aminmansour et al., 2009), it is also common in patients with subdural hematomas, intraventricular hemorrhage, and contusions (Mattioli et al., 2003; Oertel et al., 2005; Kalanuria et al., 2013). In general, subarachnoid hemorrhages are associated with extremely high rates of mortality of about 45–50%, significant morbidity exists among survivors (van Gijn et al., 2007; Bederson et al., 2009) and chronic disabilities are common (Kantor et al., 2014). It should be noted that subarachnoid hemorrhage associated with TBI accounts for about half of all cases, and subsequent cerebral vasospasm following both aneurysmal and traumatic subarachnoid hemorrhages is among the leading causes of morbidity and mortality with no proven effective treatment (Suarez et al., 2006; Amyot et al., 2015). Thus, identifying the mechanisms underlying TBI pathologies and intrinsic,

potentially protective responses involving acute reactants, such as Hp (Vejda et al., 2002; Campbell et al., 2005), will provide new insights into the development of novel strategies for TBI treatment.

Experimental and clinical data suggest that Hp phenotypes are differentially associated with occurrence of cerebral vasospasm, a common complication of subarachnoid hemorrhage, and that the patients with increased risk might be identified based on their Hp genotype (Chaichana et al., 2007, 2010; Leclerc et al., 2015). The human and mouse Hp cDNAs share homology of >80%, and although mice are homomorphic for the Hp genotype expressing only a "high" affinity Hp1–1 phenotype, Hp phenotypes were associated with differential neuropsychological outcomes after TBI; however, in this case, the Hp 1–1 phenotype, which is characterized by the highest affinity to hemoglobin, was associated with worse outcomes (Lee et al., 2002; Anderson et al., 2009).

Although Hp is implicated in the pathophysiology of different brain injuries, the published data suggest that its roles are complex, that upregulation of Hp might have either or both beneficial and detrimental effects, and that the outcomes might be predisposed by certain Hp genotypes. Thus, taking into account the heterogeneity of Hp phenotypes in humans, the goal of this preclinical study was to investigate the role of the "high" efficacy Hp phenotype in a controlled cortical impact (CCI) model of TBI using wild type (WT) C57BL/6 mice and genetically modified mice of the same background lacking the Hp gene ($Hp^{-/-}$) and comparing anatomical and gliosis outcomes in two different adult-age cohorts.

MATERIALS AND METHODS

Experimental Animals

Two matched age cohorts [i.e., 2–4 mo-old (adult) and 7–8 mo-old (older adult)] of WT and $Hp^{-/-}$ C57BL/6 male mice were used in the study. $Hp^{-/-}$ mice were maintained in the in-house facility from the breeding stock provided by Dr. E. Tolosano. The number of WT animals per group used for assessment of anatomical and immunohistochemical analyses were as follows: sham $n = 5$ and $n = 5$, and CCI $n = 8$ and $n = 10$ in the adult (2–4 mo-old) and older adult (7–8 mo-old) age cohorts, respectively. The number of $Hp^{-/-}$ mice per CCI group used for assessment of anatomical and immunohistochemical outcomes per group for analyses were $n = 9$ and $n = 4$ in the adult and older adult age cohorts, respectively. In addition, small groups of sham $Hp^{-/-}$ mice of both ages were used to confirm negligible effects of craniotomy. For assessment of the behavioral outcomes, the mice numbers were slightly inflated due to intrinsic mouse variability of the behavioral outcome measures and confirmation of behavioral test results. The numbers of animals used in the analyses are indicated in the figure legends. The experiments and procedures were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All procedures used in this study were approved by The University of Florida Institutional Animal Care and Use Committee. All surgery was performed under anesthesia, and all efforts were made to minimize the pain and distress of the experimental animals.

CCI Procedures

In this study, we used the same CCI or sham procedures as previously described (Glushakov et al., 2013). Briefly, mice were anesthetized with 4% isoflurane and maintained with 2% isoflurane during all surgery procedures. Mice were placed in the stereotaxic apparatus and the experimental contusive TBI of mild-to-moderate severity was induced using a conventional CCI model (PCI3000 PinPoint Precision Cortical Impactor, Hatteras Instruments, Cary, NC, USA) with an impact tip diameter of 3 mm, velocity of 3 m/s, and compression distance and time of 1 mm and 100 ms, respectively (Yu et al., 2009). The experimental injury in all cases was induced in the right hemisphere. Sham-injured mice underwent only the anesthesia and craniotomy surgeries. After closing the incision, the mice were removed from the stereotaxic apparatus, received an intraperitoneal injection of warm saline to prevent dehydration, and were placed into a temperature-controlled recovery chamber for at least 1 h before being transferred to the animal housing facility.

Neurobehavioral Deficits

Neurobehavioral deficits were assessed 24 and 48 h after CCI or sham procedures using a 24-point Neurological Deficit Score (NDS) scale as described in detail elsewhere (Glushakov et al., 2013). Briefly, the assessment comprised six tests, including body symmetry, gait, circling behavior, climbing on the incline plane, and tail suspension tests to access compulsory circling and front limb symmetry. Each of these individual tests was scored between 0 and 4 points for normal performance (score 0) and according to criteria of gradually increased severity from score 1 to 4; the NDS was calculated as a sum score obtained from the assessment. Prior to behavioral testing, the animals were allowed to acclimate in the testing room for about 30 min. For quantitative assessment of stereotypic activity and circling behavior, the neurobehavioral assessment was based on the moving pattern criteria used in the individual circling behavior test performed on the open bench top, which included a part of the NDS test. The circling behavior test preceded all other behavioral tests. The mouse was placed on the elevated open rectangular plane surface and allowed to move freely for at least for 2–5 min depending on the animal's moving activity. The activity was videotaped from above and analyzed off-line by a blinded examiner. The number of left and right turns was counted for at least for 2 min or for a longer time period to obtain a total number of turns of at least about 20. Preferential turns to one side are indicative of stereotypic movement or circling behavior. The activity values were calculated as a total number of left and right turns per minute, and the circling behavior values were calculated as a percentage of right turns.

Brain Histopathology and Immunohistochemistry

All mice used in the study were euthanized at 48 h and processed for quantitative stereological brain pathology using

cresyl violet staining and immunohistochemistry as described before (Glushakov et al., 2014). All slides (eight 30-μm-thick brain sections per slide cut from the same animal and spaced about 500 μm apart) were scanned using ScanScope (Aperio Technologies, Vista, CA, USA) and analyzed using ImageScope software (Aperio) in a blinded manner. The volumes of cortical lesions and tissue loss were estimated within 2-mm-thick brain segments positioned between the bregma and 2 mm posterior to the bregma (i.e., bregma coordinates from 0 to −2 mm). Cortical lesions were defined as histological alterations in the ipsilateral cortical structures within the site of impact and proximate areas characterized by abnormal cellular morphology and irregular cell density compared to the histological characteristic of their corresponding contralateral counterparts, and, in some cases, the presence of diffuse parenchymal hemorrhages and small hematomas, whereas cavitation was defined as complete loss of cortical tissue or the presence of enlarged hematomas without evident existence of cresyl violet staining. The volume of cortical injury was defined as a combined volume comprising the volumes of cortical lesions and cavitation. Hippocampal edema was assessed by measuring the volumes of ipsilateral and contralateral hippocampi as described before (Glushakov et al., 2013).

Reactive astrogliosis and microglial activation were assessed using immunohistochemistry for glial fibrillary acidic protein (anti-GFAP, 1:1000 DAKO, Carpinteria, CA, USA) and for ionized calcium-binding adapter molecule 1 (anti-Iba1, 1;100, Wako Bioproducts, Richmond, VA, USA), respectively; vector kits were then used for DAB antigen visualization (Vector Laboratories, Burlingame, CA, USA). Immunoreactivity was calculated as a sum of positive and strong positive pixels in rectangular selections (500 × 500 μm) in selected brain regions with characteristic microglial and astrocytes morphological changes. The brain regions were identified using Allen Mouse Brain Atlas. To compare the astroglial and microglial reactivity distribution in the anteroposterior directions from the midline of cortical impact (between −1 and −2 mm from the bregma) coordinates, the assessment was performed in about 1-mm-thick brain segments starting from the bregma posteriorly (from 0 to −4 bregma coordinates). To calculate relative immunoreactivity, the images of two to three sections for each segment from each brain slide (i.e., up to eight sections per animal) were analyzed separately and the mean data for each section in each animal were included in group analysis.

Statistical Analyses

The power analysis to determine approximate minimal group size was performed based on the assumption of obtaining statistical significance of anticipated differences of means equal to at least 1.5 standard deviation with a significance level of $\alpha = 0.05$ and a power of $1-ß = 0.80$. To compare the differences between anatomical and immunohistochemical outcomes and locomotor activity in mice groups with different surgical procedure (i.e., sham and CCI), genotype (i.e., WT and $HP^{-/-}$), and age (i.e., young adult and older adult), the data were analyzed using multi-factor analysis of variance (ANOVA) performed by mixed model regression data analysis and Turkeys *post-hoc* comparison test between matched groups. To compare the differences between non-parametric NDS data, a series of ANOVA on ranks and Dunn's *post-hoc* test were been performed, taking into consideration multiple aforementioned factors (i.e., surgical procedure, age, and genotype) and repeated measurements. All parametric data are presented as the mean ± standard error, and non-parametric data as median, range, and interquartile range (IQR). *P*-values less than 0.05 were considered significant (Macleod et al., 2009). Statistical analyses were performed using JMP (SAS Institute Inc., Cary, NC, USA) and GraphPad (GraphPad Software Inc., La Jolla, CA, USA) software.

RESULTS

Effects of the Hp Knockout on Neurobehavioral Outcomes Following CCI in Different Age Cohorts

Neurological deficits after experimental TBI were assessed 24 and 48 h after CCI using the NDS and the differences between multiple group factors, including surgery type and age with repeated outcome measures, which were assessed using a series non-parametric multi-factor ANOVA on ranks and *post-hoc* Dunn's rank sum test. In adult mice (2–4 mo-old), the median NDS values in the CCI group at 24 and 48 h were 5 at both time points (IQRs: 4–7 and 3–8, respectively), and these values were significantly different ($P < 0.0001$ and $P = 0.0001$) from those observed in the mice from the sham group with median values of 0 at both time points (IQRs: 0–2 and 0–1, respectively; **Figure 1A**). In older adult mice (7–8 mo-old), the median NDS values at 24 and 48 h were 7 (IQR: 5–8) and 8 (IQR: 5–10), respectively, although the NDS values in the CCI group were not significantly different from those observed in the sham group (**Figure 1B**). Although the median values were similar at both time points (7 and 8), the NDS 48 h after CCI was characterized by increased variability (IQRs: 2–5 and 0–10 24 and 48 h after surgery, respectively). In this age cohort, the same animals 48 h after surgery showed worse NDS in the CCI and sham groups, resulting in a wider NDS value range and a lack of statistical significance. As compared to the 48 h NDS scores, the median values were similar to those at the 24 h time point. In addition, further analyses of time dependency based on 24 and 48 h comparisons revealed no significant differences between NDS values at these time points in the sham or CCI group of the adult or older adult mice cohort (**Figure 1C**). The NDS values for the sham and CCI groups were higher compared to their counterpart groups of 2–4 mo-old mice. These values were significantly different ($P < 0.05$) only at the 48 h time point. No significant differences were observed between the sham groups of adult and older adult animals at any time point.

Based on the anatomical brain pathology of marked lesions and tissue loss in the ipsilateral motor cortex observed in the CCI model, we performed an analysis of locomotor activity pattern by counting the number of left and right turns of free-moving mice to quantitatively assess the circling behavior associated with

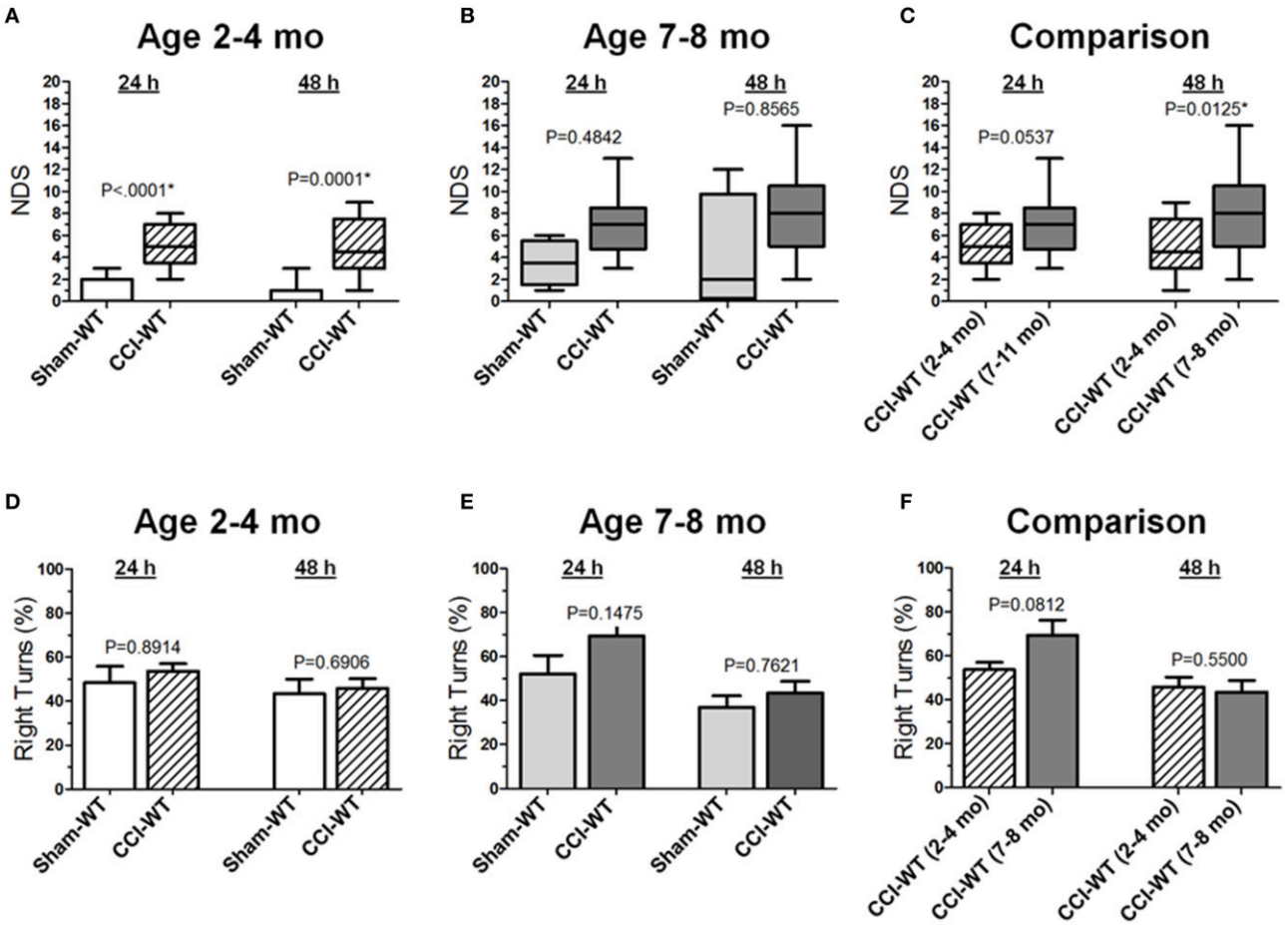

FIGURE 1 | Age-dependent effects of the experimental TBI on neurobehavioral outcomes. (A-C) Comparison of NDS assessed at 24 and 48 h after the experimental injury between sham- and CCI-injured WT mice in adult and older adult age cohorts **(A,B)**, and between CCI-injured WT mice of different ages **(C)**. Statistical comparison of combined data presented in the **(A-C)** using a series of non-parametric multi-factor ANOVA with repeated measurement structure. The numbers shown on the graphs represent *P*-values from the *post-hoc* statistical analyses performed using non-parametric Dunn's rank sum test. **(D-F)** Comparison of the circling behavior presented as the fraction of the right turns between sham- and CCI-injured WT mice in adult and older adult age cohorts **(D,E)**, and between CCI-injured WT mice of different ages **(F)**, respectively. Statistical comparison of combined data using mixed model multi-factor ANOVA with repeated measurement structure. *P*-values shown on the graphs between two groups used for pairwise comparison were obtained from the statistical analyses using the *post-hoc* Student's *t*-test. In all panels, the numbers of animals per sham and CCI experimental groups of WT mice used for analyses were as follows: $n = 6$ and $n = 18$ in the adult mice age cohort (2–4 mo-old), and $n = 5$ and $n = 13$ in the older adult mice age cohort (7–8 mo-old), respectively.

unilateral impairment of this brain region (**Figures 1D,E**). The analyses of stereotypic movement behavior and activity in the group of mice used for NDS assessment using mixed model multi-factor ANOVA revealed a significant temporal effect ($P = 0.0009$), although the *post-hoc* comparison between matching groups using Student's *t*-test revealed no significant differences between groups. There were no significant differences between proportions of left and right turns between the sham and CCI groups 48 h after experimental injury or in each group between the 24 and 48 h time points in both age cohorts. Similarly, in both age cohorts, there were no significant differences between locomotor activity calculated as the number of left and right turns or total number of left and right turns combined between the sham and CCI groups or in each group between 24 and 48 h time points (data not shown). To compare the differences between experimental groups, the values of moving symmetry were expressed as a percentage fraction of right turns. Mixed model multi-factor ANOVA revealed a significant temporal effect ($P = 0.0009$). However, *post-hoc* comparison between matching groups also revealed no statistically significant differences between outcomes in the CCI groups from adult and older adult cohorts at both 24 and 48 h time points (**Figure 1F**).

Figures 2A-C demonstrate the comparison of NDS between WT and $Hp^{-/-}$ CCI-injured mice. A series of non-parametric multi-factor ANOVA and *post-hoc* comparison between matching groups using Dunn's *t*-test revealed significant differences in NDS of the $Hp^{-/-}$ mice from the CCI group in the adult age cohort ($P = 0.0386$), whereas 48 h after injury, the differences between WT and $Hp^{-/-}$ were no longer

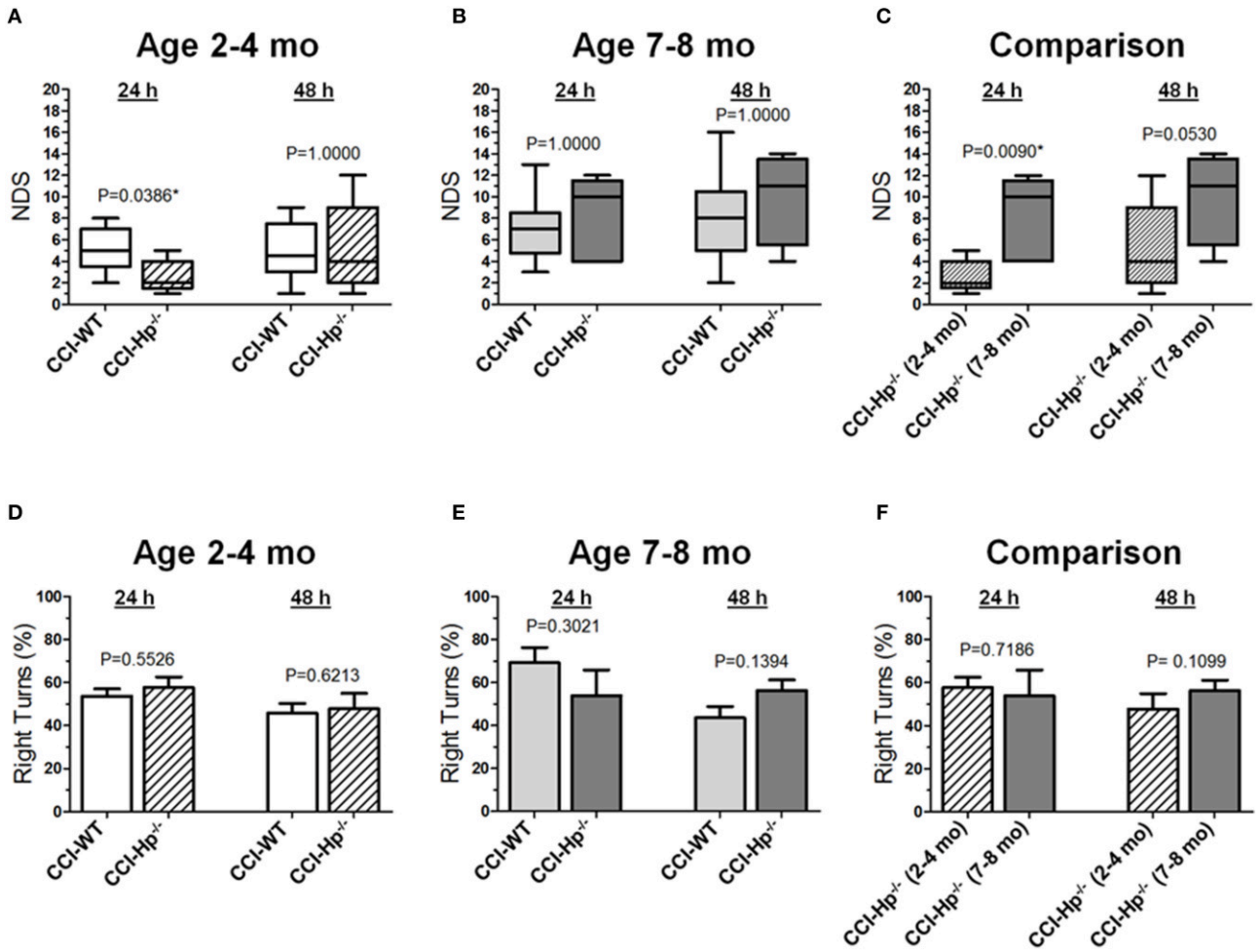

FIGURE 2 | Effects of the Hp knockout on neurobehavioral outcomes following CCI in different age cohorts. (A-C) Comparison of NDS assessed at 24 and 48 h after the experimental injury between CCI-injured WT and $HP^{-/-}$ mice in adult and older adult age cohorts **(A,B)**, and between CCI-injured $HP^{-/-}$ mice of different ages **(C)** Statistical comparison of combined data presented in the **(A-C)** using a series of non-parametric multi-factor ANOVA with repeated measurement structure. The numbers shown on the graphs represent *P*-values from the *post-hoc* statistical analyses performed using non-parametric Dunn's rank sum test. **(D-F)** Comparison of the circling behavior presented as the fraction of the right turns between CCI-injured WT and $Hp^{-/-}$ mice in adult and older adult age cohorts **(D,E)**, and between CCI-injured WT mice of different ages **(F)**, respectively. Statistical comparison of combined data using mixed model multi-factor ANOVA with repeated measurement structure. *P*-values shown on the graphs between two groups used for pairwise comparison were obtained from the statistical analyses using the *post-hoc* Student's *t*-test. In all panels, the numbers of animals per CCI experimental groups of WT and $Hp^{-/-}$ mice used for analyses were as follows: $n = 6$ and $n = 18$ in the adult mice age cohort (2–4 mo-old), and $n = 16$ and $n = 13$ in the adult mice age cohort (2–4 mo-old), and $n = 13$ and $n = 5$ in the older adult mice age cohort (7–8 mo-old), respectively.

significant, suggesting possible neurological deterioration. Significantly lower values in the $Hp^{-/-}$ group in the adult mice cohort suggest an improved neurological outcome 24 h after experimental injury, whereas no significant differences in the NDS were observed between WT and $HP^{-/-}$ CCI-injured mice in the older adult cohort at the 24 and 48 h time points (**Figure 2B**). There was a significant difference between the NDS in the CCI-injured mice from the adult and older adult cohorts 24 h after injury ($P = 0.0094$), whereas at 48 h, the increase in the NDS was also no longer significant (**Figure 2C**). In addition, to determine possible effects of surgery alone on the neurobehavioral outcomes in $Hp^{-/-}$ mice at the two ages, the NDS test was performed in small groups of animals that underwent sham surgery ($n = 4$ in adult and $n = 2$ in older adult cohorts). The data revealed no substantial increases in NDS scores in sham $Hp^{-/-}$ mice, which ranged from 0 to 1 in adult and from 0 to 4 in the older adult cohort at 24 and 48 h time points (data not shown). No circling behavior was detected in any $Hp^{-/-}$ group after CCI, and no significant differences were observed between WT and $Hp^{-/-}$ mice in both age cohorts (**Figures 2D,E**) or between different age cohorts of $Hp^{-/-}$ mice (**Figure 2F**), although a significant temporal effect ($p = 0.0009$) on the moving symmetry was revealed by mixed model multi-factor ANOVA.

Effects of the Hp Knockout on Anatomical Outcomes Following CCI in the Different Age Cohorts

In the first set of experiments, we performed tests to determine differences in anatomical deficits after CCI in 2–4 (adult) and 7–8-mo-old (older adult) WT mice; subsequent comparisons were then made between WT and $Hp^{-/-}$ mice in each age cohort. Macroscopically, the overall brain pathology from CCI with mild-to-moderate TBI parameters 48 h post-injury was characterized by neuronal death, loss of cortical tissue, and, to a lesser degree, partial loss of hippocampal tissue or hippocampal distortion with altered structural tissue integrity as compared to the contralateral hippocampus or hippocampi of animals from the sham-injury group. These anatomical brain changes are consistent with our previous published data using the same CCI parameters (Glushakov et al., 2013, 2014, 2015).

Figures 3A,B demonstrate representative microphotographs of brain sections obtained from WT sham and CCI-injured animals in the two age cohorts. At the 48 h time point in the adult and older adult age cohorts, CCI consistently produced significant anatomical pathologies in the cortex assessed by cresyl violet histo-stereological analysis, including cortical lesions and complete loss of brain tissue. Cortical lesions were characterized by morphological alternations, including characteristic changes in cellular morphology and cell density. These changes primarily reflect neurodegenerative processes, neuronal death, and the presence of diffuse parenchymal hemorrhages, which are representative of microvascular injury in cortical tissue surrounding the impacted area. In sham animals of both age cohorts, no (or only marginal) alterations were observed in the cortical tissue histology due to craniotomy surgery. **Figures 3C,D** represent analyses of distribution of contusion volume between volumes of lesioned cortical tissue and cavitation in CCI animals compared to the sham group. To determine possible changes in the patterns of brain lesions following experimental TBI, the injury volumes were analyzed by measuring and statistically comparing the volumes of cortical lesions and cavitation separately, and the data are presented as a contingency graph.

Cortical lesions were defined as histological alterations evident with cresyl violet staining, including cell loss (i.e., light stromal staining without or with markedly decreased nuclear staining), changes in cellular density and nuclear morphology (i.e., altered shape, shrinkage, and intensively stained and condensed nuclei), "penumbra" (i.e., the area adjusted to the injury core with evident signs on secondary injuries such as the aforementioned morphological changes), and small hemorrhages, whereas cavitation was defined as total loss of brain tissue or hematoma without visible cresyl violet staining. The measurements were made in brain segments between bregma coordinates from 0 to −2 mm, which covered all major brain segments including hippocampus and cortical regions associated with functional neurobehavioral outcomes. To compare the differences in cortical lesion, cavitation, and total contusion volumes between surgery groups (i.e., sham and CCI) and animal age, statistical analyses were performed using multi-factor ANOVA and *post-hoc* Student's test for pairwise comparison. The results demonstrate significant differences in all measures of cortical injury volume in CCI groups compared to sham-operated mice in both age cohorts (**Figures 3C,E**). There were significant effects of both factors (i.e., surgery type and age) on the cortical lesion and total contusion volumes (<0.0001 and $P = 0.0003$, respectively) and a significant effect of surgery factor on the cavitation volume measured ($P = 0.0001$). There was a statistically significant difference in cortical lesion volumes between CCI groups of the adult and older adult cohort ($P = 0.0248$), whereas there was no statistical difference between cavitation and total contusion volumes measured in these groups (**Figure 3E**). Interestingly, some marginal alterations in the brain that might be characterized as cortical lesions were observed in some animals from sham-surgery groups and there was a significant difference between the volumes of these lesions in sham groups of adult and older adult cohorts ($P = 0.0017$).

In most of the animals from the CCI groups in both age cohorts, morphological distortion of ipsilateral hippocampi and localized hippocampal edemas were noticeable on all brain sections where the hippocampus is present, with the most apparent presentation on the sections between about 1 and 2.5 mm posterior from the bregma. In some animals, hippocampal swelling was not obvious; ipsilateral hippocampus size was possibly reduced due to concurrent neuronal tissue degeneration (Glushakov et al., 2015). Two-way ANOVA and *post-hoc* comparison using Student's *t*-test revealed no significant changes in hippocampal volumes compared to sham in adult and older adult cohorts (**Figures 3F,G**). The analyses of hippocampal swelling has also demonstrated that the values of relative hippocampal volumes 48 h following CCI were not significantly different between WT mice from the adult and older adult cohorts (**Figure 3H**).

Figures 4A,B demonstrate examples of brain sections within the same bregma coordinates (from −1 mm to −2 mm) showing typical variability of anatomical brain pathologies between individual animals in WT and $Hp^{-/-}$ mice of adult and older adult cohorts. To compare the differences between anatomical outcome measures including cortical injury volume (i.e., cortical contusion, cortical lesions, and cavitation) and hippocampal swelling in WT and $Hp^{-/-}$ mice in two age cohorts, a multi-factor ANOVA and *post-hoc* pairwise comparison was performed using Student's test. The statistical analyses revealed the significant interaction of age and genotype in cortical lesion volume measures following CCI ($P = 0.0172$). The quantitative analyses revealed that there were no statistical differences between total contusion volumes of WT and $Hp^{-/-}$ mice in both age cohorts (**Figures 4C,D**) and between $Hp^{-/-}$ mice from adult and older adult cohorts (**Figure 4E**). Similarly, comparing hippocampal volumes in CCI-injured animals revealed no statistically significant differences between WT and $Hp^{-/-}$ mice in any age cohort or between $Hp^{-/-}$ mice from adult and older adult cohorts (**Figures 4F–H**). Interestingly, in the older adult age cohort, although total cortical lesion was not significantly different between WT and $Hp^{-/-}$ mice, the volume of cortical lesions in $Hp^{-/-}$ was significantly increased compared to WT mice ($P = 0.0365$). There was no significant difference observed

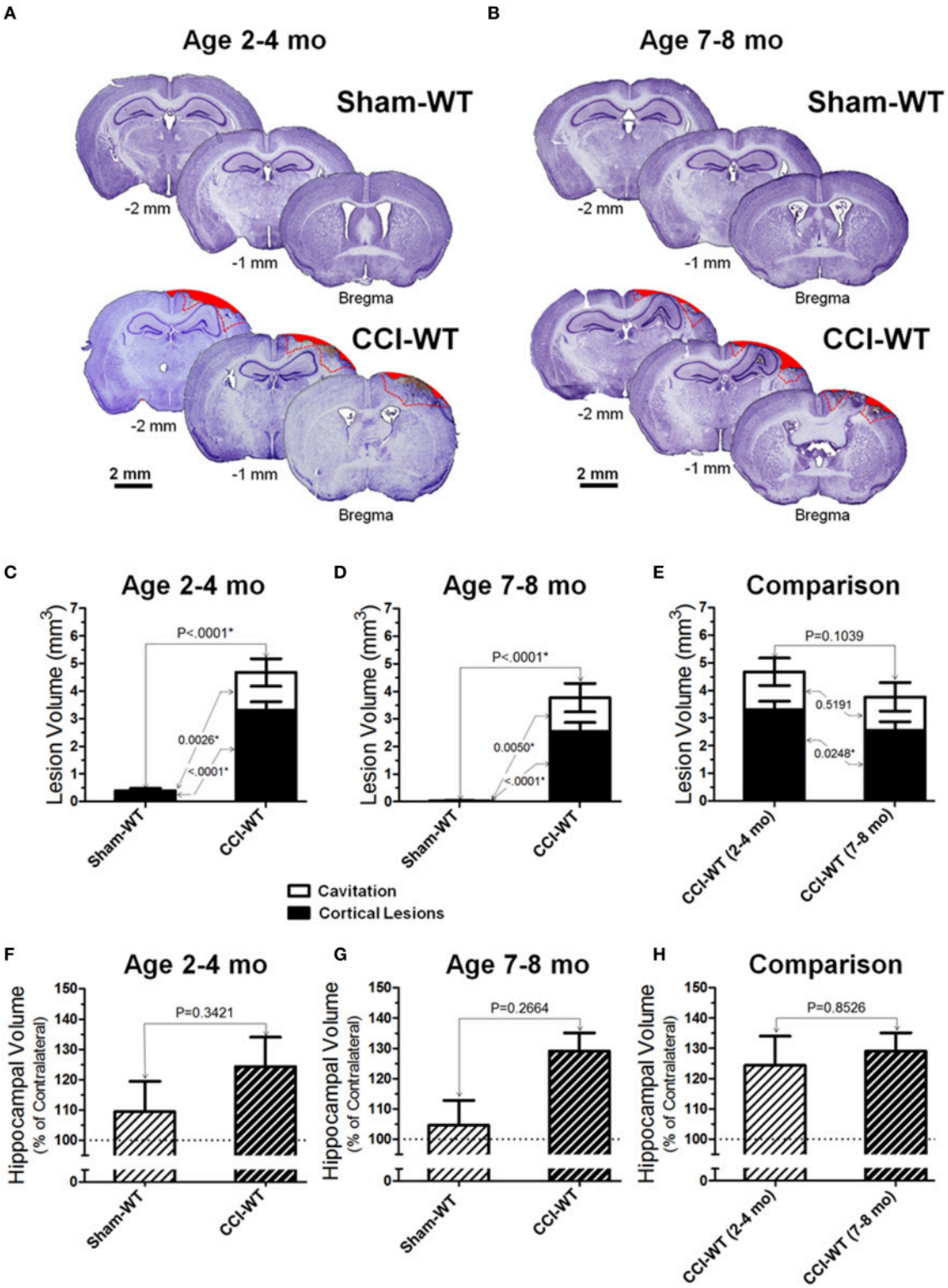

FIGURE 3 | Age-dependent effects of the experimental TBI on anatomical outcomes. (A,B) The representative microphotographs of cresyl violet-stained brain sections obtained at 48 h after CCI and sham injury in adult and older adult WT mice, respectively. In each panel, three brain sections were cut from the same mouse within 1 mm apart posteriorly from the bregma (coordinates from 0 to −4 mm). The text and numbers denotes approximate distance from the bregma in posterior direction. Areas filled with red represent cavitation and red dotted lines represent boundaries of cortical lesions and cavitation areas used for quantitative histopathological analyses. **(C-E)** Comparison of lesion volumes between sham- and CCI-injured WT mice in adult and older adult age cohorts: **(C,D)** and between

(Continued)

FIGURE 3 | Continued
CCI-injured WT mice of different ages **(C)**, respectively. **(F-H)** Comparison of relative hippocampal volumes between sham- and CCI-injured WT mice in adult and older adult age cohorts **(C,D)**, and between CCI-injured WT mice of different ages **(E)**, respectively. The numbers shown on the graphs represents *P*-values from the multi-factor ANOVA an *post-hoc* statistical analyses performed using Student's *t*-test to compare values between matched groups ($n = 5–10$).

between volumes of cavitation in these groups. Only marginal anatomical alteration was detected in sham $Hp^{-/-}$ mice in both adult and older adult cohorts (data not shown).

Effects of the Hp knockout on Astrocytic and Microglial Responses Following CCI in the Different Age Cohorts

Because the changes in glial responses to the CCI injury reflected in proliferation of glial cells and upregulation of specific markers are not definitely evident on the cresyl violet-stained sections, to study the prospective effects of Hp on the activation of reactive astrocytes and microglial cells, the GFAP and Iba1 immunostainings were performed on the brain sections obtained from WT and $Hp^{-/-}$ mice of two age cohorts, respectively. At 48 h after CCI, there was an apparent increase in immunoreactivity for both glial markers, glial cell proliferation, and changes in morphology of corresponding glial cells. **Figures 5**, **6** demonstrate representative microphotographs of GFAP- and Iba1-immunostained brain sections in two age cohorts, respectively, including zoomed selected areas of brain sections with the most extensive glial responses to the experimental TBI to demonstrate characteristic alteration in glial cell morphology. The morphological examination suggested some tendencies in the increased GFAP and Iba1 and in contralateral and ipsilateral brain regions of $Hp^{-/-}$ mice of both age cohorts. The findings of immunohistochemical experiments in adult WT mice are consistent with our previously published data showing significant increases in GFAP and Iba1 immunoreactivity in CCI compared to sham-injured animals. To assess the level of astrocytic and microglial activation, first, quantitative immunohistochemical analyses were performed separately in the major brain segments located within the CCI impact area with increments of 1 mm starting from the bregma (a total of four segments from 0 to −4 mm). However, the results revealed that there were no significant differences between relative immunoreactivities in 1-mm-thick segments within any brain region (multi-factor repeated measurement ANOVA with *post-hoc* Student's *t*-test; data not shown). Thus, we performed further analyses using the relative immunoreactivity values averaged for all four segments. **Figure 7** demonstrates a summary of the results of quantification of immunohistochemical stainings shown in **Figures 5**, **6**. The comparison of GFAP and Iba1 immunostainings between WT and $Hp^{-/-}$ mice in adult and older adult cohorts revealed that the results of statistical analyses are demonstrated in **Figure 7** as *P*-values above bar graphs between corresponding groups and above horizontal bars in selected panels to show the significant difference between groups of the same genotype from different age cohorts (ANOVA with *post-hoc* Student's *t*-test).

DISCUSSION

This study examined for the first time the potential roles of the Hp pathway in TBI using two age cohorts of WT and $Hp^{-/-}$ mice. The results suggest that the role of Hp in TBI is multi-factorial and age dependent. Because Hp is involved in clearance of generally toxic free hemoglobin and has antioxidant properties (Campbell et al., 2005), its injury-induced upregulation would contribute to the neuroprotective compensatory mechanism (Vejda et al., 2002). Although there is no clear consensus, preclinical studies from Dr. J. Aronowski and his colleagues have suggested that Hp might be expressed in the brain and its induction would be sufficient to be protective in experimental intracerebral hemorrhage (Zhao et al., 2009, 2011). The results of our study suggest that global deletion of the Hp gene is associated with statistically significant better short-term functional outcomes following experimental TBI in 2–4 mo-old (adult) mice, whereas no such effects were observed between WT and $Hp^{-/-}$ 7–8 mo-old (older adult) mice, and there were no statistical differences between volumes of total contusion, cortical lesions, and tissue loss in WT and $Hp^{-/-}$ of both age cohorts. Statistical differences were also observed in the gliosis outcomes, i.e., astrogliosis and microgliosis.

Hp is involved in the acute phase response to systemic or local tissue injuries by cytokine-triggered hepatocytic synthesis and release into circulation of several defensive proteins (Wilcockson et al., 2002; Petersen et al., 2004; Campbell et al., 2005). Although limited preclinical studies provide evidence that Hp might be expressed in the brain (Zhao et al., 2009, 2011), it is more likely that increased CSF and brain levels of Hp observed after TBI might essentially result from blood-brain-barrier breakdown (Liu and Sturner, 1988; Bell et al., 1997). It has long been recognized that elevated serum Hp after TBI is produced mainly from the liver (Bowman and Kurosky, 1982; Hoj et al., 1984; Yang et al., 2013) it peaks approximately 24 h after injury and its levels are associated with increased cytokine levels (Bell et al., 1997; Amick et al., 2001). The latter observation is also supported by a previous immunohistochemical clinical study performed in the post-mortem brain of TBI patients that provided solid evidence that the spatiotemporal profiles of increased levels of Hp and several other plasma proteins are associated with blood-brain barrier breakdown and the extravasated plasma proteins and their subsequent uptake by the glial cells (Liu and Sturner, 1988). However, there is evidence of complex roles of Hp expression and its phenotypes in different types of brain injuries. Hp is a potent antioxidant and its increased expression has been documented in different brain injuries that are not directly associated with brain hemorrhages. Experimental studies have documented significant changes in Hp expression in the plasma of rats undergoing experimental transient focal cerebral ischemia (Chen et al., 2011).

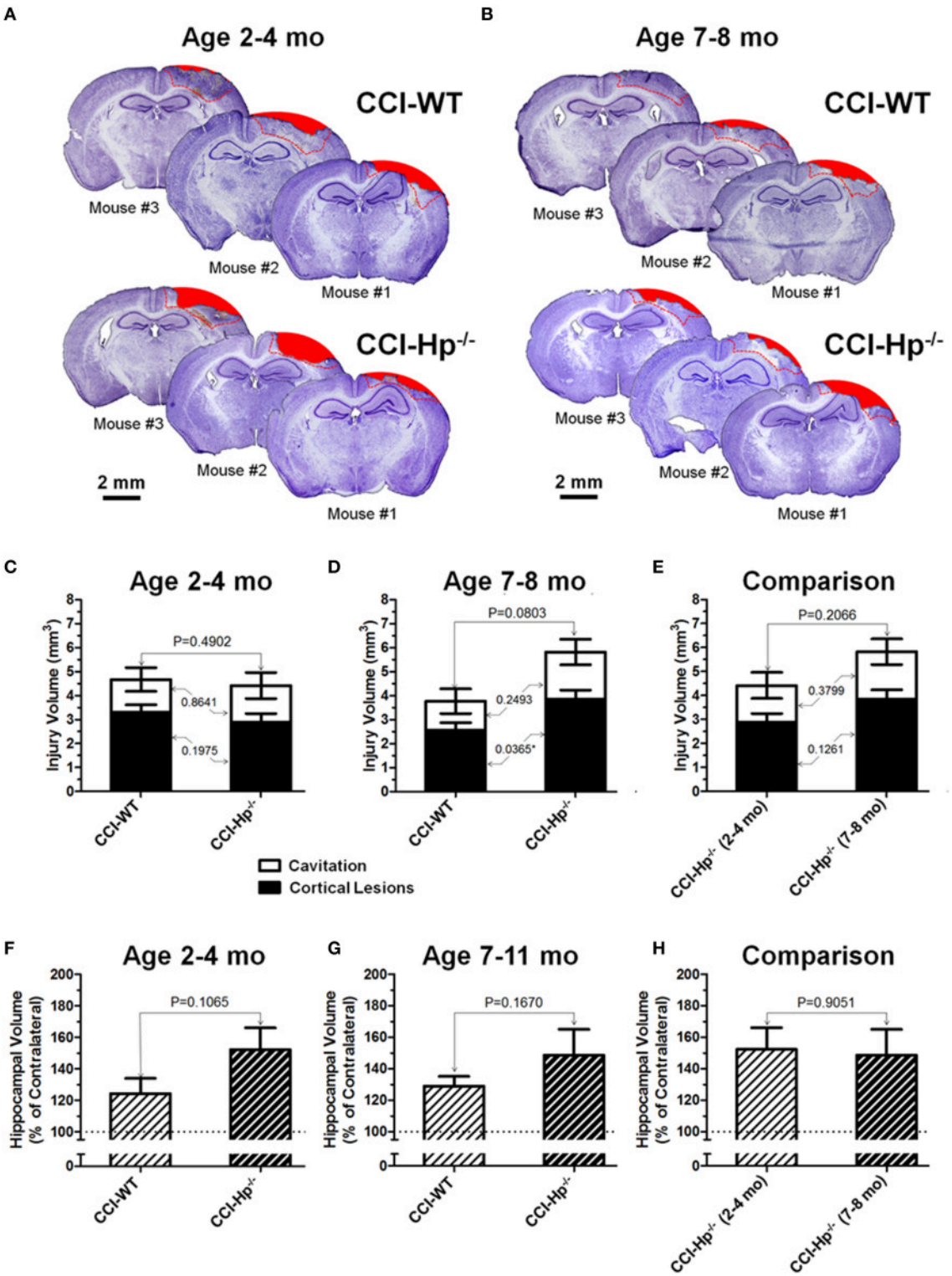

FIGURE 4 | Effects of the Hp knockout on anatomical outcomes following CCI in different age cohorts. (A,B) The representative microphotographs of cresyl violet-stained brain sections obtained at 48 h after CCI in adult and older adult WT and $Hp^{-/-}$ mice, respectively. In each panel, three examples of the brain sections from three different animals from the same experimental group (marked as Mouse #1–3). The example brain section from each mouse was cut within coordinates from −1 to −2 mm. Areas filled with red represent cavitation and red dotted lines represent boundaries of cortical areas covered by cortical lesions used for quantitative histopathological analyses. **(C-E)** Comparison of the lesion volumes between CCI-injured WT and $Hp^{-/-}$ mice in the adult and older adult age cohorts **(C,D)**, and between the CCI-injured WT mice from different age cohorts

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(E), respectively. **(F-H)** Comparison of relative hippocampal volumes between WT and $Hp^{-/-}$ CCI-injured mice in adult and older adult age cohorts **(C,D)**, and between CCI-injured $Hp^{-/-}$ mice of different ages **(E)**, respectively. The numbers shown on the graphs represents *P*-values from the multi-factor ANOVA an *post-hoc* statistical analyses performed using Student's *t*-test to compare values between matched groups ($n = 4-10$).

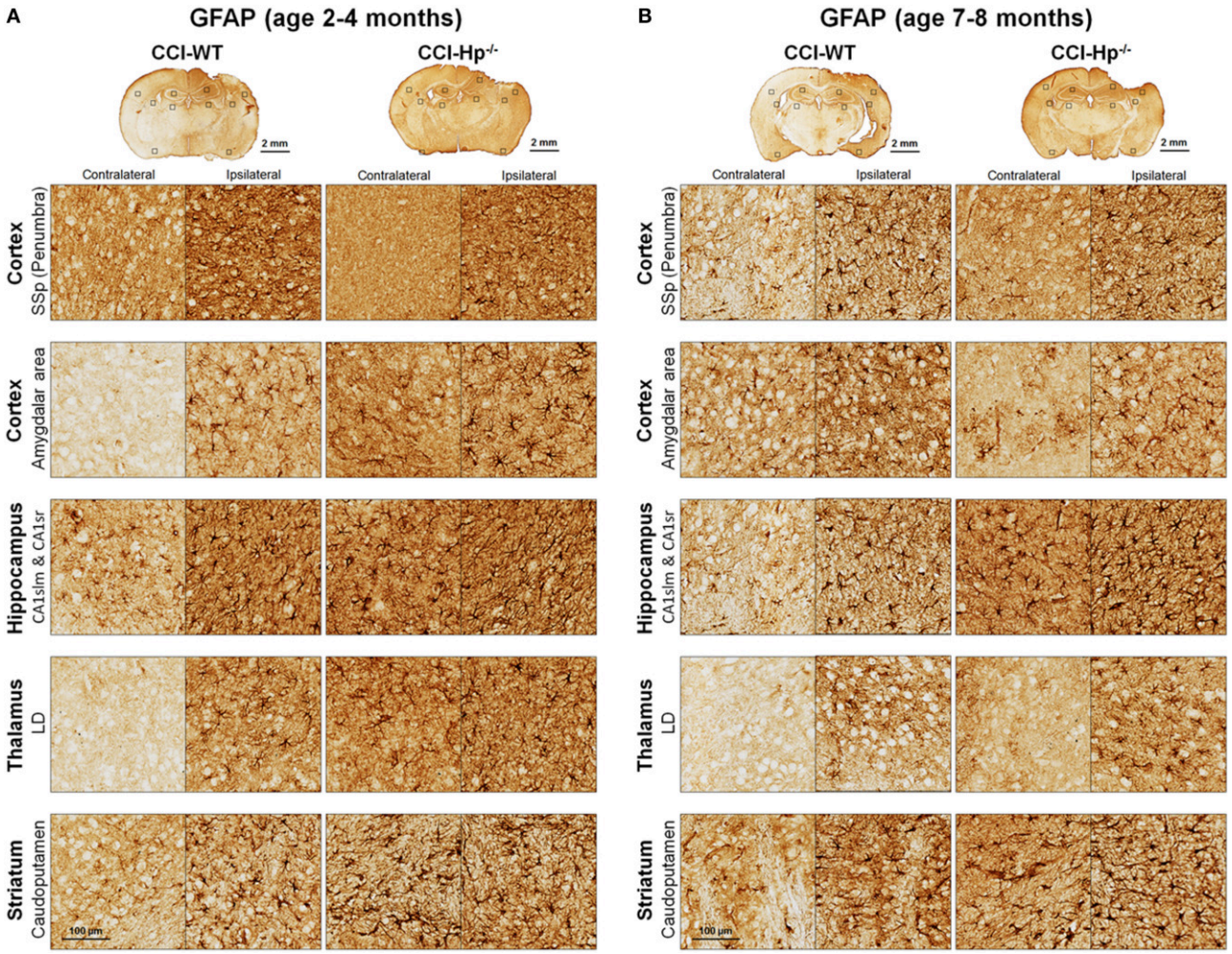

FIGURE 5 | Effects of Hp on the reactive astrocyte immunoreactivity at 48 h after CCI in 2–4 mo-old (adult) and 7–8 mo-old (older adult) mice. (A,B) The representative microphotographs of GFAP immunostainings in the "penumbral" part of the primary somatosensory area (SSp) of cerebral cortex, posterior part of cortical amigdalar areas, lacunosum-moleculare (CA1slm), and stratum radiatum (CA1sr) fields of hippocampal CA1 area, lateral dorsal nucleus (LD) of thalamus. Identification of the brain structures and usage of the histological acronyms in this and other figures were achieved using Allen Mouse Brain Atlas (Lein et al., 2007).

In a TBI model, Hp phenotypes were associated with differential neuropsychological outcomes; however, in contrast to clinical and preclinical data in subarachnoid brain hemorrhage, the "high" affinity Hp 1-1 phenotype was associated with worse outcomes (Anderson et al., 2009).

The neurological outcomes assessed using NDS and anatomical outcomes (i.e., brain lesions and cortical tissue) in adult WT mice observed in this study were consistent with our previously published data obtained in C57BL/6 mice within this age range (Glushakov et al., 2013, 2014, 2015). Not surprisingly, the NDS was increased in older adult mice of both WT and $Hp^{-/-}$ genotypes in the experiments with the same magnitude of the experimental TBI. In the sham group, WT mice from the 7–8 mo-old older adult cohort had a range of NDS that was seemingly higher compared to that observed in WT 2–4 mo-old adults from the sham group normally showing no detectable or only marginal neurological deficits, although there were no statistically significant differences between sham groups of WT mice from these two age cohorts. This observation suggests that some older adult animals have increased vulnerability to surgical procedures, including craniotomy performed in the sham groups. However, no such changes in the NDS variability

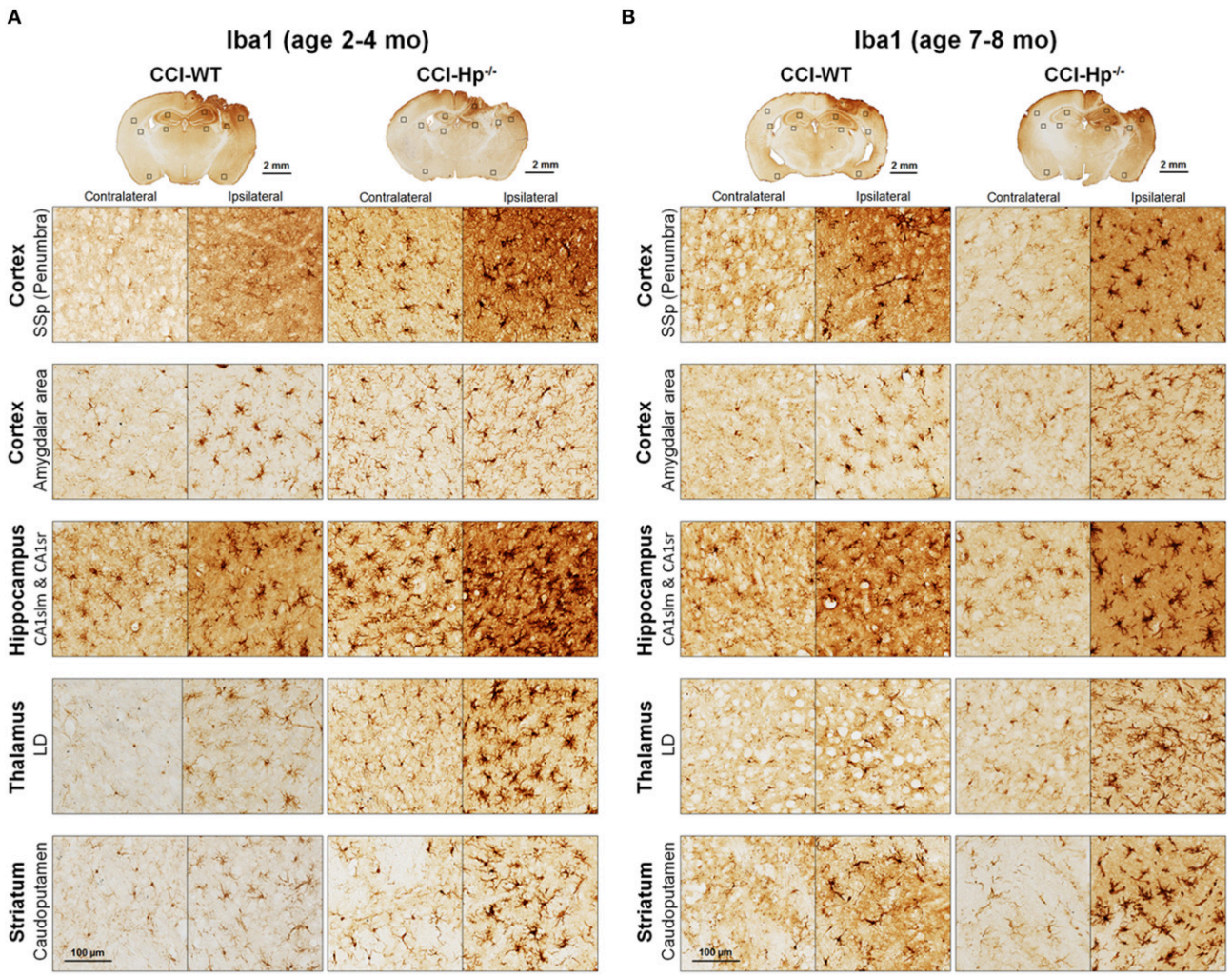

FIGURE 6 | Effects of Hp knockout on the activated microglia immunoreactivity at 48 h after CCI in 2–4 mo-old (adult) and 7–8 mo-old (older adult) mice. (A,B) The representative microphotographs of Iba1 immunostained brain sections. The acronym definitions are the same as those in the legend of **Figure 5**.

were observed in the sham $Hp^{-/-}$ mice from both age cohorts and the ranges of NDS in these mice were within the range of 2–4 mo-old WT mice from the sham group.

Based on analyses of cresyl violet-stained sections, significant cortical and hippocampal pathologies were evident at the 48 h time point used in this study in all CCI-injured groups. The anatomical cortical injury was quantified as a cortical contusion volume, which was identified by characteristic tissue loss of brain tissue, neuronal death, and alteration in cellular morphology and integrity of brain structures and intracerebral hemorrhages. As we previously reported, in the CCI model, based on evident alterations in the hippocampus that are not directly affected by impact, the overall brain lesions could be categorized into two groups with prevalence reflecting severity of secondary injuries: type I and II, which are characterized by morphopathological changes in the ipsilateral hippocampus with remaining structural organization and by a complete loss of the ipsilateral hippocampus, respectively (Glushakov et al., 2015). The cortical cavitation is characteristic of both of these lesion types, although it is more prominent with later injury-onset time points (Glushakov et al., 2013).

It is well recognized that neuronal death and loss of brain tissue reflects secondary injury predominantly resulting from necrotic calpain-induced proteolysis (Wang et al., 2006; Saatman et al., 2010). In addition, our previous data have shown that the overall brain lesions might be categorized by the changes in the hippocampal morphology with seemingly abrupt transformation from hippocampal edema to hippocampal degeneration (lesion types I and II, respectively) resulting either from increasing severity of impact (e.g., increased compression distance) or from the deleterious effect of pharmacological intervention even with mild-to-moderate initial impact reflecting the severity of secondary injuries (Glushakov et al., 2015).

CCI parameters used in this study allowed one to induce brain injury by partial direct compression of the cortical tissue with rigid impactor, whereas the brain structures located underneath

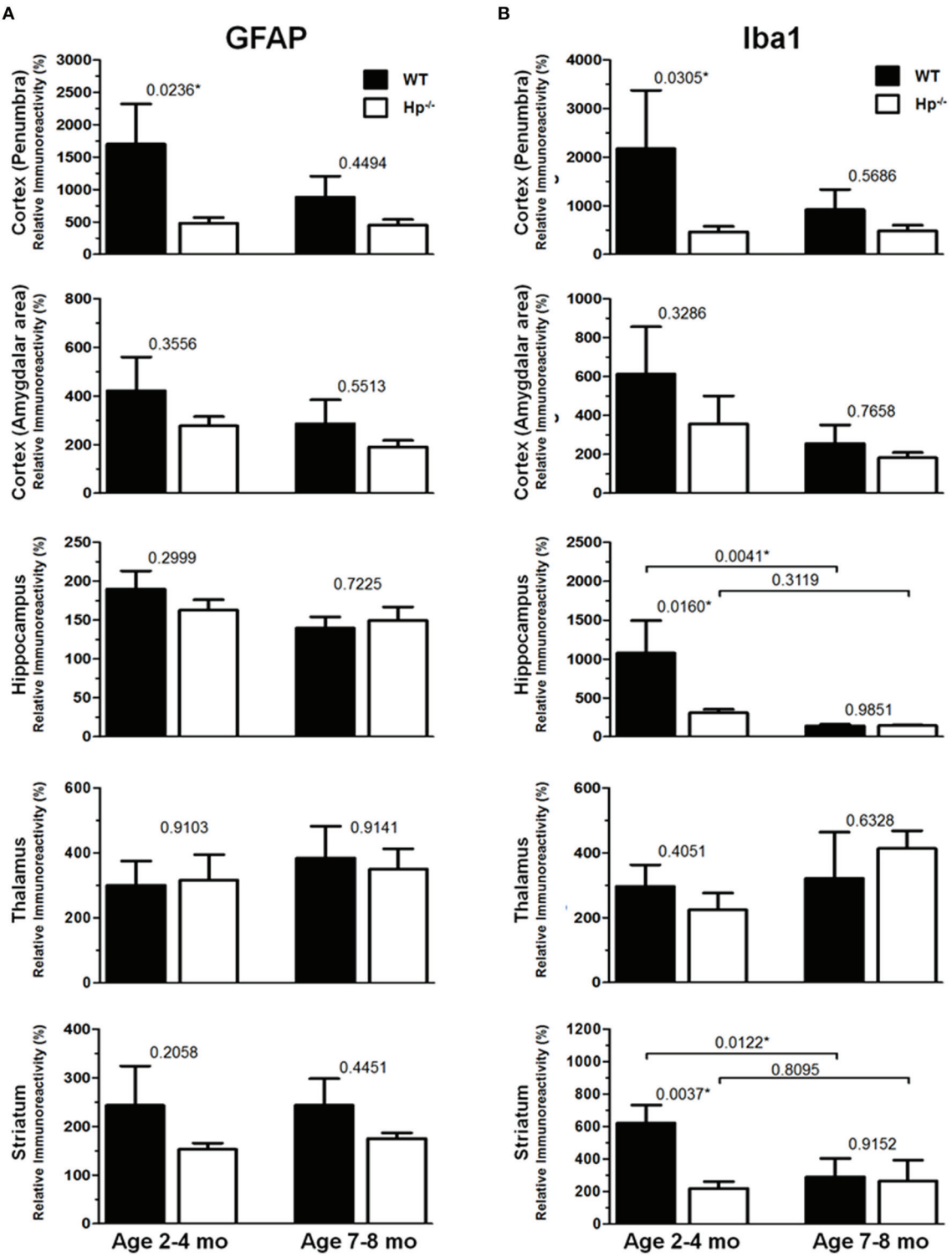

FIGURE 7 | Quantification of GFAP and Iba1 immunoreactivities at 48 h after CCI in in 2–4 mo-old (adult) and 7–8 mo-old (olde adult) mice. The bar graphs show quantitative analyses of GFAP **(A)** and Iba1 **(B)** immunostained brain sections presented as relative immunoreactivity of in the selected ipsilateral brain regions (shown in the **Figures 5**, **6**) normalized to the immunoreactivity values calculated in their contralateral counterparts. The numbers above bars in each graph represents *P*-values obtained by using multi-factor ANOVA and the *post-hoc* Student's *t*-test ($n = 4–8$). *indicates significant difference.

the cortical injury site are affected indirectly and with gradient decreasing pressure that likely would not cause meaningful mechanical damage. However, in this study, the prevalence of hippocampal alterations in most animals reflected type-I lesions, which are characterized by a marked increase in the ipsilateral hippocampal volume and distortion of hippocampal shape without substantial loss of hippocampal tissue, while in some animals, the ipsilateral hippocampi were of comparable size and shape or even had some reduction of ipsilateral hippocampal size, suggesting variability in ranges of responses to injury in individual animals.

Immunohistochemical experiments with GFAP and Iba1 stainings performed at the 48 h time point after experimental TBI revealed significant proliferation and changes in cell morphology of reactive astrocytes and activated microglial cells in selected brain regions consistent with our previous published data obtained with the same experimental parameters (Glushakov et al., 2013, 2014). The upregulation of these immunomarkers and proliferation of glial cells were observed in cortical lesioned tissue, including "penumbral" areas, located underneath the injury, and evident with histophatological examination using cresyl violet staining; and also, to a lesser extent, in the brain regions not directly affected by the CCI impact without evident histopathological changes (cresyl violet) throughout the whole ipsilateral cerebral cortex even with seemingly similar immunoreactivity intensity and cellular morphology up to distal cortical amigdalar areas; and ipsilateral brain structures that are not directly impacted, including the hippocampus, and some areas of the thalamus and striatum. The results of these experiments indicate that there were differences in microglial and astrocytic reactivity between $Hp^{-/-}$ and WT in selected brain regions of the adult but not older adult age cohort, suggesting that Hp might be involved in glial or inflammatory responses, or affecting secondary injuries to the levels that are reflected in changes in upregulation of astrocytic and microglial markers or proliferation of glial cells. There were also some apparent tendencies in the upregulation of glial cells in selected contralateral regions of $Hp^{-/-}$ mice of both age cohorts that might result from presumed impairment of overall defensive mechanisms due to deletion of Hp; establishing their potential clinical importance in brain injuries requires more detailed investigation. Although reactive astrocytes have been implicated in playing a role in the uptake of Hp and other extravasated plasma proteins (Liu and Sturner, 1988) the result of GFAP, as well as Iba1, IHC experiments in $Hp^{-/-}$ did not support the astrocytic or microglial involvement in clearance of assumed Hp-hemoglobin complexes following TBI. The roles of astrocytes microglia in brain injury are complex, including both beneficial and detrimental effects (Loane and Byrnes, 2010). On the other hand, it is also recognized that increased upregulation of astrocytic and microglial markers is associated with the extent of anatomical and behavioral deficits. In our study, the significantly decreased upregulation of GFAP and Iba1 in selected brain regions of $Hp^{-/-}$ mice may indicate involvement of Hp in these inflammatory responses. The effect of aging in experimental models of TBI is well recognized, including neurological outcomes, neuronal brain pathology, and blood-brain barrier function (Onyszchuk et al., 2008; Sandhir et al., 2008; Lee et al., 2012; Timaru-Kast et al., 2012). However, little is known about the effects of Hp expression on brain injuries, and in our study, we have made an attempt to uncover possible dependencies of Hp roles with age using WT and $Hp^{-/-}$ mice. The results of this study revealed significant associations of both age and genotype factors with several neurobehavioral, anatomical, and immunohistochemical outcome measures. Interestingly, the data suggest that improved NDS in $Hp^{-/-}$ might be associated with decreased upregulation of both GFAP and Iba1 in the cortical areas located adjacent to the injury site and Iba1 upregulation in selected hippocampal and striatal regions in adult CCI-injured mice. However, these effects of Hp deletion were lost with aging.

In terms of some limitations of this study, although we believe that using of $Hp^{-/-}$ mice is one of the currently reasonable available approaches to test our hypotheses, the use of genetically modified mice, in general, has inherent limitations due to activation of possible compensatory mechanisms to restore the organism's functions. In particular, it might be critical in case of "protective" proteins such as Hp that might potentially affect the physiological state and affect responsiveness to the experimental injury. Thus, some differences in the outcomes in adult and older adult mice might be attributed to the chronic ablation of a component of the acute phase response system. In addition, the translational potential of this study might be limited because of the heterogeneity of human Hp phenotypes in contrast to the homogeneity of Hp in mice and other animals, and thus using animal models may not reflect the whole spectrum of the various Hp-involving pathways. Another limitation of the study is using only an acute time point with the given experimental conditions reflecting only the Hp roles in short-term outcomes, whereas some results of our study suggest that long-term outcomes might be different.

In conclusion, this study provides an insight into the prospective roles of Hp in TBI and other acute brain injuries, especially those with complex mechanisms. The data of this study suggest that systemic Hp might interact with the intrinsic brain's mechanisms of hemoglobin clearance and/or antioxidative protective pathways affecting short-term clinical outcomes. The data also suggest that ablation of Hp might affect the organism's responsiveness to brain injuries and this effect is more prominent with aging. In addition, these results might be implicated in understanding the inconsistency in outcomes of clinical studies regarding the importance of Hp phenotypes in brain injuries. However, the potential roles of Hp in long-term outcomes warrant additional studies.

AUTHOR CONTRIBUTIONS

AG designed, performed, and analyzed all experiments, wrote the manuscript, and trained RA in performing behavioral and histochemical experiments and data analyses; RA performed behavioral and histochemical experiments and analyses, and edited the manuscript; ET developed and provided the breeding stock of $Hp^{-/-}$ mice, and revised the manuscript; SD designed

the experiments, assisted in the analyses, provided funding and expertise, and contributed to writing and revising the manuscript. All authors have accepted the final version of the manuscript.

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REVIEW
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Potential Roles of Adropin in Central Nervous System: Review of Current Literature

*Shima Shahjouei[1], Saeed Ansari[2], Tayebeh Pourmotabbed[3] and Ramin Zand[2,4]**

[1] Department of Neurosurgery, Tehran University of Medical Sciences, Tehran, Iran, [2] Department of Neurology, University of Tennessee Health Science Center, Memphis, TN, USA, [3] Department of Microbiology, Immunology, and Biochemistry, University of Tennessee Health Science Center, Memphis, TN, USA, [4] Biocomplexity Institute, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

Adropin is a 4.9 kDa peptide that is important for maintenance of metabolic and non-metabolic homeostasis. It regulates glucose and fatty acid metabolism and is involved in endothelial cell function and endothelial nitric oxide (NO) synthase bioactivity as well as physical activity and motor coordination. Adropin is expressed in many tissues and organs including central nervous system (CNS). This peptide plays a crucial role in the development of various CNS disorders such as stroke, schizophrenia, bipolar disorder as well as Alzheimer's, Parkinson's, and Huntington's diseases. In this comprehensive review, the potential roles of adropin in cellular signaling pathways that lead to pathogenesis and/or treatment of CNS disorders will be discussed.

Keywords: adropin, neurodegenerative disease, neuroprotection, biomarker, predictor, therapeutic, cellular signaling pathways

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Alireza Noorian,
Kaiser Permanente Orange County Stroke Program, USA
Leili Shahgholi,
University of Florida, USA
Bardia Nourbakhsh,
University of California, San Francisco, USA

****Correspondence:***
Ramin Zand
rzand@uthsc.edu

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INTRODUCTION

Adropin is a 4.9 kDa peptide encoded by Energy Homeostasis Associated gene (*Enho*) located on chromosome 9 (Kumar et al., 2008; Aydin, 2014). A variety of organs including central nervous system (neurons, neuroglial cells, pia mater, vascular area, Purkinje cells, and granular layer), heart, kidney, liver, pancreas, and human umbilical vein synthesize adropin (Lovren et al., 2010; Aydin et al., 2013, 2014).

Constantly new functions for adropin are identified. Adropin's function as a regulator of glucose and lipid homeostasis and insulin sensitivity was initially described in 2008 by Kumar et al. (2008) and later by Aydin (2014). Lovren et al. (2010) demonstrated the endothelial protective potentials of adropin in 2010. Adropin activates vascular endothelial growth factor receptor 2 (VEGFR2) and its two downstream signaling pathways—phosphatidylinositol-3 kinase/ serine, threonine kinase (PI3K/Akt) and extracellular signal-regulated kinases 1/2 (ERK 1/2) (**Figure 1**). Therefore, adropin modulates expression of endothelial nitric oxide synthase (eNOS) (Lovren et al., 2010). Also, adropin increases the endothelial cells proliferation, migration and potential to form capillary-like structures (Lovren et al., 2010). Recently, it is found that adropin reduces the endothelial permeability (Lovren et al., 2010; Yang et al., 2016).

Adropin enhances mitochondrial function and activates pyruvate dehydrogenase (PDH)—a rate-limiting enzyme in glucose oxidation. Further, adropin suppresses two key enzymes in fatty acid utilization: carnitine palmitoyltransferase-1B (CPT-1B) and Cluster of Differentiation 36 (CD36) (Gao et al., 2015); thus, it plays a role in fatty acid oxidation.

Adropin may act as a potential protective regulator of atherogenesis and cardiovascular diseases (Wu et al., 2014; Zhao et al., 2015b; Li et al., 2016). Serum adropin level is inversely associated with severity of coronary atherosclerosis and serum level of homocysteine—a potential risk factor for atherosclerosis and cardiovascular diseases (Zhao et al., 2015a). The serum adropin level is diminished in patients with cardiac syndrome X and stable coronary artery disease (Celik et al., 2013; Zhao et al., 2015b). At the onset of acute myocardial infarction, serum adropin level is usually lower than controls (Yu et al., 2014); however, it raises between 1 and 24 h following myocardial infarction (Aydin et al., 2014). Plasma adropin level has a positive association with severity of heart failure and negative correlation with left ventricular ejection fraction (Lian et al., 2011). Low level of plasma adropin is predictive of pseudoexfoliation (Oğurel et al., 2016), coronary slow flow phenomenon (Demircelik and Kurtul, 2015), saphenous vein graft occlusion following coronary artery bypass grafting (Demircelik, 2014), as well as pediatric obstructive sleep apnea in the presence of endothelial dysfunction (Gozal et al., 2013). While Gu et al. (2015) described plasma adropin level as an independent indicator of hypertension, other studies failed to show this association (Altincik and Sayin, 2015).

Adropin, as a membrane-anchored protein modulates the Notch1 signaling pathway via neural recognition molecule 3 (NB3) (**Figure 1**). NB-3 belongs to the contactin family and acts as a membrane-tethered Notch1 ligand that mediates cell surface interaction during nervous system development. An animal study demonstrated that adropin regulates locomotor activity and motor coordination via the NB3/Notch signaling pathway and plays an important role in cerebellum development (Wong et al., 2014). In this review, we discuss various roles of adropin in central nervous system pathogenesis via different intra and extra cellular signaling pathways as well as its therapeutic potentials.

Adropin and Vascular Endothelial Growth Factor Receptor 2 (VEGFR2)

VEGFR2—a tyrosine kinase receptor—is especially expressed in endothelial cells and regulates endothelial function and angiogenesis. Adropin strongly upregulates this receptor, activates PI3K/Akt and ERK1/2 pathways, and enhances eNOS thus, modulating NO bioavailability (Lovren et al., 2010; **Figure 1**). Hypoxic insults enhance hypoxia-inducible factor-1a (HIF-1a), and VEGF gene expression as its downstream signaling pathway (Mu et al., 2003). VEGF is involved in neurogenesis and has a neuroprotection function. This has been discussed under "Adropin and Neurogenesis" section in more details.

Role of Adropin in Activation of PI3K/Akt Signaling Pathway

PI3K induces the phosphorylation of Akt (also known as protein kinase B) under the effect of growth factors such as VEGF, cytokines, insulin, and other cellular stimuli (**Figure 1**). Activation of Akt requires consequent phosphorylation on Thr-308 and Ser-473. Once Ser-473 is phosphorylated, Akt is fully activated regardless of Thr-308 phosphorylation status (Wang et al., 2009). Adropin can activate Akt by stimulating Ser-473 phosphorylation (Lovren et al., 2010).

Phosphorylated-Akt provokes cell cycle progression, proliferation, differentiation, and survival (Blanco-aparicio et al., 2007; Manning and Cantley, 2007). Moreover, this pathway triggers intracellular ligands such as mammalian target of rapamycin (mTOR)—which plays an important role in angiogenesis, neuronal regeneration, synaptic plasticity, inflammatory responses, and apoptosis (Annovazzi et al., 2009; Chen et al., 2012; Li et al., 2015). Thereby, PI3K/Akt/mTOR pathway may be a target of stroke therapeutic agents (Li et al., 2015).

Neurodegenerative conditions such as Alzheimer's, Parkinson's and Huntington's diseases are associated with defective Akt signaling (Colin et al., 2005; Griffin et al., 2005; Timmons et al., 2009; Giralt et al., 2010). Similarly, damaged Akt/GSK3β (the serine/threonine kinase glycogen synthase kinase 3β) signaling pathway plays a role in the pathophysiology of neuropsychiatric disorders such as schizophrenia and bipolar disorders (Emamian et al., 2004; Jope, 2011). Since, variation in AKT1—one of the three genes encoding Akt—has been associated with schizophrenia and bipolar disorders (Ikeda et al., 2004; Karege et al., 2012), PI3K/Akt activation by adropin might also have a therapeutic potential in disorders such as Parkinson's (Burke, 2007; Timmons et al., 2009) and schizophrenia (Schwab et al., 2005) as discussed below:

Ischemic Insult

Cerebral ischemic injuries cause neural loss secondary to apoptosis or necrosis—which can be triggered by oxidative stress, metabolic compromise and disruption of calcium homeostasis at the cellular level (Mattson et al., 2001). Altintas et al. demonstrated that infarct size is positively correlated with blood adropin level in animal models of cerebral ischemia (Altintas et al., 2016). Activation of Akt by adropin can prevent neuronal and cellular death, (Chong et al., 2005) and might contribute to neuro-protective effect of ischemic postconditioning (Gao et al., 2008; Wang et al., 2009). PI3K/Akt pathway induces mTOR and also attenuates apoptotic proteins such as GSK3β and forkhead family of transcription factor. Thereby, inactivation of Akt might contribute to neuronal apoptosis and pathogenesis of ischemic stroke (Noshita et al., 2001; Franke et al., 2003; Hanumanthappa et al., 2014; Li et al., 2015).

Huntington Disease (HD)

Abnormal expansion of a polyglutamine stretch in the N terminus of protein huntingtin is responsible for neuropathology of HD (Humbert et al., 2002). Induction of Akt Ser-473 phosphorylation attenuates mutant huntingtin toxicity and makes the cell more resistance to apoptotic signals by modulating proteins such as GSK3β and FOXO1 (Humbert et al., 2002; Manning and Cantley, 2007). In addition, activated Akt decreases intranuclear inclusions of mutant huntingtin (Humbert et al., 2002). It was demonstrated that maintaining high levels of activated Akt may delay cell death and allow the recovery of neuronal viability after mutant huntingtin silencing (Canals, 2004).

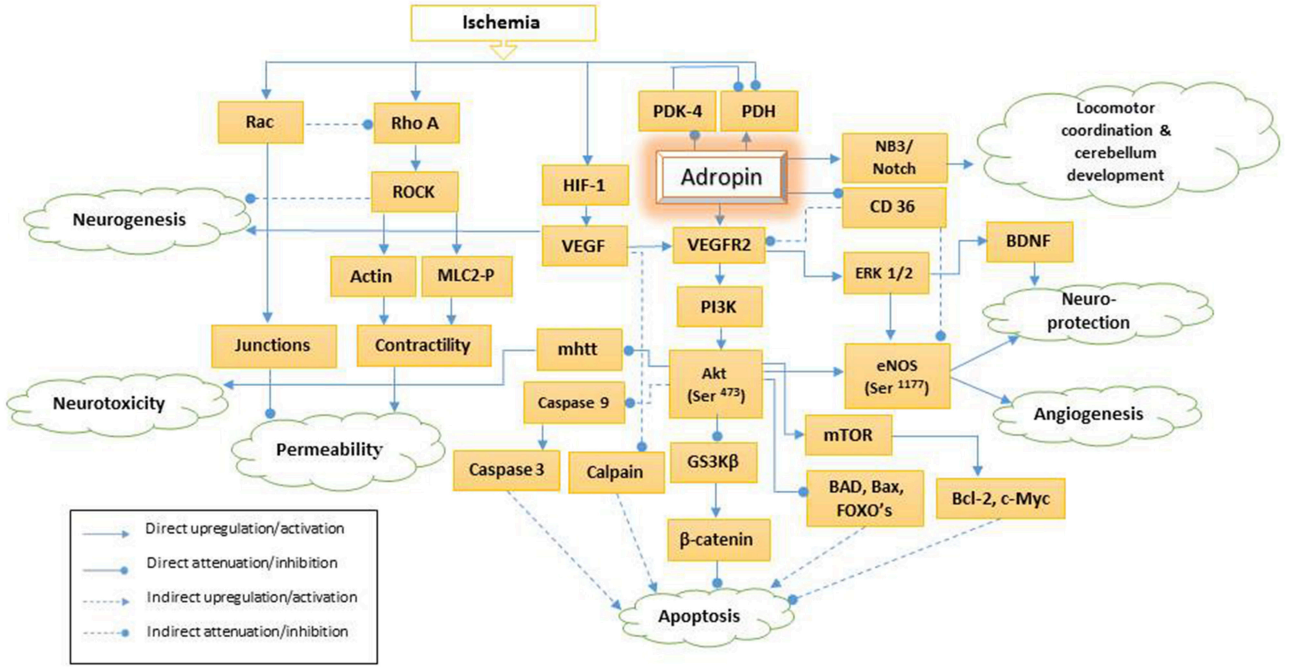

FIGURE 1 | Schematic presentation of adropin signaling pathways. PDH, pyruvate dehydrogenase; PDK-4, pyruvate dehydrogenase kinase-4; HIF-1, hypoxia-inducible factor-1a; NB3, neural recognition molecule 3; VEGF, vascular endothelial growth factor; mhtt, mutant huntingtin; VEGFR2, vascular endothelial growth factor receptor 2; PI3K, phosphatidylinositol-3 kinase; Akt (Ser-473), phosphorylation of serine 473 of serine, threonine kinase; GS3Kβ, glycogen synthase kinase 3β; CD36, Cluster of Differentiation36; ERK 1/2, extracellular signal-regulated kinases 1/2; Mtor, mammalian target of rapamycin; BAD, Bcl-2 associated death protein; eNOS (Ser-1177), phosphorylation of endothelial nitric synthase on serine 1177; BNDF, brain-derived neurotrophic factor; FOXO, Forkhead box O; Bcl-2: B-cell lymphoma 2; Bax, Bcl-2 associated X protein; ROCK, Rho-associated protein kinase; MLC2-P, Phosphorylated myosin light chain 2.

Parkinson's Disease (PD)

Timmons and colleagues showed that Akt is expressed at high levels in tyrosine hydroxylase dopaminergic neurons. Selective loss of these neurons and diminished phosphorylated Akt at Ser-473 is obvious in the brain of patients with Parkinson's disease (Timmons et al., 2009). The glial cell line-derived neurotrophic factor (GDNF) as the downstream of phosphorylated Akt has neuroprotective effect against dopaminergic neurodegeneration (Ries et al., 2006). Thus, medications like adropin that target the dopaminergic system via Akt activation or those with the potential to increase the phosphorylated Akt have neuroprotective characteristics in PD (Ries et al., 2006; Burke, 2007; Levy et al., 2009; Timmons et al., 2009).

Schizophrenia

AKT1 gene single nucleotide polymorphisms (SNPs) and haplotype studies indicated the involvement of Akt in Schizophrenia (Ikeda et al., 2004; Schwab et al., 2005; Thiselton et al., 2008). Expression or activity of AKT1 and phosphorylation of its substrate—GSK3β—is reduced in Schizophrenic patients (Emamian et al., 2004; Kalkman, 2006). As summarized by Beaulieu and colleagues, many of the antipsychotics and psychoactive substances modulate dopamine-dependent behaviors through Akt/GSK3β signaling pathway (Beaulieu et al., 2007). In addition, Schizophrenia is associated with insulin receptor deficit, disruptive insulin dependent Akt signaling and insulin resistance (Zhao et al., 2006). Adropin might be a potent therapeutic agent in Schizophrenia while it enhances Akt phosphorylation (Lovren et al., 2010) and prevents insulin resistance (Ganesh Kumar et al., 2012).

Alzheimer's Disease (AD)

Activation of PI3K/Akt/Wnt/β-catenin signaling induces neurogenesis and reverse cognitive deficit in AD animal models (Tiwari et al., 2015). In addition, reduced phospho-Akt and increased FOXO3a levels in the nuclei of neurons where proapototic genes were activated can cause adipokine dyshomeostasis, oxidative stress, mitochondrial dysfunction, and eventually neurodegeneration (Nuzzo et al., 2015). These data suggest Akt might be the link between insulin resistance, obesity, and AD.

Bipolar Disorder

Regulation of Akt/mTOR pathway is critical in synaptic neurotransmission and plasticity, as well as modulating cell proliferation and migration. There is evidence of excitotoxicity, neuroinflammation, and brain atrophy in BD due to apoptosis and disturbed synaptic function. A cadaver study on BD postmortem prefrontal cortex demonstrated an elevation in protein and mRNA levels of the pro-apoptotic factors (Bax, BAD, caspase-9 and caspase-3) and reduction in anti-apoptotic factors (BDNF and Bcl-2) and the synaptic markers (synaptophysin

and drebrin) (Kim et al., 2010). The Bax/Bcl-2 ratio appeared to be crucial in deciding the life or death of a cell and was increased in the above study. In another study, blood AKT1and mTOR mRNA expression decreased in BD during depressive episodes comparing to healthy controls, supporting an integrated Akt/mTOR signaling pathway activity in the pathogenesis of BD (Machado-Vieira et al., 2015). In accordance, activation of mTOR by N-methyl-D-aspartate (NMDA) antagonists results in rapid antidepressant effect in animal models (Li et al., 2010).

Study on animals under high-fat diet showed that obesity may desensitize serotonin-dependent Akt/GSK3β signaling and impair cell proliferation in the dentate gyrus of the hippocampus, and cause depression (Papazoglou et al., 2014). Available evidence support the notion that enhancing the inhibitory control of Akt/GSK3β is a key component of the therapeutic actions of drugs used to treat mood disorders (Li and Jope, 2010).

Adropin and Extracellular Signal-Regulated Kinases 1/2 (ERK1/2)

ERK 1/2 is a member of the mitogen-activated protein kinase family. Adropin via VEGFR2 can activate ERK 1/2 and its downstream cascades of substances such as brain-derived neurotrophic factor (BDNF) (**Figure 1**; Lovren et al., 2010). BNDF promotes neuronal development, differentiation, survival and neurological function improvement following brain injury and ischemia (Zhu et al., 2013; Zhao et al., 2014; Wu et al., 2015). Ischemic postconditioning, both early and delayed, may further reduce reperfusion injury via ERK 1/2 and BDNF activation (Wu et al., 2015). In contrast, post-ischemic inhibition of ERK 1/2 in diabetic rats may mitigate DNA repairing ability, accelerated apoptosis and aggravate neuronal loss (Zhao et al., 2014). In addition, ERK 1/2 activation induces nuclear factor erythroid 2-related factor2 (Nrf2) and protects neurons against beta-amyloid-induced cell death and oxidative stress.

Adropin and Nitric Oxide Synthase (NOS)

One of the endothelial protective functions of adropin is regulation of nitric oxide (NO) bioavailability (Lovren et al., 2010). NO promotes angiogenesis, reparative vasculogenesis and acts as an anti-atherosclerotic, anti-inflammatory and anti-thrombotic factor.

NO is generated by nitric oxide synthase (NOS) that is upregulated by PI3K/Akt and ERK 1/2 signaling pathways **(Figure 1)** (Lovren et al., 2010; Peng et al., 2012). NOS polymorphisms and diminished endothelial NOS expression are associated with spontaneous cerebral thrombosis and infarction, progressive cerebral amyloid angiopathy, blood brain barrier breakdown, and cognitive impairment—characteristics of cerebral small vessel disease, stroke and neurodegenerative diseases such as Alzheimer's disease (Hassan, 2004; Jeynes and Provias, 2009; Tan et al., 2015). Additionally, Tan et al. evidenced that this vascular occlusion occurs exclusively at the same hypoperfused areas identified in preclinical Alzheimer's disease (temporoparietal and retrosplenial granular cortexes, and hippocampus; Tan et al., 2015).

Adropin directly upregulates NOS expression in both *in-vivo* and *in-vitro* endothelial cells resulting in proliferation, migration, and capillary-like tube formation and diminished permeability and apoptosis of these cells (Lovren et al., 2010). Moreover, upregulation of NOS increases cerebral blood flow and prevents stress-induced hypotension, inflammation, apoptosis and cerebral ischemia (Lin et al., 2010). Thus, early administration of nitric oxide or its precursor to patients with acute stroke has been shown to affect lesion size, cerebral blood flow, mood, cognition and quality of life (Willmot et al., 2005; Woodhouse et al., 2015).

Adropin and Cluster of Differentiation 36 (CD36)

CD36 is a member of the class B scavenger receptor family and is activated by various ligands with diverse cellular responses—such as the production of free radicals, induction of inflammatory responses, and endothelial dysfunction (Cho, 2005, 2012). CD36 has anti-angiogenic nature and downregulates VEGFR2 phosphorylation, (Primo et al., 2005) and through its ligands such as oxLDL (a major factor in the development of atherosclerosis) causes endothelial cell stiffness and atherosclerosis (Shentu et al., 2010). Adropin downregulates CD36 gene expression and cell surface CD36 protein levels which indicate a potential reduction of muscle fatty acid uptake (Gao et al., 2015). Alongside, adropin treatment has been shown to downregulate peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC-1α) that regulates expression of CD36 (Gao et al., 2015).

CD36 is known to be one of the underlying causes of cerebrovascular and neurodegenerative diseases. Accumulation of β-Amyloid (a CD36 ligand) in the vicinity of plaques of Alzheimer's disease, and in the cerebrovascular wall of hemorrhagic stroke had been described (Winkler et al., 2001; Hernandez-Guillamon et al., 2012). Increased CD36 gene expression following blood-brain barrier damage and circulating amyloid β protein following ischemic insult might contribute to the pathogenesis of vascular dementia and bridge the gap between vascular dementia and Alzheimer's disease (Lee et al., 2005; Ueno et al., 2016).

Adropin and Glucose Oxidation

Adropin upregulates glucose oxidation via decreasing acetylation of pyruvate dehydrogenase complex (PDHC, a rate-limiting enzyme in glucose oxidation) and down-regulating pyruvate dehydrogenase kinase-4 (PDK-4)- a PDHC inhibitor. PDHC is a mitochondrial matrix enzyme complex that catalyzes oxidative decarboxylation of pyruvate to produce acetyl-CoA, which plays a critical role in cerebral aerobic energy metabolism (Cardell et al., 1989; Martin et al., 2005). Impaired cerebral energy metabolism and PDHC activity are seen in acute brain injury and chronic neurodegenerative conditions such as Alzheimer's disease and Wernicke-Korsakoff syndrome (Martin et al., 2005). PDHC activity is attenuated after brain ischemia (Cardell et al., 1989; Martin et al., 2005). This reperfusion dependent suppression might be due to the depressed activity of pyruvate dehydrogenase phosphatase or oxidative stress (because of hyperoxic resuscitation) (Martin et al., 2005). Inactivation of PDHC can be a possible cause of post-ischemic metabolic depression, prolonged intracellular lactic acidosis, and secondary

tissue energy depletion, which contribute to neuronal injury and neurological impairment (Cardell et al., 1989; Martin et al., 2005). In addition, compensating the enzyme activity by administration of acetyl-L-carnitine which is converted to acetyl-Co or dichloroacetate (DCA) improves neurologic outcome (Rosenthal et al., 1992; Martin et al., 2005). Adropin treatment in animal studies increases the ratio of CoA/acetyl-CoA which directly promote PDHC activity and pyruvate oxidation (Gao et al., 2015).

Adropin and Endothelial Permeability

The involvement of Adropin in endothelial permeability was originally described by Lovren and coworkers in 2010 (Lovren et al., 2010). Adropin attenuates the hypoxic/low glycemic induced paracellular permeability by inhibiting ROCK/MLC2 signaling pathway **(Figure 1**; Yang et al., 2016). As described by Wojciak-Stothard and Ridley, the endothelial permeability is determined by intercellular junctions integrity and basal intracellular actinomyosin contractility (Wojciak-Stothard and Ridley, 2002). Rho GTPases such as Rac 1 and Rho A act antagonistically to regulate endothelial permeability (Wojciak-Stothard and Ridley, 2002; Wojciak-Stothard et al., 2006). Rac 1 enhances the cellular junction and adherence, (Wojciak-Stothard et al., 2006) and inhibits Rho under chronic ischemia (Wojciak-Stothard et al., 2005). In contrast, Rho A and its downstream Rho-associated protein kinase (ROCK) enhance the marginal cell isometric tension and actinomysin contractility (Wojciak-Stothard et al., 2006). Hypoxic/hypoglycemic condition induces activation of Rho/ROCK signaling pathway by stimulating K-ras effector pathways independent of HIF-1 (Mizukami et al., 2006; Wojciak-Stothard et al., 2006; Yang et al., 2016) **(Figure 1)**. Activated ROCK promotes direct phosphorylation of myosin light chain 2 (MLC2) at Ser19 site and inhibition of myosin light chain phosphatase (MLCP). Phosphorylated MLC2 enhances actinomyosin contractility, intracellular tension and increases cellular permeability (Yang et al., 2016). In addition, down regulation of Rac 1 induces actin formation via Rho activation and intensifies contractility (Wojciak-Stothard et al., 2006; Weidemann et al., 2013).

Adropin and Neurogenesis

Induction of mesenchymal cells with inhibitors of prolyl hydroxylase—a key enzyme in HIF-1α degradation—promotes mesenchymal cells differentiation to morphologically neuron-like cells (Pacary et al., 2006). HIF-1α production under ischemic conditions induces potentially neurogenic factors—EPO (erythropoietin), p21 and VEGF (Jin et al., 2002; Yu et al., 2002; Pacary et al., 2006). Animal models of ischemic stroke demonstrated functions for VEGF in neuroprotection (better neurological outcomes and smaller infarct volume), neurogenesis (in both early and delayed phases in neuronal precursors) and in angiogenesis (endothelial cell proliferation, migration, survival and vascular permeability) (Jin et al., 2001; Sun et al., 2003; Shimotake et al., 2010). Although neurogenesis and angiogenesis are known to be coupled, the neurotrophic potential of VEGF might be independent of angiogenesis: VEGF induces axonal outgrowth—by acting on growing axons and nerve cell bodies—and suppresses the cell-death pathways mediated by calpain-dependent and caspase-3-dependent mechanisms (Sondell et al., 2000; Jin et al., 2001; Shimotake et al., 2010).

Recent studies demonstrated that inhibition of Rho/ROCK signaling pathway enhances HIF-1 activity and upregulates EPO, VEGF and p21, and consequently potentiates neurogenesis (Pacary et al., 2007, 2008). Adropin might be a novel candidate to promote neurogenesis as it can inhibit the Rho/ ROCK pathway without affecting VEGF level (Yang et al., 2016).

Adropin and Orphan G Protein-Coupled Receptor (GPR19)

Stein et al. discovered GPR19 as a potential adropin receptor (Stein et al., 2016). GPR19 is a transmembrane receptor similar to the neuropeptide Y receptors and the dopamine D2 receptor family (O'Dowd et al., 1996). GPR19 is more likely expressed in cerebellum, caudate, putamen, thalamus, hypothalamus, hippocampus, frontal cortex and olfactory bulb (O'Dowd et al., 1996; Hoffmeister-Ullerich et al., 2004). Transcripts of GPR19 can be detected in neuroectodermal origin tissues in early embryogenesis, and they are gradually restricted to the regions of the developing brain (Hoffmeister-Ullerich et al., 2004). Signal transduction through GPR19 enhances ERK and Akt phosphorylation in cerebral neurons (Hossain et al., 2016). Recently, Stein et al. described the adropin function in water intake inhibition through GPR19 (Stein et al., 2016). However, the distribution of GPR19 and potency of its downstream signaling pathways suggest more critical actions for adropin in neuronal development and protection.

CONCLUSION

Studies regarding the effects of adropin in different organs are still in infancy stage, but increasing evidence suggest that this peptide has unique effects on endothelial cell function via upregulating eNOS expression through the VEGFR2-PI3K-Akt, VEGFR2-ERK 1/2 pathways and inhibition of Rho/ROCK pathway. However, our current knowledge mainly comes from animal studies or treatment with the putative secreted domain of adropin. Whether these findings are transferable to clinical studies needs to be determined. Moreover, adropin may be utilized as a promising biomarker for CNS disease risk stratification or diagnosis, and/or a potential therapeutic candidate in CNS injuries. Although adropin seems to be a novel target to limit vascular diseases, in parallel with the documented effects on metabolic modulation, further investigations are needed to elucidate the specific mechanism underlying the association between adropin and CNS diseases.

AUTHOR CONTRIBUTIONS

Study concept and design: SA, RZ. Acquisition of data: SS, SA, TP, RZ. Drafting and critical revision of manuscript: SS, SA, TP, RZ. Study supervision: TP, RZ.

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REVIEW
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Advances in Stem Cell Research- A Ray of Hope in Better Diagnosis and Prognosis in Neurodegenerative Diseases

Shripriya Singh[1,2], Akriti Srivastava[1], Pranay Srivastava[1], Yogesh K. Dhuriya[1,2], Ankita Pandey[1], Dipak Kumar[1,2] and Chetan S. Rajpurohit[1,2]*

[1] System Toxicology and Health Risk Assessment Group, Council of Scientific and Industrial Research-Indian Institute of Toxicology Research, Lucknow, India, [2] Academy of Scientific and Innovative Research, Lucknow, India

Neurodegeneration and neurodegenerative disorders have been a global health issue affecting the aging population worldwide. Recent advances in stem cell biology have changed the current face of neurodegenerative disease modeling, diagnosis, and transplantation therapeutics. Stem cells also serve the purpose of a simple *in-vitro* tool for screening therapeutic drugs and chemicals. We present the application of stem cells and induced pluripotent stem cells (iPSCs) in the field of neurodegeneration and address the issues of diagnosis, modeling, and therapeutic transplantation strategies for the most prevalent neurodegenerative disorders. We have discussed the progress made in the last decade and have largely focused on the various applications of stem cells in the neurodegenerative research arena.

Keywords: neurodegenerative disorders, stem cells, Induced pluripotent stem cells (iPSCs), disease modeling, diagnosis

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Vimal Kishor Singh,
Delhi Technological University, India
Abhijit Biswas,
University of Notre Dame, USA

****Correspondence:***
Shripriya Singh
shripriyas9@rediffmail.com;
shripriya.singh@iitr.res.in

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INTRODUCTION

Progress in the field of clinical research and medicine has decreased global mortality drastically. The developed countries have extended the life span of their aging population. However, the modern world is now faced with the issues of aging and age related disorders. Neurodegeneration and neurodegenerative disorders are one of the major health implications faced by the aging population. Neurodegeneration studies have largely benefited from neuropathology and *in-vivo* research (Agrawal and Biswas, 2015). Neurodegenerative disorders have been thoroughly investigated using animal models, primary cultures, and post mortem human brain tissues (Marchetto et al., 2011). Though informative, these approaches have some limitations. Data obtained from animal models fails to directly correlate with that of humans because a rodent brain is not an exact mimic of a human brain. Despite being highly conserved evolutionarily, mammalian genomes are not identical. The embryonic development of mice and humans are considerably different and almost 20% genetic variability is accounted for (Strachan et al., 1997). Therefore species' difference prevents the animal data from successful validation during clinical field trials which poses a severe economic burden. A study reported the failure of therapeutic drugs for treating amyotrophic lateral sclerosis in human beings, which had earlier proved effective in case of rodents (Takahashi and Yamanaka, 2013). Preclinical studies often do not efficiently translate to the clinic and the clinical trial failures have been reported time and again (Prinz et al., 2011; Begley and Ellis, 2012). Primary

culture of neurons is challenging because these are the post mitotic differentiated cells which are difficult to sustain in the *in-vitro* conditions. Ethical constraints have held back human based research and thus the best possible source of human samples are the postmortem brain tissues. However, these autopsied samples depict the end stages of the disease and do not give much insight into the intricacies of the disease' developing stages (Marchetto et al., 2011). Researchers are not willing to subject the human beings to untested interventions, but the choices have been limited so far.

Majority of neurodegenerative disorders have been incurable (Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic lateral sclerosis) so far but timely diagnosis can help in the management and symptom alleviation. However, researchers across the world are continuously striving to achieve the cure and hope to achieve fruitful results in the near future. Neurodegeneration studies are largely divided into two major categories. One is the experimental modeling strategy which allows for a comprehensive understanding of the disease such as the etiology, pathophysiology, genotypic-phenotypic interactions, symptomatic, and mechanistic insights. The second is the medical approach which deals with the treatment, therapy, and disease management. Stem cells and iPSCs find widespread application for both, disease modeling as well as transplantation and regenerative therapeutics. In the present review we shall discuss the applicability of stem cell research in the field of neurodegenerative disease modeling and provide the current updates of how stem cell and induced pluripotent stem cell based studies have been employed to address the diagnosis and therapy of the most common neurodegenerative disorders. We shall briefly touch upon the advances and preferable methodologies employing stem cell and iPSC culture such as the three dimensional (3D) culture which has revolutionized the current trend of *in-vitro* studies. The article intends to highlight the fact, that though animal based *in-vivo* research is absolutely necessary for the neuroscience research, one cannot wholly and solely depend upon it and human based stem cell driven research has and will open newer avenues for the neurodegenerative disorders′ modeling and treatment.

STEM CELLS AND INDUCED PLURIPOTENT STEM CELLS (IPSCS) IN NEURODEGENERATION: WHY THE CHOICE?

It is easier to say that cells of human origin can be directly employed to generate a clearer picture of the neurodegenerative diseases but practically the approach is not as simple as it seems. The *in-vitro* scenario is devoid of an intact organ system, organ-organ interactions are missing and the blood supply and connective tissues are lacking. Every disease has its characteristic cellular, molecular, anatomical, genotypic, and phenotypic attributes. If one has to model these various aspects *in-vitro*, very specific cell types expressing the disease phenotypes are required. Sustaining the culture of such specific cells is another challenging task that requires standardized protocols, select growth conditions, and expertise. For example, Parkinson's disease requires the culture of dopaminergic neurons, ALS requires the culture of glial cells, motor neurons, and astrocytes and Huntington's disease requires the culture of medium spiny and striatal projection neurons as discussed in the later sections. All these requirements have been largely met by the use of stem cell technology.

Stem cells in brief are the naïve cells of the body with an exceptional ability to self-renew, proliferate, differentiate, and get programmed for multi-lineage commitment (Cananzi and De Coppi, 2012; Liu et al., 2013; Kumar et al., 2015). Their origin can either be fetal, embryonic, or adult tissues of the body (Nam et al., 2015; Singh et al., 2015). Despite a few ethical concerns stem cell biology is finding widespread applicability in the field of research and medicine. Stem cells can be practically converted into any possible cell type and thus are being routinely used to model diseases. Monogenic disorders with a clear cut cellular phenotype and high penetrance are comparatively easier to model than the late onset diseases involving a number of genes and showing less penetrance. In case of the monogenic disorders the disease associated gene is deliberately mutated via gene editing to obtain the stem cell models. Embryonic stem cells (ESCs) harboring the chromosomal aberrations are used for the modeling of the chromosomal diseases. The late onset complex diseases which cannot be prenatally diagnosed are modeled using patient derived iPSCs more effectively (Avior et al., 2016).

In-vivo animal models have so far been used to experimentally model diseases however, the data generated fails to recapitulate the human diseases and thus cannot be directly extrapolated (Yamanaka, 2009). This forms a major limitation of the various animal based studies. Only samples of human origin can be employed to overcome this major hurdle. Neurodegeneration leads to a gradual loss of brain functionality via an irreversible gradual loss of neurons and other cells of the central nervous system (Peng and Zeng, 2011). In this regard transplantation therapy is employed to restore and repair the damaged circuitry of the brain as well as to replenish the lost neuronal population (Thompson and Björklund, 2015). Successful commitment of stem cells toward the neuronal lineage is widely reported and myriad of protocols are available to achieve the same (Nikoletopoulou and Tavernarakis, 2012; Ferroni et al., 2013; Lu et al., 2015).

Diseases such as brain ischemia (Ju et al., 2014), spinal muscular atrophy (Frattini et al., 2015), spinal cord injury (Lukovic et al., 2015), amyotrophic lateral sclerosis (Nicaise et al., 2015), Machado-Joseph disease (Mendonça et al., 2015), and many more have been studied and stem cell therapy has been effectively employed for the same. Embryonic stem cells are pluripotent in nature and hold an excellent potential for restoring brain injuries and neurodegeneration via transplantation therapy, however tumor formation restricts their widespread application (Aleynik et al., 2014). Mesenchymal stem cells which are multipotent also find widespread application because they are immunomodulating in nature. Immunomodulation simply refers to the unique ability to escape the host's immune system surveillance thereby leading to successful transplants without eliciting an adverse immune response (Glenn and

Whartenby, 2014). Neural progenitor cells or NSCs isolated from fetal brain are again multipotent in nature and are stringently committed toward the neuronal lineage. They are suitable because of a reduced risk of tumor formation however they are difficult to procure and usually few in number (Jiang et al., 2012). Apart from naïve stem cells, iPSCs derived via a reverse programming of somatic cells finds widespread application now a days (Takahashi and Yamanaka, 2006). Induced pluripotent stem cells are being produced in bulk and various iPSC cell lines are commercially made available. The use of Parp1 i.e., poly(ADP-ribose) polymerase 1 for the production of iPSCs is now well reported and this has also reduced the tumor forming risk sufficiently (Chiou et al., 2013). However, the formation of teratomas has not been completely eliminated. Production of iPSCs is well reported from various somatic cells of the body such as the peripheral blood cells, hepatocytes, stomach cells (Okano et al., 2013), and keratinocytes (Aasen et al., 2008). It is now well reported that just 10 μl of capillary blood drawn from finger tips can be used to generate iPSCs (Tan et al., 2014). Patient derived induced pluripotent stem cells are widely used cells of human origin which can directly be used to model the various human neurodegenerative diseases (Sterneckert et al., 2014). Scientific groups have reported disease specific iPSCs cell lines (Dimos et al., 2008). Stem cells and induced pluripotent stem cells have been widely used for modeling several neurodegenerative diseases as well as used in transplantation therapy. Spinal muscular atrophy has been efficiently modeled *in-vitro* using patient derived iPSCs (Sareen et al., 2012; Wang et al., 2013). The first of their kind, these iPSCs derived models efficiently depicted the disease phenotypes. However, iPSCs too have a few limitations. Neurodegenerative disorders are generally the late-onset diseases and symptoms begin to manifest with increasing age. Thus modeling such diseases via the animal models is not only time consuming but also cost heavy. It is generally assumed and even reported that patient derived iPSCs harbor the disease mutations and carry the epigenetic background of the patient, thus making them an excellent choice as the *in–vitro* disease models. However, when somatic cells are reprogrammed to an induced pluripotent state they lose the age associated features, undergo rejuvenation, and their embryonic age is reestablished. It is well reported that even aged donor derived iPSCs are rejuvenated and age reversal is evident as there is a loss/decrease in the markers of senescence, enhanced mitochondrial fitness, and an increased telomere length (Lapasset et al., 2011; Freije and López-Otín, 2012). Thus even these patient derived iPSCs do not effectively model the late onset neurodegenerative diseases as they lack the age related phenotypes. However, this hurdle has been largely overcome by progerin induced aging in the iPSCs (Miller et al., 2013). Progerin is a truncated transcript of lamin A (nuclear envelop protein) formed by mutation in the gene LMNA. Accumulation of progerin in the nuclear membrane results in the dysfunction of lamin A resulting in interrupted chromatin organization, cell cycle, telomere maintenance, and DNA damage response. High progerin levels are associated with aging. Age related phenotypes have been observed in the progerin exposed iPSCs such as degeneration of dendrites, neuromelanin accumulation, AKT deregulation, mitochondrial swelling, and reduction in the TH-positive neurons (Miller et al., 2013). These iPSCs harboring the aging markers will thereby mimic the neurodegenerative disorders more efficiently. In the upcoming sections we shall discuss the application of stem cells and iPSCs for the most common and globally prevalent neurodegenerative disorders. **Figure 1** shows a diagrammatic representation of progress made in the field of stem cell research in context with neuroscience.

ALZHEIMER'S DISEASE (AD)

Alzheimer's disease (AD) is described as "Presenile dementia" by German psychiatrist Alois Alzheimer and is one of the most prevalent neurodegenerative disorders of the world. It is a leading cause of dementia in the aging population and has lately been declared the sixth major reason for death. Patients having AD are reflected with cognitive deficits, memory loss, and behavioral changes and these changes are inherently associated with neurodegeneration (Blundell and Shah, 2015). Hippocampus, amygdala, neocortex, and basal forebrain regions of the brain are adversely affected leading to the severe impairment of cognition and memory. Neurofibrillary tangles (NFT) and β-amyloid plaques are the pathological hallmarks of AD (Peng and Zeng, 2011). Hyperphosphorylation of tau proteins and amyloid peptide aggregates are responsible for the formation of NFT and β-amyloid plaques respectively. Alzheimer genetics involves the mutated forms of presenilin 1 (PSEN1), presenilin 2 (PSEN2), Amyloid Precursor Protein (APP), and apolipoprotein E. There are still no permanent treatments available for AD except acetylcholinesterase (AChE) inhibitors which provide only temporary relief (Birks, 2006; Lindvall and Kokaia, 2006). Several drugs serve as potent acetylcholinesterase inhibitors such as tacrine, tacrine derivatives, donepizel, rivastigmine, galantamine, and the glutamate receptor agonist memantine (Romero et al., 2013; Cecilia Rodrigues Simoes et al., 2014; Ehret and Chamberlin, 2015). These FDA approved pharmacological interventions provide only symptomatic relief for a limited period and may also have side effects in the long run. Removal of the Amyloid β levels from the brain is considered an effective therapy for AD and physiologically, the enzyme neprilysin has been reported to be involved in the clearance of the Amyloid β plaques by degrading it (Iwata et al., 2001). Other proteinases such as cathepsin B (Mueller-Steiner et al., 2006) and plasmin (Melchor et al., 2003) too have a similar role and are used to decrease the levels of Aβ thus acting as potent therapeutic agents for AD. A number of past studies show the relevance of nerve growth factor (NGF) in the prevention of neurodegeneration and amyloid toxicity (Tuszynski et al., 2005; Tuszynski, 2007) but a severe limitation with NGF is that it is unable to cross the blood brain barrier and therefore cannot be delivered peripherally.

Transgenic animal models of AD carrying the disease mutations have given ample insight into the etiology and pathophysiology of the disease but have failed to entirely recapitulate the formation of NFT and β-amyloid plaques together. Human disease pathophysiology has not been completely depicted by mouse models so far (D'avanzo et al.,

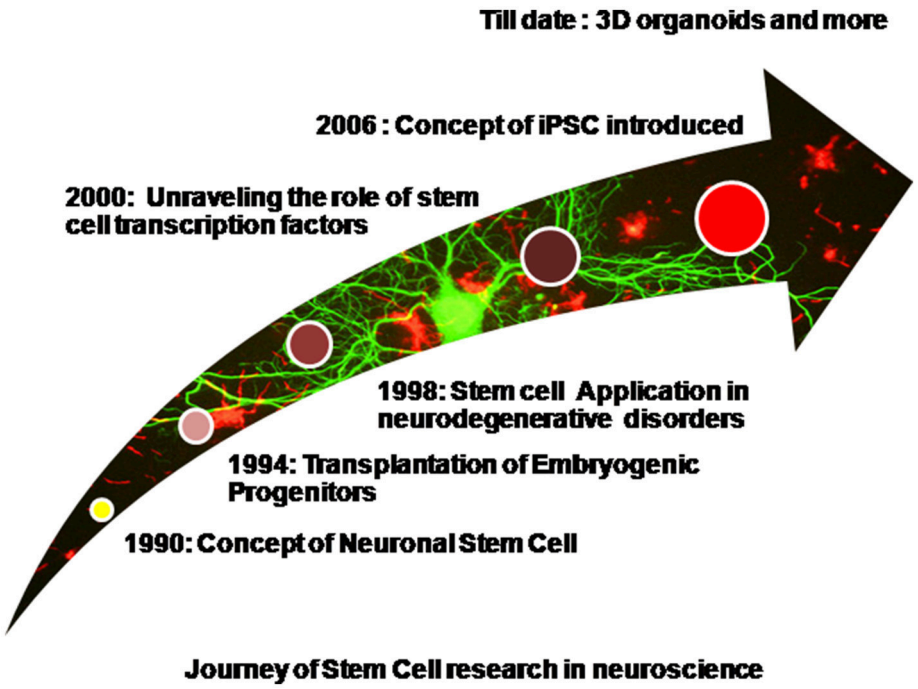

FIGURE 1 | Diagrammatic representation of progress made in the field of stem cell research in context with neuroscience.

2015; Pistollato et al., 2015) and clinical failure of drugs has also been reported (Cavanaugh et al., 2014; Langley, 2014). Past studies demonstrate that the implantation of genetically modified fibroblast cells into the forebrain of the patients led to decreased neurodegeneration and restored the cognitive deficits associated with AD (Tuszynski et al., 1994). However, fibroblasts are immobile in nature and cannot migrate efficiently within the different brain regions, but on the other hand transplanted stem cells can effectively migrate and release growth factors to the damaged sites. Thus, positional stem cell transplantation therapy may prove more fruitful in this regard (Flax et al., 1998).

Many published studies report the successful use of stem cell transplantation strategies that have helped in the management of AD. One study showed that ESCs derived neural stem cells (NSCs) when transplanted into the mouse model of AD served as a better tool for the treatment for AD in comparison to using ESCs alone. The transplanted NSCs were stable, successfully differentiated into the cholinergic neurons and memory was found to be increased. On the other hand the vehicle group which received ESCs alone developed teratomas (Wang et al., 2006). Another study was carried out on the mouse brain that expressed aggregates of plaques and tangles. Genetically modified NSCs for expression of BDNF were successfully transplanted into the rodent brain which led to improved learning and memory. These NSCs did not decrease the level of the amyloid plaques instead the increased levels of BDNF helped in the formation a number of new synapses (Blurton-Jones et al., 2009).

Stem cells and iPSCs have been extensively employed to study the human specific responses and unravel the complexities of AD. In 2011, Yagi et al. first derived neurons from patient iPSCs which carried mutant (PSEN2) and (PSEN1) (Yagi et al., 2011). Since then a number of studies have been dedicated toward this approach of patient specific iPSC derived AD modeling. The catalytic subunit of the enzyme gamma-secretase is encoded by the gene (PSEN1) and mutated form of this gene results in the manifestation of the early stages of FAD (familial Alzheimer's disease). Stem cells models of the disease have largely targeted the involvement of gamma-secretase activity in the elucidation of sporadic as well as familial AD. For example, studies report that the Aβ42/40 ratios were higher in neurons derived from patient iPSCs (derived from PSEN1 mutant fibroblasts) in comparison to the healthy controls (Livesey, 2014). Similarly mutations in APP have also led to a similar increase in the Aβ42/40 ratios in the neurons of the human forebrain. A couple of studies have modeled FAD using iPSCs carrying mutations in the APP, such as dominant V717L and recessive E963Δ bearing mutation (Kondo et al., 2013) and the V717I mutation bearing iPSCs (Muratore et al., 2014). A study carried by Israel et al. demonstrated that inhibition of gamma or β-secretase activity led to a decreased production of Aβ40, however γ-secretase inhibition did not prevent phosporylation of tau proteins (Israel et al., 2012).

Stem cell derived neurons and astrocytes have been widely used to model FAD *in-vitro*. Gene mutations bring about observable changes in the cellular phenotype such as the Aβ peptide changes. However, how APP processing is interlinked with tau phosphorylation is an aspect which has not been efficiently modeled so far. The onset and initiation of AD is largely attributed to the amyloid hypothesis. APP is a single

pass transmembrane protein and its proteolytic cleavage forms short Aβ peptides (Livesey, 2014). Mutation in the genes that are involved in the proteolysis of APP play a pivotal role in FAD. Aβ42 is the longer form of amino acid and its accumulation brings about neurodegeneration and cell death (Sproul et al., 2014). Post translational and cellular localization changes in the microtubule-associated tau protein play the second biggest role in AD progression. Tau changes and amyloid plaques if modeled together via stem cells will provide the best models of AD (Moore et al., 2015). Simple monogenic iPSCs' derived neurons are not sufficient to model amyloidosis since they accumulate low levels of toxic β-amyloid plaques. To overcome this hurdle stem cell lines bearing multiple mutations have been generated, which also over express the mutated genes such as APP and PSEN1.

PARKINSON'S DISEASE (PD)

Parkinson's disease ranks second after AD in being the most common and widely prevalent neurodegenerative disorder inflicting almost one percent of the aging population globally. Dopaminergic neuron loss from the nigrostriatum and substantia nigra pars compacta brain regions is the major characteristic of the disease (Marchetto et al., 2010). The other major hallmark being the presence of lewy bodies (α-synuclein aggregates) (Spillantini et al., 1998) which further promotes neuronal death due to the altered firing pattern of the neurons (Janezic et al., 2013). The genetic involvement of ubiquitin carboxy terminal hydrolase L1, serine threonine kinase 1, parkin, DJ-1, α-synuclein, and leucine-rich repeat kinase 2 have been reported in the case of genetically acquired familial PD (Dauer and Przedborski, 2003). Environmental influence in conjunction with age, genetic polymorphisms and chemical exposure predispose an individual toward sporadic PD, however the complex etiology is yet to be fully understood (Adami et al., 2014). Fitzmaurice et al. showed that variation in Aldehyde dehydrogenase enhances the pesticide effect related to PD thereby proving that environmental influences work in conjunction with genetics (Fitzmaurice et al., 2014).

Rigidity, resting tremor and bradykinesia are the major symptoms which make PD the most common movement disorder of the world affecting individuals post 65 years of age (Fu et al., 2015). Mechanistic and pathophysiological studies have given us a deep understanding of the disease. PD is generally associated with a disrupted calcium homeostasis, inflammation, disrupted kinase pathways, generation of reactive oxygen species, and dysfunctional mitochondria (Schapira et al., 2014; Xiao et al., 2016). Animal and cellular models have given us a deep understanding of the disease but data generated is not fully applicable to human subjects due to difference in disease pathogenesis of animals and humans (Devine et al., 2011).

PD so far has been managed using monoamine oxidase inhibitors, dopamine agonists, levodopa, and deep brain stimulation (Politis and Lindvall, 2012). The latter employs stimulation of the ventral intermediate nucleus, a part of the thalamus which can greatly reduce the symptoms of tremor. Other symptoms like rigidity and bradykinesia are also alleviated after the stimulation of the subthalamic nucleus or internal segment of globus pallldius. However, these treatments fail to repair the damaged brain region and the oral drugs are not effective beyond 5 years. Administration of L-DOPA (L-dihydroxy-phenyl alanine) can induce dyskinesis and fails to halt the disease progression (Politis and Lindvall, 2012).

The earliest transplantation studies employed the use of fetal ventral mesencephalic tissue of human origin (hfVM) which were engrafted in the striatum of the PD patients and laid the basis for cell therapy for PD. The attempts were successful and symptomatic relief was provided for almost 16 years in the successful cases. However, the clinical trials produced anomalies and the success of the approach was further challenged by the presence of side effects such as GIDs (graft induced dyskinesis). The reason was the presence of the serotonergic neuroblasts in the hfVM that led to an imbalanced serotonin/DA transporter ratios and false DA release (Politis and Lindvall, 2012). Studies also indicated that the survival of the transplanted fetal mesenchymal cells was very low and ethical issues further blurred the scope of this therapy. Since PD is characterized by a regional loss of dopaminergic neurons, transplantation, and replenishment therapy employing stem cell and iPSC derived dopaminergic neurons (yielding a pure population) in the SN region provides an excellent alternative.

Neurons with the DA phenotype have been developed from ESCs by using sonic hedgehog (Shh) and fibroblast growth factor-8 (FGF8) or the over expression of Nurr1 by using genetically modified NSCs (Kim et al., 2003). Co-culture of mouse bone marrow stromal and monkey ESCs have successfully yielded dopaminergic neurons in the past (Takagi et al., 2005). Studies further demonstrate the successful intrastriatal transplantation of fetal brain human NSCs in the MPTP lesioned monkeys which brought about improved behavioral changes (Redmond et al., 2007). A study reported that when NSCs were isolated from the patient brain, converted into dopaminergic neurons and re-implanted into the patient brain, the symptoms of trembling and rigidity were substantially reduced. The brain scans revealed an increase in the dopamine production by almost 58% and even when the levels did not increase further the symptoms did not revert back, hinting at a possible restorative potential of stem cell derived dopaminergic neurons (Hassan et al., 2010).

Transition from pluripotent stem cells to iPSCs has shown promising results and has opened new avenues for the modeling of PD. Dopaminergic neurons derived from patient specific iPSCs were successfully transplanted into a Parkinsonian rat striatum and showed a considerable reduction in motor asymmetry (Hargus et al., 2010).The role of mitophagy has been strongly implicated in PD. The mitochondrion targeted kinase PINK1 accumulates on the outer membrane of mitochondria on depolarization and further recruits Parkin which is instrumental in initiating mitophagy (Van Laar et al., 2010; Cai et al., 2012). The data generated on animal models so far has generated conflicts regarding the direct involvement of mitophagy in PD. However, PINK1 mutated dopaminergic neurons from human patient derived iPSCs have given us a clearer understanding of the mitophagy theory, thus proving once again that stem cell derived human disease models are far superior and far more edifying than

any animal model (Seibler et al., 2011). It is also implicated that mitophagy may be a result of aging and may not have a direct correlation with the disease; however the hypothesis is still under contradiction. Progerin expression and long term culture have been employed to produce artificial aging of neurons in culture. These aged neurons have been treated as a model to study the late onset of PD and to elucidate the disease phenotypes (Miller et al., 2013).

As mentioned earlier, apart from disease modeling, stem cells, and iPSCs hold great promise as the *in-vitro* screening tools for therapeutic agents, drugs, and compounds. It has been reported that rapamycin, GW5074 (LRRK2 kinase inhibitor) and coenzyme Q10 diminish the cytotoxic effect of concanamycin A also known as valinomycin in patient derived iPSC neurons. It has been clearly seen that GW5074 does not reduce oxidative stress in the healthy neurons from control subjects whereas effectively lowers oxidative stress in the patient derived neurons bearing mutated PINK1 (Cooper et al., 2012). This difference underlines the importance of therapeutic compound screening in disease mutation bearing cells as well as the healthy cells. Another example where stem cells have been employed to study the PD physiology is the mutation correction of A53T α-synuclein via genome editing which diminished the formation of lewy bodies in iPSC derived dopaminergic neurons (Ryan et al., 2013). Mutation bearing iPSCs will thus serve as an excellent tool for screening and assessing the biosafety of drugs and compounds as well as identifying the underlying signaling cascades and novel therapeutic targets. The generation and characterization of iPSCs is cumbersome and the differentiated population of dopaminergic neurons may contain traces of the undifferentiated cells which may lead to teratoma formation. Thus, if patient derived fibroblasts are directly converted to dopaminergic neurons, the limitations with iPSCs can be overcome (Han et al., 2015). The successful differentiation of fibroblasts into the DA neurons are reported in literature and has paved way for potential disease modeling (Caiazzo et al., 2011; Kim et al., 2014). The stem cell technology can be used to identify the biochemical markers of the disease and can thus help in the diagnosis of early PD (Xiao et al., 2016).

AMYOTROPHIC LATERAL SCLEROSIS (ALS)

A fatal neurodegenerative disease ALS is caused due to the motor neuron degeneration in the spinal cord, brain stem, and the primary motor cortex (Thomsen et al., 2014) which results in muscle wasting, paralysis and eventually death due to respiratory failure (Hedges et al., 2016). First described by Charcot in 1874, ALS is also known as Lou Gehrig's disease (Marchetto et al., 2010). ALS has been linked with FTD (frontotemporal lobar dementia) due to symptomatic, clinical, genetic, and pathological overlap. ALS and FTD when occur together further shorten the life span of a patient further. The two diseases are often considered the two ends of the same disease spectrum. Ling et al. gave evidence at the genetic level by reporting that FTD-ALS and ALS patients carry similar mutated genes (Lee and Huang, 2015). Many gene mutations are responsible for causing familial ALS such as mutations in PFN1, FUS/TLS (fused in sarcoma/translocation in liposarcoma), TARDBP or TDP-43 (TAR-DNA-binding protein 43), UBQLN2, C9ORF72, SOD1 (superoxide dismutase 1), HNRNPA1, OPTN, and VCP (Adami et al., 2014). Recently a new gene named TBK1 has been discovered which plays a crucial role in inflammation & autophagy which are inherently associated with ALS pathogenesis (Cirulli et al., 2015). Pathogenesis of sporadic ALS is attributed to glutamate excitotoxicity, protein mitochondrial dysfunction, aggregation, oxidative stress, deficiency of neurotrophic factors, glial cell dysfunction, and impaired axonal transport, all of these together eventually lead to the accumulation of intracellular neurofilaments (Kiernan et al., 2011; Robberecht and Philips, 2013). Riluzole is the only therapeutic drug commercially available which helps in the disease management, but its effect does not last beyond 6 months (Cetin et al., 2015). ALS has been widely studied using animal models; however reported failure of clinical trials has somehow restricted the sole dependency on *in-vivo* research (Gordon and Meininger, 2011). Patient specific iPSC banks hold promise for personalized medicine and are a good alternative for screening the efficacy of a number of drugs and compounds for the treatment of ALS (Giri and Bader, 2015).

Transplantation therapy employing stem cells can be effectively used as a therapeutic measure to deal with the devastating disease. Mesenchymal stem cells and hematopoietic stem cells have been efficiently employed as transplants in the affected spinal cord and have favorably supported ALS management (Mazzini et al., 2012). However, studies were conducted on a small group of patients and thus thorough research continues so as to be applicable for a larger pool of patients. Neural stem cells (NSCs) ESCs, glial-restricted progenitor cells (GRPs), and induced pluripotent stem cells (iPSCs) also offer a potential alternative for transplantation approaches and can be used (Traub et al., 2011). It is hypothesized that when donor cells are engrafted near the damaged motor neurons, they not only have an immunomodulatory effect but secrete trophic factors which improves the overall therapeutic potential of the transplant. Such transplants can effectively delay the progression and even the initiation of the disease (Teng et al., 2012).

The direct or peripheral injection of MSCs into the spinal cord of patients serves as a potent treatment for ALS and several studies have reported the therapeutic potential of MSCs (Mao et al., 2015). Several studies report the beneficial aspects of transplantable MSCs, which are used to deliver the required neurotrophic factors aiding in the prevention of motor neuron loss, improve survival of experimental animals, and delay the disease progression (Zhao et al., 2007; Vercelli et al., 2008; Uccelli et al., 2012; Krakora et al., 2013). Genetically manipulated MSCs which secrete GDNF (glial-derived neurotrophic factor) have been reported to increase the life of ALS rats by rescuing motor neuron loss (Suzuki et al., 2008). "NurOwn" developed by BrainStorm Cell Therapeutics are specialized MSCs which can secrete neurotrophic factors, can successfully differentiate into neuronal cells and can be used for ALS treatment. The cells are

under the clinical trial phase (Therapeutics, 2015). A number of clinical trials have been conducted to assess the efficacy and safety of MSCs and many are still in process.

Neural stem cells have a specific lineage commitment for the cells of the CNS and find widespread applicability in the neurodegenerative disorders. When engrafted in the animal models of ALS, NSCs have been reported to exert a protective effect on the adjacent motor neurons (Hefferan et al., 2012). Stem cells not only provide symptomatic relief but help in restoration of the brain damage via repair and neurogenesis which is triggered in the affected spinal cord. Successful transplantation of neural stem cells from aborted fetuses into the patient's spinal cord has been reported (Xu et al., 2012). Results of the phase I clinical trials have been documented and show that stem cell therapy is a reasonably safe and can be used to treat a large enough pool of patients (Mazzini et al., 2015).

The familial cases of ALS can be modeled by ESCs harboring the disease mutations but the sporadic cases require the use of patient specific iPSCs. Literature reports the use of iPSCs for ALS modeling and hints at a possibility that motor neurons derived from patient specific iPSCs can be employed for the recapitulation of disease phenotypes. ALS exhibits a complex physiology and thus requires use of more than one cell type for its modeling. It was shown that SOD1 bearing astrocytes (from ESCs) exerted toxicity on the adjacent motor neurons (Wada et al., 2012) whereas iPSCs derived astrocyte bearing the TDP-43 mutation exerted no toxicity (Serio et al., 2013), hinting at the relevance of the co-culture of cells for modeling the complex disease. One drawback with *in-vitro* disease modeling of ALS is the short survival duration of motor neurons in culture which limits the study of phenotypic signs occurring in the aged diseased tissues. However, if iPSC derived motor neurons are grafted in the animal model it will increase the survival of these cells. The grafted cells can be recovered lately to visualize the disease phenotypes in the post mortem rodent tissues, thus offering a possible solution (Coatti et al., 2015).

Studies report that iPSC derived glial rich population of neural progenitors can be successfully transplanted into the spinal cord of mice suffering from ALS. These transplanted cells show a good survival, differentiation potential, and also enhanced the life span of the treated animals (Kondo et al., 2014). Stem cell therapy has been an area of debate for a long time. The beneficial aspects cannot be overlooked, but extensive clinical trials are in progress so as to generate an effective treatment and possible cure for ALS in the near future.

HUNTINGTON'S DISEASE (HD)

Huntington's disease is a fatal genetic neurodegenerative disorder with no cure so far. Genetically acquired in an autosomal dominant manner, the disease is caused by an increased trinucleotide CAG (encoding polyglutamine) repeat in the ITI5 huntington gene. The CAG repeats are normally less than 36 in a healthy individual however a repeat of more than 40 will predispose an individual toward the disease. The striatum, cortex, substantia nigra pars compacta, substantia nigra pars reticulata, globus pallidus are the major brain regions which are subjected to severe degeneration (Carter and Chan, 2012). Enkephalin and gamma-aminobutyric acid containing medium spiny neurons, glutaminergic, GABAergic, and parvalbuminergic striatal projection neurons are severely affected. Mutated Huntington protein forms aggregates in the nuclei and cytoplasm of the brain tissues. Being a Cognitive, movement, psychiatric disorder the disease brings about mitochondrial, synaptic, and axonal transport dysfunction. The disease leads to a severe transcriptional dysregulation, proteolysis, and excitotoxicity (Peng and Zeng, 2011).

The disease, though incurable can be managed via gene therapy and drugs to alleviate symptoms. Drugs provide temporary symptomatic relief and largely target the motor aspects of HD. Tetrabenazine is dopamine depleting in nature and is used to reduce chorea, however severe side effects restricts its widespread use as a drug of choice for HD (Frank, 2014). The pros and cons of the various animal models of HD have been reviewed by Pouladi et al. In 2013 (Pouladi et al., 2013). HD has been widely modeled via rodent models *in-vivo*, however here too the success of the clinical trials has been limited. The problem lies not only with the animal model chosen but also with the robustness of the preclinical studies (Menalled and Brunner, 2014).

Gene therapy employs RNAi mediated silencing and reduction in the mutated HTT protein translation. However, gene therapy is effective only at the earliest stage of the disease but the symptoms are not visible until the later stages by which the major damage is already done. Replenishing the stratial neuron population by intracranial transplantation is an alternative cell therapy approach. Fetal striatal tissue grafts have been employed in the non human primate and rodent HD models as a means of transplantation therapy (Chen et al., 2014). These grafted tissues have a low survival rate and since they are procured from aborted fetuses, ethical constraints are high. Limited availability of donors further restricts the scope of this promising strategy. Neural progenitor cells (NPCs) derived from stem cells and iPSCs are a good alternative in this regard.

Successful differentiation of human ESCs into the GABAergic and DARPP32 positive medium spiny neurons (MSNs) is well reported. NPCs transplanted into the chemical lesion mouse model of HD showed efficient connectivity with the host neurons and also alleviated motor deficits (Ma et al., 2012). Another study by Carri et al. reports the successful differentiation of ESCs and iPSCs into MSNs carrying adenosine and dopamine receptors (Carri et al., 2013). However, NPCs transplantation has been more successful in chemical lesion HD mouse models as compared to the transgenic (R6/2) mouse model, the reason speculated being the short life span of the transgenic mouse (El-Akabawy et al., 2012). Along with disease modeling and regenerative transplantation therapy, stem cells and iPSCs can be simply used as an *in-vitro* tool for screening the efficacy and biosafety of drugs and small molecule compounds that help in relieving the symptoms of the disease as well as bring about a reduction in neurodegeneration (Carter and Chan, 2012). HD patient specific cell lines have been generated which recapitulate the disease specific phenotypes more closely (Consortium, 2012).

The gene therapy for HD involves targeting the mutant HTT allele (mHTT) transcript by RNA interference and ASOs (antisense oligodeoxynucleotides) specifically without disturbing the normal HTT allele transcript (Carroll et al., 2011; Hu et al., 2012). The exact role HTT in normal conditions is still not clearly understood but a study reports that HTT knockout mice do not survive and are lethal embryonically. Thus targeting only the mutant allele is preferable. ASOs target complimentary mRNA via RNase H mediated degradation and are important in research as they are capable of targeting the SNPs (single nucleotide polymorphisms). These SNPs are responsible for the difference between normal and mutated HTT gene alleles (Carroll et al., 2011). Studies have been carried out in the non human primate and rodent models of HD and disease alleviation was observed (Kordasiewicz et al., 2012). This paves way for a possibility that patient derived iPSCs carrying the disease mutations can be effectively corrected via ASOs and gene silencing and the NPCs thus derived can be successfully transplanted. Thus stem cell therapy in amalgamation with gene therapy can help in the management of the incurable disease in future (Chen et al., 2014).

THREE DIMENSIONAL (3D) STEM CELL BASED STUDIES: APPLICATION IN NEURODEGENERATION AND NEURODEGENERATIVE DISORDERS

In-vitro stem cell based studies are the best possible alternative to animal models because cells of human origin are used and thus data generated can be easily extrapolated to human subjects. However, two dimensional *in-vitro* studies have many limitations. The 2D culture comprise a homogenous population of cells, complex intercellular interactions are lacking, the cultures do not represent the complex microenvironment of an organ like the brain and the intact organ physiology is lacking. In this regard 3D cultures have played a pivotal role in overcoming the hurdles of typical 2D studies and also find widespread application in the field of neuroscience. Advances have been made to recapitulate the brain development and literature supports successful formation of stem cell derived 3D cortical and cerebral organoids (Lancaster et al., 2013; Sasai, 2013; Lancaster and Knoblich, 2014).

AD has been extensively modeled using stem cell and iPSC derived 2D neuronal cell culture however, a major limitation being the diffusion of Aβ aggregates, which are gradually, washed off with successive media changes. Therefore, it is hypothesized that 3D stem cell models of AD will provide a more compact and comprehensive brain microenvironment where the local niche will allow a sufficient Aβ aggregation (D'avanzo et al., 2015). This theory has been confirmed lately, where 3D matrigel system has been used for the culture of ReN cells to model familial AD. The 3D stem cell model of AD showed a significant deposit of β-amyloid plaques in a 6 week differentiated culture. The cells further showed elevated levels of phosphorylated tau protein thereby confirming that 3D based stem cell models are more efficient in recapitulating the disease pathophysiology (Choi et al., 2014). A study by Zhang et al. reported the successful recapitulation of *in-vivo* like responses in a 3D culture model of AD. The study comprises neuroepithelial stem cell derived neuronal culture in a PuraMatrix hydrogel comprising self assembling peptide matrix and laminin. The study confirmed that 3D microenvironment allowed Aβ sensing via p21-activated kinase (Zhang et al., 2014). Genetically engineered hNPCs production and 3D cell culture protocols for AD modeling have been standardized and published (Kim et al., 2015).

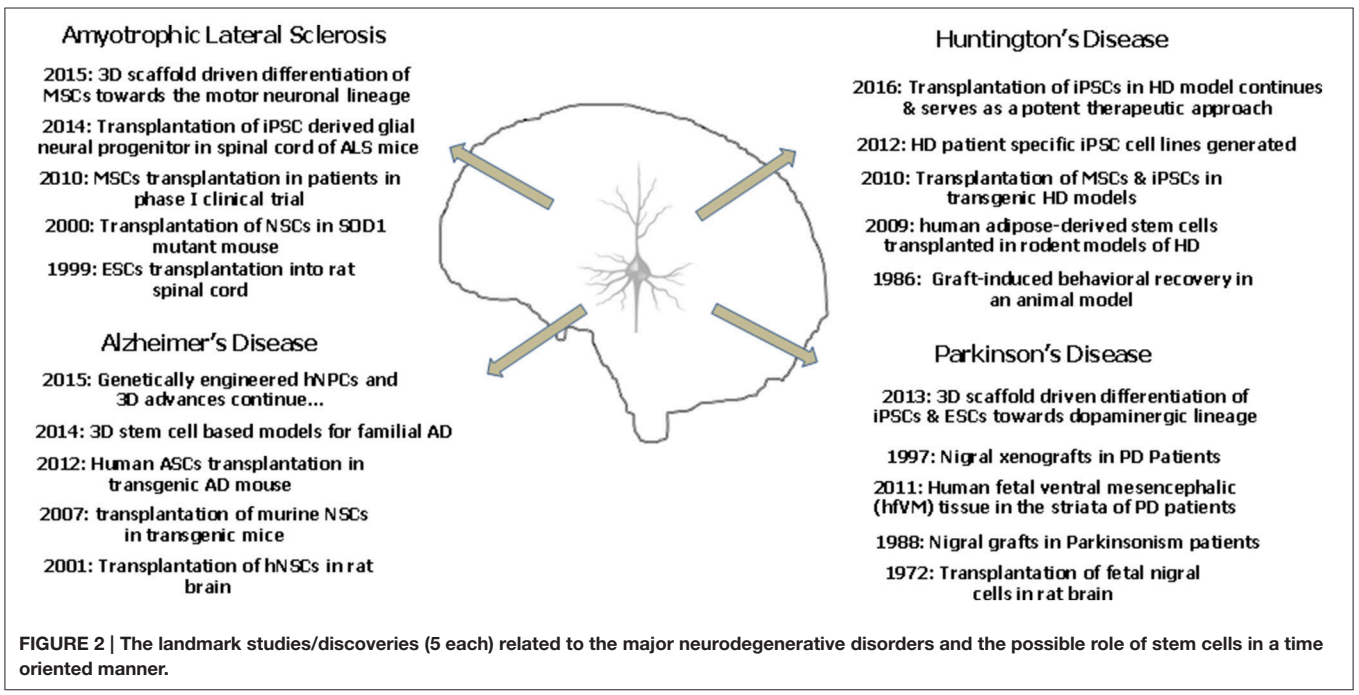

FIGURE 2 | The landmark studies/discoveries (5 each) related to the major neurodegenerative disorders and the possible role of stem cells in a time oriented manner.

It is well reported that mouse iPSCs and ESCs show a better dopaminergic differentiation potential in a three dimensional peptide derived nanofibre scaffold. The 3D culture provides a better environment for the development of the DA neurons which showed appropriate action potential firing and expressed the specific markers as well (Ni et al., 2013). Successful differentiation of chorion derived MSCs into motor neurons has been recently reported in 3D nanofibrous gelatin scaffold. These motor neurons shall provide a possibility to model ALS in a three dimensional scenario (Faghihi et al., 2016). **Figure 2** depicts the landmark studies/discoveries (5 each) related to the major neurodegenerative disorders and the possible role of stem cells in a time oriented manner.

CONCLUDING REMARKS

The above discussion so far clearly sheds light on the widespread applicability of stem cells and induced pluripotent stem cells in the field of neurodegeneration. Disease modeling, transplantation therapy, restoration of lost brain functionality due to injury and aging and regenerative therapeutics are some of the areas where stem cells have been abundantly used. The article highlights the advances made especially in the past 5 years as envisaging the entire applicability of stem cells in neurodegenerative medicine is beyond the scope of the present discussion. Human stem cells and patient derived iPSCs have been instrumental in overcoming the major limitations of animal based research providing a more profound understanding of the neurodegenerative disorders. Patient derived iPSCs are even better models for understanding the disease pathophysiology and mechanistics because they carry the patient's genotype, bear the disease mutations and also account for the environmental influences. Stem cells have also been employed as simplistic *in-vitro* tools for screening of therapeutics and drugs. Three dimensional stem cell based studies and stem cell derived organoids have further contributed by providing a more *in-vivo* like microenvironment which is the closest possible mimic of a live animal. With technological advancements and efficient imaging techniques have revolutionized the concept of 3D stem cell based organoid research. Pharmacological intervention utilizing natural agents like curcumin has shown neuroprotective efficacy in clinical and experimental models of neurotoxicity and can provide beneficial effects in the neurodegenerative disorders in future (Srivastava et al., 2014). However, the current prevalent pharmacological treatments provide symptomatic relief only for a limited period of time and the drugs administered may have side effects. The advent of stem cell therapy has laid the foundation keystone for a possible cure with minimized side effects. Personalized medical treatment using iPSCs is the current face of modern medicine and constant efforts are being made to scale down the cost and increase the efficacy of the approach. Animal based clinical field trials cannot be completely surpassed and transplantation therapies will require validation. However, if cells of human origin are employed for the preliminary disease modeling and therapeutic screening, a lot shall be saved in terms of funds, resource, time and even animal lives. It would not be an exaggeration to say that with the proficient and judicious use of stem cells and iPSCs lesser animals shall be sacrificed and the rate of clinical trial failure shall be curbed. This shall lessen the moral as well as the economic burden. We still hope that future research will come up with effective cures for the fatal neurodegenerative disorders, until then efficient and affordable disease management and treatment can ensure a longer and healthier life for the aging population. **Figure 3** is a diagrammatic illustration of possible therapeutic strategies against the prevalent neurodegenerative disorders (ND). The nature of ND is progressive in nature and limits the clinical utility of pharmacological drugs which provide only symptomatic relief. Stem cell therapy and iPSC technology could harness neurorestorative and neuro regenerative relief for the patients suffering from neurodegenerative disorders as well as pave way for a possible cure in future.

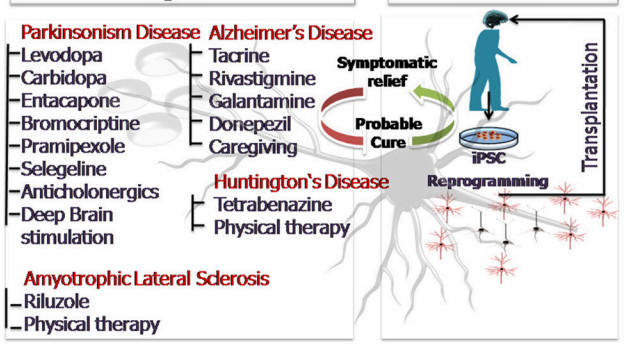

FIGURE 3 | The diagrammatic illustration of possible therapeutic strategies against the prevalent neurodegenerative disorders (ND). The nature of ND is progressive in nature and limits the clinical utility of pharmacological drugs which provide only symptomatic relief. Stem cell therapy and iPSC technology could harness neurorestorative and neuro regenerative relief for the patients suffering from neurodegenerative disorders as well as pave way for a possible cure in future.

AUTHOR CONTRIBUTIONS

SS drafted and prepared the manuscript, AS reviewed the draft, PS, YD, and CR prepared graphics. AP and DK helped in compilation of literature. All the authors have discussed the manuscript and agree to be accountable for the content of the work.

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GENERAL COMMENTARY
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Commentary: The C9orf72 Repeat Expansion Disrupts Nucleocytoplasmic Transport

Tudor Munteanu and Tim Lynch*

Dublin Neurological Institute at Mater Misericordiae Hospital, Dublin, Ireland

Keywords: ALS, neurodegeneration, pathophysiology, nuclear pore complex proteins, RanGAP1

A commentary on

The C9orf72 repeat expansion disrupts nucleocytoplasmic transport
*by Zhang K, Donnelly CJ, Haeusler AR, Grima JC, Machamer JB, Steinwald P, et al. Nature (2015) **525**(7567):56–61. doi: 10.1038/nature14973*

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that affects the upper and lower motor neurons. It has a focal onset but gradually spreads, leading to disability and, eventually, death. About 5–10% of ALS is inherited, usually following a dominant pattern. Pathological analysis revealed that motor neuron degeneration and death in the familial and sporadic forms is closely connected to protein aggregation and deposition, abnormal level and function of RNA molecules, abnormal neuronal cytoarchitecture, and non-neuronal cell death (1).

The GGGGCC hexanucleotide expansion (G_4C_2 HRE) in chromosome 9 open reading frame 72 (C9orf72) gene is found in 40% of the familial ALS cases. It is also found in other neurodegenerative disorders, including frontotemporal dementias (FTDs) (2) and atypical parkinsonian syndromes (3).

The molecular mechanisms of C9orf72 ALS neurodegeneration are currently a subject of controversy. Two main pathophysiological models have been hypothesized with experimental evidence supporting both (4). The first is transcription of the HRE segments of the abnormal gene into abnormal RNA strands, which assemble into G-quadruplex structures, which directly interact with proteins and alter their function. The second is translation of this abnormal RNA into dipeptide repeat proteins (DPRs), which in turn can adversely influence cell function. Both of the above described mechanisms have the potential to influence the nuclear pore function by altering its protein complexes.

Zhang et al. have recently investigated the complex mechanisms that lead to impairment of the normal trafficking through the nuclear pore complex (NPC) associated with G_4C_2 HRE (5). They demonstrated that overexpression of RanGAP gene (which encodes a NPC protein – RanGAP1) rescued certain phenotypic traits associated with G_4C_2 HRE-mediated neurodegeneration by using $(G_4C_2)_{30}$ *Drosophila* models. Different phenotypes were obtained by expressing the abnormal gene at different stages of fly development. If expressed in 1-day-old flies, the HRE sequence caused progressive defects in the photoreceptor organization, suggesting age-dependent neurodegeneration. Locomotor defects were noted in 15-day-old $(G_4C_2)_{30}$ flies pointing toward motor neuron pathology. Both of these phenotypes were rescued either by RanGAP overexpression or by using a heterozygous RanGAP gain-of-function mutation. Conversely, photoreceptor degeneration was accelerated by RanGAP knockdown using RNA interference. When the $(G_4C_2)_{30}$ HRE was expressed in larval motor neurons, it caused severe neuromuscular junction defects that were not rescued by RanGAP overexpression. These results suggested that RanGAP overactivity suppressed HRE-mediated neurodegeneration in adult *Drosophila*, but HRE-mediated neurotoxicity in the larval stage is RanGAP independent.

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Edited by:
Megha Agrawal,
University of Illinois at Chicago, USA

Reviewed by:
Vivek Kumar,
CSIR-Indian Institute of Toxicology Research, India
Bryan Traynor,
NIH, USA

****Correspondence:***
Tudor Munteanu
tudormunteanu@physicians.ie

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The authors also studied the RanGAP/G_4C_2 RNA interaction in induced pluripotent stem cell (iPSC) neurons, derived from multiple C9orf72 ALS patients and noted that RanGAP G_4C_2 RNA can colocalize in these cells. This interaction seems to lead to RanGAP loss-of-function, which was shown by a disrupted Ran nucleocytoplasmic gradient in cells expressing G_4C_2 HRE. HRE toxicity also appeared to be modulated by nucleocytoplasmic transport, and the data suggested that genetically enhancing nuclear import and/or inhibiting nuclear export can suppress G_4C_2-repeat-mediated degeneration in both *Drosophila* and human cells.

In order to prove that abnormal G_4C_2 HRE RNA is involved in the neurodegenerative pathophysiological pathway, the investigators treated C9 ALS iPSC neurons with antisense oligonucleotides targeting these molecules. The treatment rescued the disrupted Ran gradient, suggesting that the abnormal RNA may be responsible for the nucleocytoplasmic transport deficits. Neurodegenerative phenotype suppression also occurred using a nuclear export inhibitor, suggesting that nuclear export inhibition may compensate for disrupted nuclear import.

In summary, G_4C_2 HRE disrupts nucleocytoplasmic transport in *Drosophila* models and in human cells. RanGAP seems to be a key link in the pathophysiological chain, but other nuclear pore proteins may also be involved. Nuclear pore transport was previously implicated in both ALS and FTD pathophysiology, and data from this study suggest that the RNA fragments cause nucleocytoplasmic trafficking defects by direct interaction with NPC proteins. However, other authors argued that DPRs can cause a reversible dysfunction of the NPC in the absence of abnormal C9orf72 RNA (4). These mechanisms can be complementary, and further work is needed to elucidate a potential link between them. The results presented suggest that nuclear pore dysfunction is key to neurodegeneration in C9orf72 ALS. The abnormal phenotype is reversible by modifying the RanGAP gene, suggesting a potential therapeutic approach.

AUTHOR CONTRIBUTIONS

TM – summarized the original article, selecting important points for presentation, and wrote the manuscript. TL – manuscript review.

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REVIEW
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Copy Number Variations in the *Survival Motor Neuron* Genes: Implications for Spinal Muscular Atrophy and Other Neurodegenerative Diseases

Matthew E. R. Butchbach [1, 2, 3, 4]*

[1] Center for Applied Clinical Genomics, Nemours Biomedical Research, Nemours Alfred I. duPont Hospital for Children, Wilmington, DE, USA, [2] Center for Pediatric Research, Nemours Biomedical Research, Nemours Alfred I. duPont Hospital for Children, Wilmington, DE, USA, [3] Department of Biological Sciences, University of Delaware, Newark, DE, USA, [4] Department of Pediatrics, Thomas Jefferson University, Philadelphia, PA, USA

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Edited by:
Megha Agrawal, University of Arkansas at Little Rock, USA

Reviewed by:
Alessandro Vercelli, University of Torino, Italy
Michal Mielcarek, Imperial College London, UK

***Correspondence:**
Matthew E. R. Butchbach
butchbach@nemoursresearch.org

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Proximal spinal muscular atrophy (SMA), a leading genetic cause of infant death worldwide, is an early-onset, autosomal recessive neurodegenerative disease characterized by the loss of spinal α-motor neurons. This loss of α-motor neurons is associated with muscle weakness and atrophy. SMA can be classified into five clinical grades based on age of onset and severity of the disease. Regardless of clinical grade, proximal SMA results from the loss or mutation of *SMN1* (*survival motor neuron 1*) on chromosome 5q13. In humans a large tandem chromosomal duplication has lead to a second copy of the *SMN* gene locus known as *SMN2*. *SMN2* is distinguishable from *SMN1* by a single nucleotide difference that disrupts an exonic splice enhancer in exon 7. As a result, most of *SMN2* mRNAs lack exon 7 (*SMNΔ7*) and produce a protein that is both unstable and less than fully functional. Although only 10–20% of the *SMN2* gene product is fully functional, increased genomic copies of *SMN2* inversely correlates with disease severity among individuals with SMA. Because *SMN2* copy number influences disease severity in SMA, there is prognostic value in accurate measurement of *SMN2* copy number from patients being evaluated for SMA. This prognostic value is especially important given that *SMN2* copy number is now being used as an inclusion criterion for SMA clinical trials. In addition to SMA, copy number variations (CNVs) in the *SMN* genes can affect the clinical severity of other neurological disorders including amyotrophic lateral sclerosis (ALS) and progressive muscular atrophy (PMA). This review will discuss how *SMN1* and *SMN2* CNVs are detected and why accurate measurement of *SMN1* and *SMN2* copy numbers is relevant for SMA and other neurodegenerative diseases.

Keywords: spinal muscular atrophy, amyotrophic lateral sclerosis, progressive muscular atrophy, neurodegenerative disease, copy number variation, *SMN1, SMN2*

INTRODUCTION

Proximal spinal muscular atrophy (SMA) is a leading genetic cause of infant death worldwide, alongside cystic fibrosis. The incidence of SMA is 1 in 6000–10,000 live births (Pearn, 1978; Cuscó et al., 2002; Sugarman et al., 2012). The carrier frequency for SMA is 1:25–50 in most populations (Ben-Shachar et al., 2011; Su et al., 2011; Lyahyai et al., 2012; Sugarman et al., 2012) though it is lower for some ethnicities (Zaldívar et al., 2005; Labrum et al., 2007; Hendrickson et al., 2009; Sangaré et al., 2014).

SMA is an early-onset neurodegenerative disease characterized by the loss of α-motor neurons in the anterior horn of the spinal cord, i.e., lower motor neurons (LMNs; Crawford and Pardo, 1996; Kolb and Kissel, 2015). This loss of α-motor neurons is associated with muscle weakness and atrophy. In SMA, those muscles that are proximally innervated are preferentially affected over distal muscles. SMA can be classified into five clinical grades based on age of onset and severity of the disease (Munsat and Davies, 1992; Russman, 2007; **Table 1**). Type 0 SMA infants present with very severe hypotonia and require respiratory support from birth. These SMA infants cannot survive beyond 6 months. Type I SMA [listed in the Online Inheritance in Man (OMIM) database under accession number 253300; http://www.omim.org/entry/253300] patients have an age of onset before 6 months. They show hypotonia and weakness in limbs; they are unable to sit independently. Type I SMA infants show a bell-shaped chest due to weakness in the intercostals muscles but sparing of the diaphragm; this bell-shaped chest results in abnormal breathing patterns. These patients typically live <2 years. Type II SMA (http://www.omim.org/entry/253500) patients have an age of onset before 18 months. They are poor crawlers and weak sitters; most of these patients can rarely stand and only with support. Their legs are generally weaker than their arms. Due in part to better supportive care, these patients generally have a life expectancy into early adulthood. Type III SMA (http://www.omim.org/entry/253400) patients have an age of onset >18 months. These patients are able to walk with difficulty (waddling gait) and the legs are weaker than the arms. Type III patients usually have a normal lifespan. Adult-onset (type IV) SMA (http://www.omim.org/entry/271150) patients have an age of onset of 18–21 years. Type IV SMA exhibits as a slowly progressive limb weakness. The disease is fairly benign in these patients.

Abbreviations: ALS, amyotrophic lateral sclerosis; CNV, copy number variation; dPCR, digital polymerase chain reaction; FUS, fused in sarcoma; GTF2H2, general transcription factor IIH; LMN, lower motor neuron; MLPA, multiple ligation-dependent probe amplification; NAIP, neuronal apoptosis inhibitory protein; OMIM, Online Mendelian Inheritance in Man; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; PMA, progressive muscular atrophy; SERF1A, small EDRK-rich factor 1A; SMA, spinal muscular atrophy; SMNΔ7, survival motor neuron lacking exon 7; SMN1, survival motor neuron 1; SMN2, survival motor neuron 2; snRNP, small nuclear ribonucleoprotein particle; SOD1, superoxide dismutase 1; TDP-43, TAR DNA binding protein-43kDa; UMN, upper motor neuron.

GENETICS OF SMA

SMA is an autosomal recessive disorder (Brandt, 1949). Linkage analysis (Brzustowicz et al., 1990; Gilliam et al., 1990; Melki et al., 1990a,b) along with genetic and physical mapping studies (reviewed in Morrison, 1996) identified the SMA locus on the long arm of chromosome 5, specifically in the 5q13 region. There is a 500 kilobase (kb) inverted segmental duplication within this region of chromosome 5 that is unique to humans (Courseaux et al., 2003; Schmutz et al., 2004). Four protein-coding genes have been identified within this region (**Figure 1**): *SMN1* [*survival motor neuron 1*, *telomeric SMN* (*SMN*^{T}; Lefebvre et al., 1995)], *NAIP* [*neuronal apoptosis inhibitor protein* (Roy et al., 1995)], *GTF2H2A* [*general transcription factor IIH*, *p44* (Bürglen et al., 1997; Carter et al., 1997)], and *SERF1A* [*small EDRK-rich factor 1A*, *H4F5A* (Scharf et al., 1998)]. The duplicated genes are either identical to their partner gene (*SERF1B*), different in a small number of nucleotides [*SMN2* or *centromeric SMN* (*SMN*^{C})] or are pseudogenes (*ΨGTF2H2B* and *ΨNAIPΔ5*).

In more than 95% of cases, proximal SMA results from the loss of *SMN1* but retention of *SMN2*, regardless of clinical grade (Lefebvre et al., 1995). Large-scale deletions in chromosome 5q13 that include *SMN1*, *NAIP*, *SERF1A,* and *GTF2H2A* are observed in patients with type I SMA (Wirth et al., 1995; Burlet et al., 1996; Rodrigues et al., 1996; Velasco et al., 1996; Bürglen et al., 1997; Carter et al., 1997). Smaller deletions only involving *SMN1* have also been observed in type I SMA patients demonstrating that *SMN1* is the most likely causative gene for SMA. In addition, the identification of intragenic SMA mutations in *SMN1* (Lefebvre et al., 1995; see Burghes and Beattie, 2009 for a comprehensive listing of SMA-associated point mutations in *SMN1*) provides additional evidence to support *SMN1* as the gene responsible for SMA. To date, no intragenic mutations in the other genes within this segmental duplication have been associated with SMA.

As mentioned earlier, the *SMN* gene is duplicated in humans to give rise to *SMN1* and *SMN2*. This duplication of *SMN* is unique to humans (Rochette et al., 2001). What is the difference between *SMN1* and *SMN2*? The major difference between these two *SMN* genes is a C-to-T transition in exon 7(*SMN2 c.850C>T*; Lorson et al., 1999; Monani et al., 1999). This nucleotide change is translationally silent. This position on exon 7 is in the middle of an exonic splicing enhancer (ESS) sequence that regulated the inclusion of exon 7 in SMN transcripts (**Figure 2**). For *SMN1*, the C at this position promotes inclusion of exon 7 in *SMN1*-derived mRNAs which leads to the production of full-length SMN protein. Full-length SMN protein is able to form functional complexes. For *SMN2*, the T at this position disrupts this ESS which results in the exclusion of exon 7 (SMNΔ7) from the majority of *SMN2*-derived mRNAs. As a result, a truncated SMNΔ7 protein is produced by the majority (∼90%) of *SMN2*-derived mRNAs; this SMNΔ7 protein is unstable and is unable to associate with itself (Lorson and Androphy, 2000; Burnett et al., 2009; Cho and Dreyfuss, 2010). The SMNΔ7 protein is still partially functional given that transgenic overexpression of SMNΔ7 in severe SMA mice partially ameliorates their phenotype since these mice die at 14–15 days as opposed to 5–8 days (Le et al., 2005). About

TABLE 1 | Clinical classification of spinal muscular atrophy (SMA).

Type	Age of onset	Requires respiratory support at birth	Able to sit	Able to stand	Able to walk	Life expectancy	Predicted *SMN2* copy number
0	Prenatal	Yes	No	No	No	<6 months	1
1	<6 months	No	No	No	No	<2 years	2
2	6–18 months	No	Yes	No	No	10–40 years	3
3	>18 months	No	Yes	Yes	Assisted	Adult	3–4
4	>5 years	No	Yes	Yes	Yes	Adult	>4

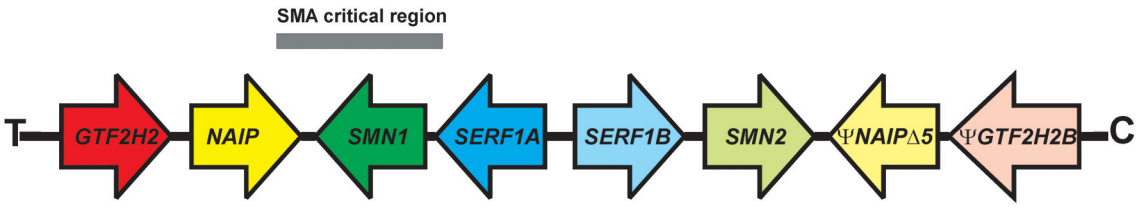

FIGURE 1 | Organization of the inverted duplication locus on 5q13. 4 protein-coding genes—*GTF2H2* (*general transcription factor IIH*), *NAIP* (*neuronal apoptosis inhibitory protein*), *SMN1* (*survival motor neuron 1*), and *SERF1A* (*small EDRK-rich factor 1A*)—are present within the 500-kilobase inverted duplication on the long arm of chromosome 5 (5q13.2). The duplicated genes are *ΨGTF2H2B* (*GTF2H2 pseudogene*), *ΨNAIPΔ5* (*NAIP pseudogene with loss of exon 5*), *SMN2*, and *SERF1B*. The SMA critical region is under the gray bar. C, centromeric end; T, telomeric end.

10% of the mRNAs from *SMN2* contain exon 7 and these full-length mRNAs can produce some full-length, functional SMN protein.

BIOLOGY OF SMN

SMN is a ubiquitously expressed protein whose expression is reduced in SMA. There is a strong inverse correlation between SMN protein levels and disease severity in SMA fibroblasts and lymphoblastoid cells as well (Coovert et al., 1997; Lefebvre et al., 1997; Kolb et al., 2006). Changes in SMN mRNA and protein levels observed in SMA patient-derived PBMCs mirror those observed in SMA cell lines (Sumner et al., 2006; Simard et al., 2007; Vezain et al., 2007; Tiziano et al., 2010; Crawford et al., 2012). SMN protein is present within the nuclei in discreet foci known as gems (Liu and Dreyfuss, 1996). In SMA fibroblasts, the number of SMN-positive subnuclear gems is higher in cells derived from mild SMA individuals than in those from children with more severe forms of SMA (Coovert et al., 1997).

SMN is required for the assembly of the small nuclear ribonucleoprotein (snRNP) complexes that mediate splicing (Pellizzoni, 2007; Burghes and Beattie, 2009). snRNP assembly is defective in SMN-deficient SMA cells (Wan et al., 2005). Since snRNP assembly is required for all cell types, why are motor neurons primarily affected in SMA? snRNP assembly is defective in tissues from mouse models for SMA and that the extent of reduced snRNP assembly correlates with phenotypic severity of these SMA mice (Gabanella et al., 2005; Zhang et al., 2008). snRNP assembly is more markedly reduced in SMA mouse neural tissues than in other tissues like the kidney (Gabanella et al., 2005) suggesting that motor neurons are more sensitive to deficits in snRNP assembly. SMN may also have a function that is unique to motor neurons. Axonal defects in *Smn*-knocked down zebrafish embryos (McWhorter et al., 2003) are corrected by overexpression of mutant SMNs which are incapable of snRNP assembly (Carrel et al., 2006).

SMN2 CNV IN SMA

The number of *SMN2* copies in the genome varies between 0 and 8. Numerous studies have demonstrated an inverse relationship between *SMN2* copy number and disease severity among in SMA (Lefebvre et al., 1995, 1997; van der Steege et al., 1995; Coovert et al., 1997; McAndrew et al., 1997; Taylor et al., 1998; Feldkötter et al., 2002; Mailman et al., 2002; Anhuf et al., 2003; Gérard et al., 2004; Prior et al., 2005; Su et al., 2005; Swoboda et al., 2005; Arkblad et al., 2006; Scarciolla et al., 2006; Wirth et al., 2006; Gómez-Curet et al., 2007; Huang et al., 2007; Tiziano et al., 2007; Elsheikh et al., 2009; Wang et al., 2010a,b, 2014a; Alías et al., 2011; Amara et al., 2012; Crawford et al., 2012; Dobrowolski et al., 2012; Kirwin et al., 2013; Qu et al., 2014; Brkušanin et al., 2015; Fang et al., 2015; Stabley et al., 2015). Patients with milder forms of SMA have higher *SMN2* copy numbers than severe SMA patients (**Table 1**).

Mice have only 1 *SMN* gene, *mSmn*, which is orthologous to *SMN1* (DiDonato et al., 1997a; Viollet et al., 1997). Loss of *mSmn* leads to embryonic lethality (Schrank et al., 1997). Conditional loss of *mSmn* in specific cell types like neurons, myofibers and hepatocytes results in loss of those cells *in vivo* (Cifuentes-Diaz et al., 2002; Nicole et al., 2003; Vitte et al., 2004). Transgenic insertion of *SMN2* rescues the embryonic lethality observed in *mSmn* nullizygous mice (Hsieh-Li et al., 2000; Monani et al., 2000; Michaud et al., 2010). While two copies of *SMN2* rescues embryonic lethality in *mSmn*-deficient mice, these mice develop

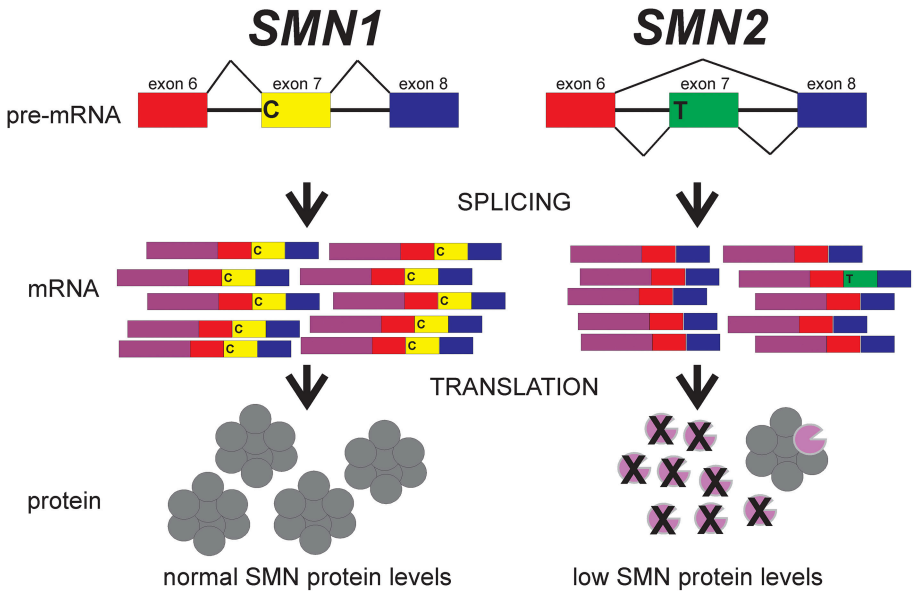

FIGURE 2 | The effect of the C-to-T transition in exon 7 between *SMN1* and *SMN2* on splicing. Adapted from Butchbach and Burghes (2004).

a very severe motor phenotype and die within 8 days after birth (Hsieh-Li et al., 2000; Monani et al., 2000). Those *mSmn*-deficient mice with 3-4 *SMN2* copies exhibit a milder SMA phenotype than the two copy *SMN2* SMA mice (Hsieh-Li et al., 2000; Michaud et al., 2010). If the *SMN2* copy number is high (i.e., 8), then the resultant *mSmn*-deficient mice exhibit no signs of SMA and are phenotypically normal (Monani et al., 2000). *SMN2* CNV, therefore, is a major modifier of disease severity in SMA in mice as well as in humans.

Gene conversion is one mechanism to account for increased *SMN2* copy number in the absence of *SMN1* in SMA (Burghes, 1997). In this scenario, the *SMN1* gene actually contains part of *SMN2*, in particular within exon 7 (Wirth et al., 1995; Devriendt et al., 1996; Hahnen et al., 1996; van der Steege et al., 1996; Campbell et al., 1997; DiDonato et al., 1997b). It is hypothesized that type I SMA patients have deletions of *SMN1* on both chromosomes. Type II SMA patients have an *SMN1* deletion on one chromosome and a *SMN1*-to-*SMN2* conversion on the other chromosome (three copies of *SMN2*). Type III SMA patients have *SMN1*-to-*SMN2* converted genes on both chromosomes (four copies).

While the inverse relationship between *SMN2* copy number and disease severity generally holds true in SMA, there are some exceptions. For example, there are cases of type II and III SMA patients who harbor only two copies of *SMN2* instead of the predicted three or four copies (Prior et al., 2009; Bernal et al., 2010; Vezain et al., 2010). Sequencing of *SMN2* in these cases revealed the presence of a rare single nucleotide variant (*SMN2 c.859G>C*) in exon 7 (Prior et al., 2009; Bernal et al., 2010; Vezain et al., 2010). This variant regulates the splicing of *SMN2* pre-mRNAs so that a greater proportion of *SMN2* transcripts contain exon 7. This variant may either create an additional SF2/ASF binding to promote exon 7 inclusion (Prior et al., 2009) or disrupt a hnRNPA1-dependent splicing silencer element (Vezain et al., 2010).

Intrafamilial variability in clinical presentation has been reported in SMA families with more than one affected sibling (Burghes et al., 1994; Cobben et al., 1995; Hahnen et al., 1995; McAndrew et al., 1997; Cuscó et al., 2006). Even though the siblings are haploidentical with respect to *SMN2* copy number, they have differing clinical presentations. This would suggest that there are *SMN2*-independent modifiers of disease severity for SMA. *Plastin-3* (*PLS3*) mRNA levels were higher in females with milder SMA than in discordant siblings with a more severe SMA clinical presentation (Oprea et al., 2008; Stratigopoulos et al., 2010; Bernal et al., 2011; Yanyan et al., 2014). In some families, however, female siblings with a more severe SMA phenotype show high *PLS3* mRNA levels (Bernal et al., 2011). It is possible that the modifier property of *PLS3* is age- and sex-dependent as well as incompletely penetrant; alternatively, *PLS3* may not actually be a major modifier of SMA phenotype. There may be other non-*SMN2* molecular modifiers of disease severity in SMA. It is important to identify and characterize these novel modifiers for the development of novel SMA biomarkers and targets for the development of therapeutic strategies for SMA as well as for the planning of current and future clinical trials in SMA (Wirth et al., 2013).

MEASURING *SMN1* AND *SMN2* CNVs

Because *SMN2* copy number influences disease severity in SMA, there is prognostic value in accurate measurement of *SMN2* copy number from patients being evaluated for SMA. Molecular diagnosis of SMA— i.e., loss of *SMN1*—has historically been made using a polymerase chain reaction (PCR)-based

assay followed by digestion of the PCR product with specific restriction endonucleases (PCR-RFLP; Lefebvre et al., 1995; van der Steege et al., 1995). Numerous assays have since been developed to quantify *SMN2* copy number in DNA samples from SMA patients. These assays include radioactive PCR (Coovert et al., 1997; McAndrew et al., 1997), fluorescent PCR (Taylor et al., 1998), quantitative (real-time) PCR (qPCR; Feldkötter et al., 2002; Anhuf et al., 2003; Gómez-Curet et al., 2007), competitive PCR/primer extension (Gérard et al., 2004), denaturing high performance liquid chromatography (Su et al., 2005), multiplex ligation-dependent probe amplification (MLPA; Arkblad et al., 2006; Scarciolla et al., 2006; Huang et al., 2007; Alías et al., 2011; Fang et al., 2015), quantitative capillary electrophoresis fragment analysis (Kirwin et al., 2013), short-amplicon melt profiling (Dobrowolski et al., 2012), fluorescent multiplex PCR/capillary electrophoresis (Wang et al., 2010a,b), and universal fluorescent triprobe ligation (Wang et al., 2014a). An important limitation of these established PCR-based copy number assays is the requirement for a parallel-run calibration curve to assign a breakpoint necessary that identifies placement of an ordinal *SMN2* value. Additionally, these techniques cannot easily distinguish unit differences in *SMN1* or *SMN2* when the copy number is >3 (Gómez-Curet et al., 2007; Alías et al., 2011; Prior et al., 2011).

To overcome some of the limitations associated with the PCR-based assays described above, digital PCR (dPCR) distributed across a large number of partitions by limited dilution so that some partitions will lack the template DNA (Sykes et al., 1992; Vogelstein and Kinzler, 1999). The absolute abundance of the target gene can be measured by counting the number of positive partitions and the number of negative partitions. dPCR can reliably and accurately measure *SMN1* and *SMN2* copy numbers over a wide range, i.e., between 0 and 6 copies (Zhong et al., 2011; Stabley et al., 2015).

SMN1 AND *SMN2* CNVs IN ALS

Amyotrophic lateral sclerosis (ALS) is a mostly adult-onset motor neuron disease characterized by a progressive loss of motor function leading to paralysis and respiratory failure (Boylan, 2015; Statland et al., 2015). Unlike SMA, ALS is caused by degeneration of LMNs as well as upper motor neurons (UMNs). ALS is usually fatal within 3–5 years after disease onset but there is considerable variability with respect to duration as well as phenotypic presentation (Swinnen and Robberecht, 2014). Most cases of ALS are sporadic in nature since there is no apparent family history. Approximately 10% of ALS is considered familial since either a causative gene has been identified or there is strong family history. With the recent advents of whole exome and whole genome sequencing, the genetic bases of almost 70% of familial ALS and 10% of sporadic ALS have been identified (Renton et al., 2014).

There are many case studies reporting the co-occurrence of SMA and ALS within a family (Appelbaum et al., 1992; Camu and Billiard, 1993; Orrell et al., 1997; Corcia et al., 2002a) which suggests that *SMN1* deficiency may lead to ALS in addition to SMA. *SMN1* deletions, however, have not been observed in either familial or sporadic ALS patients (Orrell et al., 1997; Corcia et al., 2002a). Furthermore, no intragenic point mutations in *SMN1* have been reported in the ALS population (Blauw et al., 2012). The intrafamilial coexistence of SMA and ALS, therefore, occurs by chance.

Even though loss of *SMN1* is not associated with ALS, CNVs in the *SMN* genes may modulate the clinical severity of ALS in addition to SMA. Multiple studies suggest that deletion of *SMN2* leads to increased risk of the sporadic forms of amyotrophic lateral sclerosis (ALS) (Veldink et al., 2001, 2005; Kim et al., 2010; Corcia et al., 2012; Lee et al., 2012). Additionally, atypical *SMN1* copy number—in other words, any number other than two—can affect the risk of ALS (Corcia et al., 2002b, 2006; Blauw et al., 2012; Wang et al., 2014b). Other studies, however, have shown no association between deletion of either *SMN1* or *SMN2* in ALS (Jackson et al., 1996; Moulard et al., 1998; Parboosingh et al., 1999; Crawford and Skolasky, 2002; Gamez et al., 2002). The discrepant results from these studies may be due, in part, to different assays used to assess *SMN1* and *SMN2* CNVs as some reports using quantitative PCR while others used MLPA or RFLP.

SMN and some ALS-associated proteins are involved in common biochemical pathways. Both familial and sporadic ALS have been linked to mutations in *fused in sarcoma* (*FUS)* (Kwiatkowski et al., 2009; Vance et al., 2009) (OMIM #608030) as well as in *TAR DNA binding protein-43 kDa* (*TDP-43*) (Kabashi et al., 2008; Sreedharan et al., 2008) (OMIM #612069). Both FUS and TDP-43 colocalize with SMN in subnuclear gems and ALS-associated mutations in *FUS* and *TDP-43* reduce gem localization of SMN (Shan et al., 2010; Yamazaki et al., 2012; Gerbino et al., 2013; Groen et al., 2013; Ishihara et al., 2013; Sun et al., 2015). Gem localization of SMN, however, is not altered in other forms of sporadic ALS (Kariya et al., 2014). These mutant proteins also disrupt the SMN-mediated assembly of the splicing machinery by disrupting the interaction between SMN and U1-snRNPs (small nuclear ribonucleoprotein particles; Gerbino et al., 2013; Tsuiji et al., 2013; Sun et al., 2015; Yu et al., 2015). Additionally, ALS-associated FUS mutations disrupt the localization of SMN to axons (Groen et al., 2013). The SMN function, therefore, may be disrupted in certain forms of ALS.

Ectopic overexpression of SMN protects NSC34 motor neuron-like cells from cell death induced by ALS-associated mutant *superoxide dismutase 1* (*SOD1*) (OMIM #105400) (Zou et al., 2007). The SOD1(G93A) transgenic mouse model for ALS that also harbors a knockout of 1 *mSmn* allele ($SOD1(G93A)^{+/-};mSmn^{+/-}$) exhibits a more severe ALS phenotype than SOD1(G93A) ALS mice ($SOD1(G93A)^{+/-};mSmn^{+/+}$) (Turner et al., 2009). Furthermore, ectopic overexpression of SMN in neurons and glia improves motor function of and delays motor neuron loss in SOD1(G93A) ALS mice (Turner et al., 2014). Strong transgenic overexpression of *SMN2*—in other words, eight copies of *SMN2*—delayed disease onset in the SOD1(G86R) mouse model for ALS (Kariya et al., 2012). Thesestudies suggest that increasing SMN expression may modulate disease severity in ALS. It will be interesting to determine the effect of *SMN2* overexpression on disease severity in FUS- and TDP-43-associated ALS.

SMN1 AND *SMN2* CNVs IN PMA

Progressive muscular atrophy (PMA) is an adult-onset motor neuron disease characterized by loss of LMNs (Rowland, 2010; Liewluck and Saperstein, 2015). It is a rare and sporadic disorder that is clinically distinct from ALS even though subclinical involvement of UMNs has been observed in many PMA patients. Those PMA patients exhibiting a more severe clinical presentation tend to harbor higher *SMN1* copy numbers (Kuzma-Kozakiewicz et al., 2013). No relationship between *SMN2* copy number and disease severity was noted in these PMA patients. Moulard et al. (1998) noted that the frequency of *SMN2* deletion was higher in a small group of patients with sporadic LMN disease.

CONCLUSIONS

CNVs in the *SMN* genes can modulate disease severity in SMA as well as in other motor neurons. It is well-established that *SMN2* copy number is inversely correlated with disease severity in SMA. Because of this relationship, *SMN2* is a primary target for the development of therapeutics for SMA (Arnold and Burghes, 2013; Cherry et al., 2014). Numerous approaches including promoter activation, increased inclusion of exon 7 and protein stabilization are currently being developed to increase *SMN2* expression. With respect to other motor neuron diseases, it is presently unclear whether increasing *SMN1* or *SMN2* expression would be beneficial or detrimental. On one hand, increasing SMN expression provides neuroprotective benefit to cell culture and transgenic mouse models for ALS; however, some genetic studies suggest that duplication of *SMN1* increases the risk of sporadic ALS. Future studies will assess the relationship between *SMN1* and *SMN2* CNVs and disease risk and progression in ALS and PMA as well as in other disorders affecting motor neurons.

SMN2 copy number is becoming an inclusion criterion for many clinical trials for SMA. Additionally, *SMN2* copy number can be used to help guide the type of care SMA patients will receive. It is, therefore, essential to be able to accurately and reliably measure *SMN2* CNVs in SMA patient samples, especially for those individuals harboring more than three copies of *SMN2*. Newly developed technologies like dPCR offer a means to accurately determine *SMN2* copy number over a wider range.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Elevated Neuronal Excitability Due to Modulation of the Voltage-Gated Sodium Channel Nav1.6 by $A\beta_{1-42}$

Xi Wang[1†], Xiao-Gang Zhang[1†], Ting-Ting Zhou[2], Na Li[1], Chun-Yan Jang[1], Zhi-Cheng Xiao[3], Quan-Hong Ma[4] and Shao Li[1]*

[1] Department of Physiology, Dalian Medical University, Dalian, China, [2] Department of Neurology, the First Affiliated Hospital of Dalian Medical University, Dalian, China, [3] The Key Laboratory of Stem Cell and Regenerative Medicine, Kunming Medical College, Institute of Molecular and Clinical Medicine, Kunming, China, [4] Jiangsu Key Laboratory of Translational Research and Therapy for Neuro-Psycho-Diseases, Institute of Neuroscience, Second Affiliated Hospital, Soochow University, Suzhou, China

Aberrant increases in neuronal network excitability may contribute to the cognitive deficits in Alzheimer's disease (AD). However, the mechanisms underlying hyperexcitability are not fully understood. Such overexcitation of neuronal networks has been detected in the brains of APP/PS1 mice. In the present study, using current-clamp recording techniques, we observed that 12 days *in vitro* (DIV) primary cultured pyramidal neurons from P0 APP/PS1 mice exhibited a more prominent action potential burst and a lower threshold than WT littermates. Moreover, after treatment with $A\beta_{1-42}$ peptide, 12 DIV primary cultured neurons showed similar changes, to a greater degree than in controls. Voltage-clamp recordings revealed that the voltage-dependent sodium current density of neurons incubated with $A\beta_{1-42}$ was significantly increased, without change in the voltage-dependent sodium channel kinetic characteristics. Immunohistochemistry and western blot results showed that, after treatment with $A\beta_{1-42}$, expressions of Nav and Nav1.6 subtype increased in cultured neurons or APP/PS1 brains compared to control groups. The intrinsic neuronal hyperexcitability of APP/PS1 mice might thus be due to an increased expression of voltage-dependent sodium channels induced by $A\beta_{1-42}$. These results may illuminate the mechanism of aberrant neuronal networks in AD.

Keywords: Alzheimer's disease, beta-amyloid peptide, excitability, voltage-gated sodium channel, neurodegeneration

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M. Heather West Greenlee,
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****Correspondence:***
Shao Li
lishao89@hotmail.com

[†]These authors have contributed equally to this work.

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INTRODUCTION

Alzheimer's disease (AD) is the most frequent neurodegenerative disease and a common cause of dementia in elderly individuals. Various evidence suggests, that β-amyloid (Aβ) peptides play a causal role in AD's pathogenesis, but the underlying mechanisms remain unclear (Palop and Mucke, 2010). In prodromal AD patients, functional MRI has revealed increased activities in neural networks, rather than loss of activity (Putcha et al., 2011). Other evidence supports the view, that Aβ-induced excitotoxicity could be critically involved in the pathogenesis of AD (Ong et al., 2013). In Aβ-induced excitotoxicity, high levels of glutamate overexcite neurons and cause cell death (Choi, 1988; Olney et al., 1997). Aβ also enhances the sensitivity of neuron to glutamate, which increases the activity of neuronal networks, resulting in excitatory potentials and Ca^{2+} influx (Brorson et al., 1995). However, these changes cannot

explain patients with early AD frequently alternating between sober and confused, because synaptic disruption, or regeneration cannot repeatedly occur within such short time periods. This phenomenon is more likely due to the abnormal neural network excitability.

Voltage-gated sodium channels (Nav) play an essential role in excitable cells. They are necessary components required to generate and propagate action potentials (Goldin et al., 2000; Yu and Catterall, 2003; Catterall et al., 2005). The 260 kDa α subunit is the main component of the voltage-gated sodium channel. Nine α subtypes, named Nav1.1-Nav1.9, are expressed in excitable cells (Goldin et al., 2000; Ragsdale, 2008). Among them, the Nav1.1, Nav1.2, and Nav1.6 subtypes are expressed in the adult brain and regulate voltage-dependent sodium currents across the plasma membrane. Nav1.1 is primarily localized in the neuronal somata of GABAergic neurons (Yu et al., 2006; Ogiwara et al., 2007). Nav1.2 shows preferentially high expression in unmyelinated fibers (Ragsdale, 2008). The Nav1.6 subtype, encoded by the gene SCN8A, is conspicuously expressed at the nodes of Ranvier and axon initial segments (Trimmer and Rhodes, 2004). Unique features of Nav1.6 include its contribution to the persistent current, resurgent current, and repetitive neuronal firing.

Our previous results showed that Nav1.6 interacts with amyloid precursor protein (APP), which undergoes abnormal proteolytic processing to generate Aβ (Xu et al., 2014). APP also increases the surface expression of sodium channels through a G_o protein-coupled JNK pathway (Liu et al., 2015). Due to this effect of APP, we hypothesized that the upregulatory effect of Aβ on neuronal excitability might be partially based on modulating the expression of sodium channels. As we expected, in cultured cortical neurons, $A\beta_{1-42}$ increased the expression of sodium channels, particularly the Nav1.6 subtype. The increased voltage-dependent sodium current could decrease action potential threshold and increase the probability of action potential generation in response to synaptic excitation, thus increasing the excitability of the neuron.

MATERIALS AND METHODS

Animals

Newborn C57BL/6J mice were obtained from the Animal Center of Dalian Medical University. APP/PS1 transgenic mice were purchased from Jackson Laboratory (stock number 004462) and were maintained on a C57BL/6J background by crossing heterozygous transgenic mice with C57BL/6J breeders. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

Preparation of $A\beta_{1-42}$

Lyophilized $A\beta_{1-42}$ peptide (SIGMA, A9810) and reverse peptide $A\beta_{42-1}$ (SIGMA, SCP0048) were diluted to 1 mg/ml using sterile PBS and incubated at 37°C, 220 rpm for 48 h allowed to aggregate as described (Jones et al., 2013). For concrete forms of $A\beta_{1-42}$ peptide, see **Supplementary Figure S1**. In a further set of experiments primary cultured neurons were incubated with $A\beta_{1-42}$ (5.0 μM) or reverse peptide $A\beta_{42-1}$ (5.0 μM) for 24 h at 37°C.

Cell Culture

P0 (post-natal day 0–1) C57BL/6J mice or APP/PS1 mice and their littermates were sacrificed by CO_2 inhalation and the cortices were rapidly dissected under sterile conditions in cold PBS. The tissue was digested with 0.125% trypsin-EDTA at 37°C for 30 min. The trypsin solution was replaced with 2 ml 10% DMEM (DMEM with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin solution). The digested tissue was gently triturated by suction using a glass pipette flamed on the tip to avoid cellular damage. The cell suspension was filtered through a 74 μm screen mesh, plated on poly-L-lysine-coated coverslips or 6/12-well-plates and incubated in a 37°C, 5% CO_2 incubator. After 2 h, the medium was changed to Neurobasal (Gibco, 21103–049) supplemented with 2% B27, 1% L-glutamine, and 1% penicillin/streptomycin. In accordance with the routine culture, the medium was changed every 2 days until use.

MTT Assay

Cell viability of neurons was determined by the MTT assay. Neurons were plated in 96 well-plates. $A\beta_{42-1}$ and $A\beta_{1-42}$ peptides were added to wells for 24 h. After cell treatments the medium was removed and the cells were incubated with red free medium and MTT solution (0.5 mg/ml) for 4 h at 37°C. Finally, the medium was removed and formazan particles were dissolved in DMSO. Cell viability, defined as the relative amount of MTT reduction was determined by spectrophotometry at 570 nm.

Electrophysiological Recordings

Electrophysiological measurements were performed on pyramidal cells. Action potentials or sodium currents were recorded at room temperature using whole-cell patch-clampings. The extracellular solution contained the following (in mM): NaCl 150, KCl 5, $MgCl_2$ 1.1, $CaCl_2$ 2.6, HEPES 10, glucose 10, pH adjusted to 7.4 with NaOH. Patch pipettes were made from borosilicate glass capillaries (1.5 mm outer diameter, 0.8 mm inner diameter) using a micropipette puller (Narishige, PP 830, Japan). Pipette resistance ranged from 3 to 5 MΩ. Stimulation and data acquisition were performed using the EPC-10 patch-clamp amplifier and Pulse program (HEKA Electronik, Germany). Membrane currents were filtered at 2 kHz and digitized at 10 kHz.

Action potentials recordings were made using the current-clamp mode. The intracellular solution contained the following (in mM): KCl 65, KOH 5.0, KF 80, HEPES 10, EGTA 10, Na_2ATP 2, pH adjusted to 7.2 with KOH. Cells were held at −70 mV, then peak amplitude (80 pA, 10 ms), threshold (80 pA, 10 ms), and action potential firing (200 pA, 500 ms) were recorded and measured.

Sodium currents were recorded using the voltage-clamp mode. The intracellular solution contained the following (in mM): CsCl 140, $MgCl_2$ 2, Na_2ATP 2, EGTA 10, HEPES 20, pH adjusted to 7.2 with Tris-HCl. Cells were held at −70 mV and stepped to a range of potentials (−60 to +60 mV in 10 mV

increments) for 12 ms each. Peak inward currents (I) were plotted as a function of depolarizing potential to generate I–V curves. Activation curves were obtained by converting current (I) to conductance (G) at each voltage (V) using the equation $G = I/(V - V_{rev})$, where V_{rev} is the reversal potential, which was determined for each cell individually. Activation curves were then fit with the Boltzmann function in the form of $G/G_{max} = 1/\{1 + \exp[(V_{1/2} - V)/\kappa]\}$, where G_{max} is the maximal sodium conductance, $V_{1/2}$ is the half-maximal activation potential, V is the test potential, and κ is the slope factor. Steady-state fast inactivation was achieved with a series of 500 ms prepulses (−120 to −10 mV in 10 mV increments), and the remaining available channels were activated by a 12 ms test pulse to 0 mV. Peak inward currents obtained from steady-state fast-inactivation protocols were normalized to the maximal peak current (I_{max}) and fit with Boltzmann functions: $I/I_{max} = 1/\{1 + \exp[(V - V_{1/2})/\kappa]\}$, where V represents the inactivating prepulse potential, and $V_{1/2}$ represents the mid-point of inactivation curve.

Data Analysis

Data were analyzed using Pulsefit 8.6 and Origin 7.5, and presented as means ± SEM. The Kruskal–Wallis non-parametric test was used to analyze current density data. One-way ANOVA was used to assess the statistical significance of changes in characteristics of channel activation and inactivation. Statistical comparisons were performed by Student's *t*-test.

Western Blot

All cells were lysed in RIPA buffer (with 1% PMSF), and total protein concentrations were determined with a BCA Protein Assay Kit (TransGen). Total protein (10–20 mg) for each sample was loaded into precast 8% SDS-PAGE gels and run with running buffer. Gels were transferred onto PVDF membranes (Millipore). Antigen-specific primary antibodies (Pan sodium channel, Chemicon-AB5210; Nav1.6, Chemicon-AB5580 and Abcam-ab65166; β-tubulin, Abcam-ab6046; β-actin, Abcam-ab6276) were incubated overnight at 4°C and detected with species-specific horseradish-peroxidase-labeled secondary antibodies. An ECL Western Blotting Detection kit (TIANGEN) was used to obtain a chemiluminescence signal, which was detected using Gel Imaging System (Bio-Rad). Band quantification was performed using a Gel-Pro software. Bands of interest were normalized to actin- or tubulin- for a loading control.

RT-PCR

Total RNA of cultured neurons was prepared by using the Trizol Reagent (Invitrogen, USA). The Superscript TM-III kit (Invitrogen, USA) was used for reverse-transcribed with oligo dT and 2.5 mg total RNA. Primer sequences were as follows: NM_019266 (SCN8A), and NM_031144 (β-actin). SCN8A forward 5′-CTG GAG AAT GGA GGC ACA CAC-3′, reverse 5′-ACG CTG CTG CTT CTC CTT GTC-3′; and β-actin forward 5′-CGT TGA CAT CCG TAA AGA CCT-3′, reverse 5′-TCA GGA GGA GCA ATG ATC TTG-3′. The resulting cDNA PCR amplification was performed by using the following protocol: 95°C for 10 s followed by 50 cycles of 95°C for 5 s and 60°C for 31 s, and verified by 2.0% agarose gel electrophoresis. Images were captured by using Gel Imaging System (Bio-Rad). The amplicon size of each gene was108 and 144 bp, respectively.

Immunofluorescence Staining and Immunohistochemistry

Cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton-x-100 for 5 min, and incubated with 5% BSA for 60 min at room temperature to block non-specific binding. Without washing, the diluted primary antibodies (MAP2, Abcam-ab32454; Nav1.6, Abcam-ab65166) were added and incubated at 4°C overnight. After three washes with PBS, cells were incubated with the corresponding secondary antibodies at room temperature for 1 h. For immunohistochemistry, cells were stained with DAB kit (Vector Laboratories) according to the instructions of the manufacturer for peroxidase labeling. Images were acquired from a fluorescence microscopy (Leica Microsystems DM400B, Germany).

Image Analysis and Quantification

Quantitative analysis of mean fluorescence intensities (MFIs) of immunoreactive neurons was performed using Image J software (National Institutes of Health). MFI per square micrometer was calculated by dividing the MFI units by the area of outlined regions.

RESULTS

Elevated Excitability in APP/PS1 Mice

To investigate change in intrinsic excitability in APP/PS1 mice, whole-cell patch-clamp recordings were performed on 12 DIV (days *in vitro*) primary cultured neurons obtained from P0 APP/PS1 mice and littermate wide type mice in current-clamp mode. Frequency, threshold, and peak amplitude of action potentials (AP) were examined by different protocols. AP firing frequencies (f) elicited by increasing depolarizing currents (200 pA, 500 ms) were significantly increased in neurons from APP/PS1 mice compared to WT mice (**Figures 1A,D**; $f_{WT} = 15.375 \pm 3.428$ Hz, $n = 12$; $f_{APP/PS1} = 23.25 \pm 7.264$ Hz, $n = 10$; $p = 0.0128$). We used a depolarizing current (80 pA, 10 ms) to induce a single firing of an AP. Threshold potential ($V_{threshold}$) and amplitude of action potential (V_{peak}) were recorded and compared with WT. We found that thresholds of neurons from APP/PS1 mice were significantly decreased compared to WT mice (**Figures 1B,E**; $V_{threshold,\ WT} = -29.001 \pm 2.304$ mV, $n = 9$; $V_{threshold,\ APP/PS1} = -41.601 \pm 1.965$ mV, $n = 9$; $p = 0.0012$), but the peak amplitude was not (**Figures 1C,F**; $V_{peak,\ WT} = 46.159 \pm 2.663$ mV, $n = 9$; $V_{peak,\ APP/PS1} = 47.236 \pm 3.849$ mV, $n = 9$; $p = 0.6475$). These results suggested that the excitability of mature neurons obtained from APP/PS1 mice was increased.

$A\beta_{1-42}$ Increases Excitability in Cultured Cortical Neurons from Mice

It is likely that diverse factors contribute to the pathogenesis of AD patients or mice (Blennow et al., 2006; Bertram and Tanzi, 2008; Mucke, 2009). Among them, Aβ stands out on the basis of overwhelming genetic evidence and strong experimental

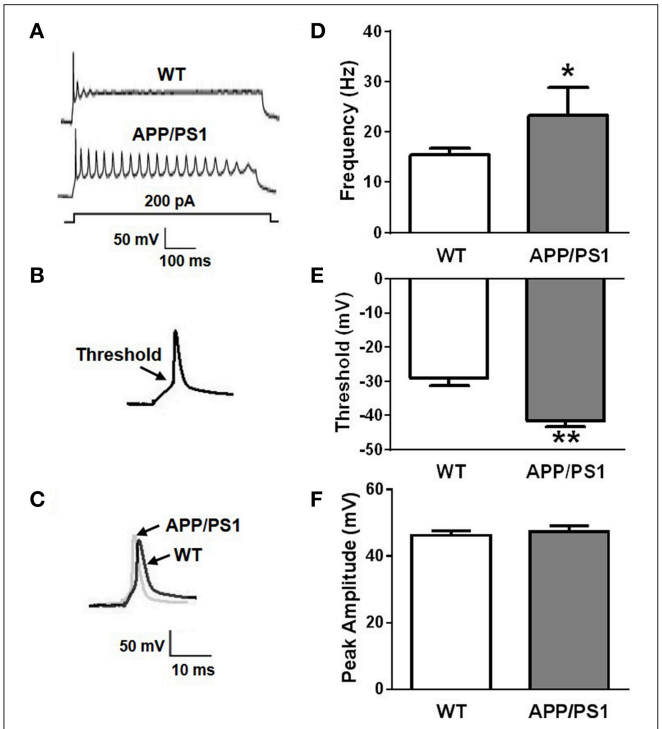

FIGURE 1 | Increased neuronal excitability in APP/PS1 mice. (A) Representative recording traces of action potential repetitive firing. **(B)** The schematic of threshold. **(C)** Representative recording traces of action potential peak amplitude. **(D)** The mean number of APs elicited by 200 pA depolarizing current. Neurons from APP/PS1 mice ($n = 10$) fired significantly more APs than WT ($n = 12$). **(E)** AP threshold in APP/PS1 mice cultured pyramidal neurons was significantly lower than WT mice ($n = 9$ per group). **(F)** The peak amplitude of APs in WT and APP/PS1 ($n = 9$ per group) mice cultured pyramidal neurons didn't show significantly difference. Mean ± SEM was displayed. *Presents p < 0.05; **presents $p < 0.01$.

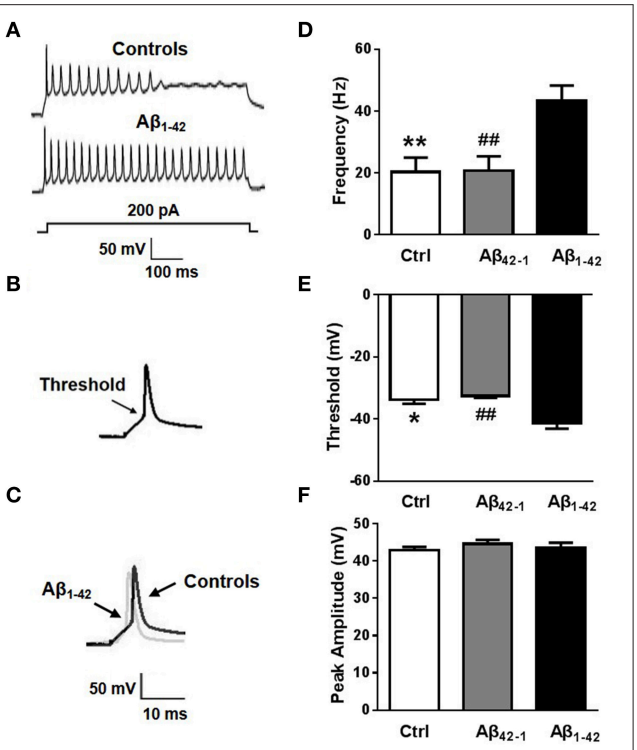

FIGURE 2 | Aβ affects neuronal excitability. (A) Representative recording traces of action potential repetitive firing. **(B)** The schematic of threshold. **(C)** Representative recording traces of action potential peak amplitude. **(D)** The mean number of APs elicited by 200 pA depolarizing current. Neurons after $A\beta_{1-42}$ treatment ($n = 8$) fired significantly more APs than controls (both negative control group and reverse peptide $A\beta_{42-1}$ group, $n = 8$). **(E)** AP threshold in pyramidal neurons treated with $A\beta_{1-42}$ ($n = 7$) was significantly lower than controls ($n = 7$). **(F)** The peak amplitude of APs in pyramidal neurons treated with $A\beta_{1-42}$ ($n = 7$) didn't show significantly difference with controls ($n = 7$). Mean ± SEM was displayed. *Presents $p < 0.05$ vs. control group; **presents $p < 0.01$ vs. control group; ## presents $p < 0.01$ vs. $A\beta_{1-42}$ group.

data (Farrer et al., 1997; Hardy and Selkoe, 2002; Tanzi and Bertram, 2005; Mahley and Huang, 2006). Accordingly, we attempted to investigate whether $A\beta_{1-42}$ contributed to intrinsic excitability. Cell viability was firstly determined by MTT assay in primary cultured neurons treated with 5 μM $A\beta_{42-1}$ or 5 μM $A\beta_{1-42}$ for 24 h. We found that $A\beta_{1-42}$ slightly induced loss of neuron viability (**Supplementary Figure S2**; $n = 3$, means three independent experiments; $p = 0.047$). As described previously, whole-cell patch-clamp recordings were performed in current-clamp mode on normal morphological primary neurons which were incubated with $A\beta_{1-42}$ or $A\beta_{42-1}$ for 24 h. Frequency, threshold, and peak amplitude of AP were examined. We found AP firing frequencies were significantly increased in neurons after treatment with $A\beta_{1-42}$ peptide compared to controls (**Figures 2A,D**; $f_{control} = 20.25 \pm 4.742$ Hz, $n = 8$, $p = 0.0050$; $f_{A\beta42-1} = 20.75 \pm 4.644$ Hz, $n = 8$, $p = 0.0054$; $f_{A\beta1-42} = 43.25 \pm 5.028$ Hz, $n = 8$). $A\beta_{1-42}$ also significantly decreased the threshold (**Figures 2B,E**; $V_{threshold,\ control} = -33.83 \pm 1.207$ mV, $n = 7$, $p = 0.0126$; $V_{threshold,\ A\beta42-1} = -32.55 \pm 0.6600$ mV, $n = 7$, $p = 0.0035$; $V_{threshold,\ A\beta1-42} = -41.37 \pm 1.771$ mV, $n = 7$). But it had no effect on the peak amplitude (**Figures 2C,F**; $V_{peak,\ control} = 42.83 \pm 0.9437$ mV, $n = 7$, $p = 0.6579$; $V_{peak,\ A\beta42-1} = 44.60 \pm 1.051$ mV, $n = 7$, $p = 0.5633$; $V_{peak,\ A\beta1-42} = 43.58 \pm 1.305$ mV, $n = 7$). These results suggested that incubation in $A\beta_{1-42}$ increases neuronal excitability *in vitro*.

Increased Neuronal Excitability Induced by $A\beta_{1-42}$ Due to an Up-Regulation of Nav Current

In mammalian neurons, dense clusters of voltage-gated sodium channels (Nav) at the axonal initial segment and nodes of Ranvier underlie action potential generation and fast propagation (Leterrier et al., 2011). In addition, non-inactivating persistent sodium currents support maintained depolarization during and between action potentials. Finally, the resurgent persistent sodium current is triggered upon repolarization and supports repetitive firing in some types of neurons (Raman et al., 1997). We therefore hypothesized that the increased excitability induced by $A\beta_{1-42}$ could be due, at least in part, to an upregulation of Nav current. To test this hypothesis, we evaluated the magnitude

of Nav currents in voltage-clamp mode after incubation with $A\beta_{1-42}$ or reverse peptide for 24 h. Representative traces of the total inward current recorded in response to voltage steps from −60 to +60 mV are shown in **Figure 3A**. Current density curves (**Figure 3B**) and peak current densities (**Figure 3C**) were significantly increased after $A\beta_{1-42}$ treatment compared to controls ($Peak_{control} = 79.18 \pm 11.60$ pA/pF, $n = 12$, $p = 0.0028$; $Peak_{A\beta42-1} = 74.14 \pm 13.54$ pA/pF, $n = 12$, $p = 0.0031$; $Peak_{A\beta1-42} = 146.3 \pm 10.70$pA/pF, $n = 12$).

We next investigated the effect of $A\beta_{1-42}$ on sodium channel kinetic characteristics (**Figures 3D,E**). We found that $A\beta_{1-42}$ did not significantly shift the voltage-dependent sodium activation curve (**Figure 3D**). Single Boltzmann distribution fits showed a $V_{1/2,\ control} = -41.62 \pm 1.462$ mV, and a κ of 4.165 ± 1.548 mV ($n = 12$); a $V_{1/2,\ A\beta42-1} = -41.71 \pm 1.768$ mV and a κ of 4.795 ± 1.825 mV ($n = 12$); and a $V_{1/2,\ A\beta1-42} = -41.19 \pm 1.052$ mV and a κ of 4.155 ± 1.184 mV ($n = 12$). For steady-state inactivation curves (**Figure 3E**), the $V_{1/2}$ and κ of inactivation also did not change significantly ($V_{1/2,\ control} = -51.47 \pm 1.040$ mV and $\kappa = 6.992 \pm 1.017$ mV, $n = 12$; $V_{1/2,\ A\beta42-1} = -51.90 \pm 0.8201$ mV and $\kappa = 8.384 \pm 0.8552$ mV, $n = 12$; $V_{1/2,\ A\beta1-42} = -51.40 \pm 0.4131$ mV and $\kappa = 7.723 \pm 0.4178$ mV, $n = 12$).

Overall, these results demonstrated that exposure of neurons to $A\beta_{1-42}$ leads to an increased neuronal excitability, likely through a Nav current-mediated mechanism.

Increased Expression of Nav in Cultured Neurons and APP/PS1 Mice

Although, the experiments described above revealed that $A\beta_{1-42}$ increased neuronal excitability, perhaps dues to increasing Nav currents, it remained unclear how the Nav currents increased. There was no observable difference in sodium channel kinetic characteristics after $A\beta_{1-42}$ incubation (**Figures 3D,E**). We then examined the expression of Nav in neurons after $A\beta_{1-42}$ treatment. Using Western blot analysis of cultured cortical neurons, we found that incubation with $A\beta_{1-42}$ significantly increased the expression of Nav [**Figures 4A,B**; $n = 3$; $p_{Ctrl\ vs.\ A\beta1-42} = 0.0146$; $p_{A\beta42-1\ vs.\ A\beta1-42} = 0.0358$]. We also investigated the Nav1.6 subtype, which plays a major role in the transmission of subthreshold currents, namely the persistent and resurgent currents (Raman et al., 1997), and the electrophysiological properties of Nav1.6 make these channels especially suited for the sustained repetitive firing of neurons (Van Wart and Matthews, 2006). As shown in **Figures 4C,D**, Nav1.6 displayed a significant increase after $A\beta_{1-42}$ treatment

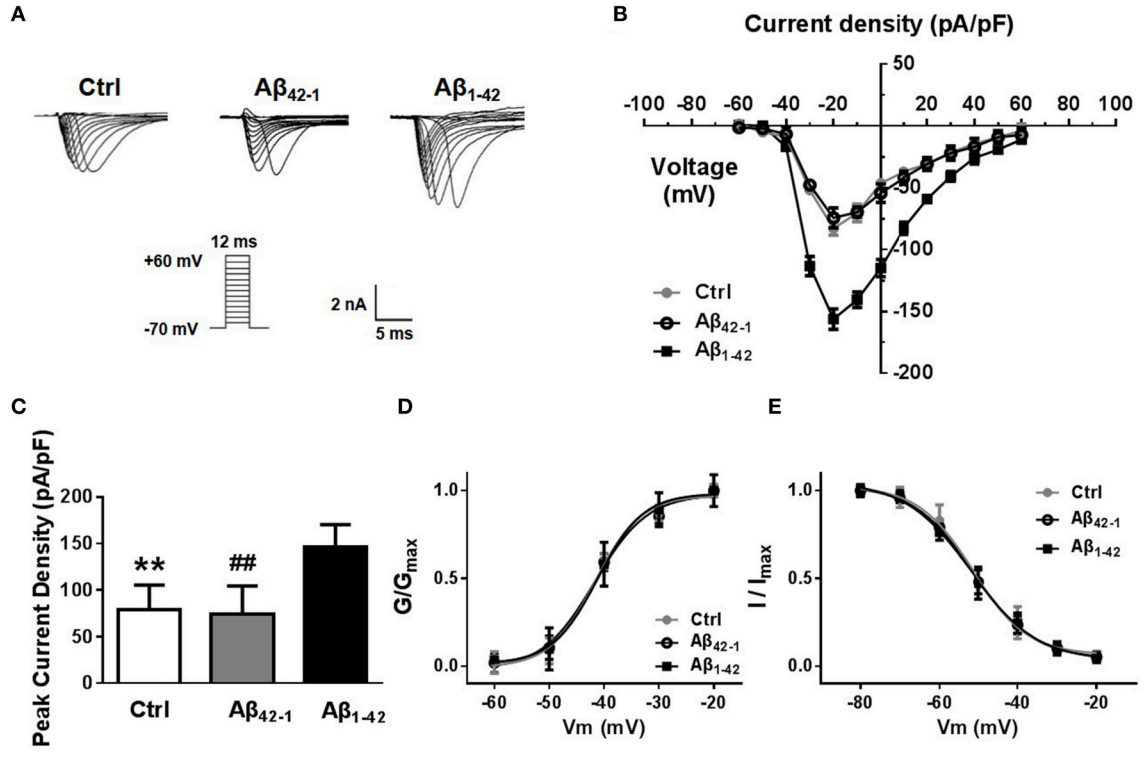

FIGURE 3 | $A\beta_{1-42}$ up-regulates voltage-dependent sodium current. (A) Representative traces of voltage gated sodium currents recorded in response to voltage steps from −60 to +60 mV. **(B)** Current density-voltage relationship, illustrates that Na^+ current density recorded from $A\beta_{1-42}$ treatment pyramidal neurons was significantly bigger than counterparts (negative control group and reverse peptide $A\beta_{42-1}$ group, $n = 12$). **(C)** Graph, data showed peak current density recorded from $A\beta_{1-42}$ treatment neurons was significantly bigger than counterparts ($n = 12$). **(D)** Normalized steady-state activation curves for Na^+ currents recorded from cultured pyramidal neurons after $A\beta_{1-42}$ treatment and counterparts ($n = 12$). The activation curves were not significantly shift after $A\beta_{1-42}$ treatment. **(E)** Normalized steady-state inactivation curves for Na^+ currents recorded from these three groups, and the inactivation curve was not significantly shift after $A\beta_{1-42}$ treatment either. Mean ± SEM was displayed. **Presents $p < 0.01$ vs. $A\beta_{1-42}$ group; ## presents $p < 0.01$ vs. $A\beta_{1-42}$ group.

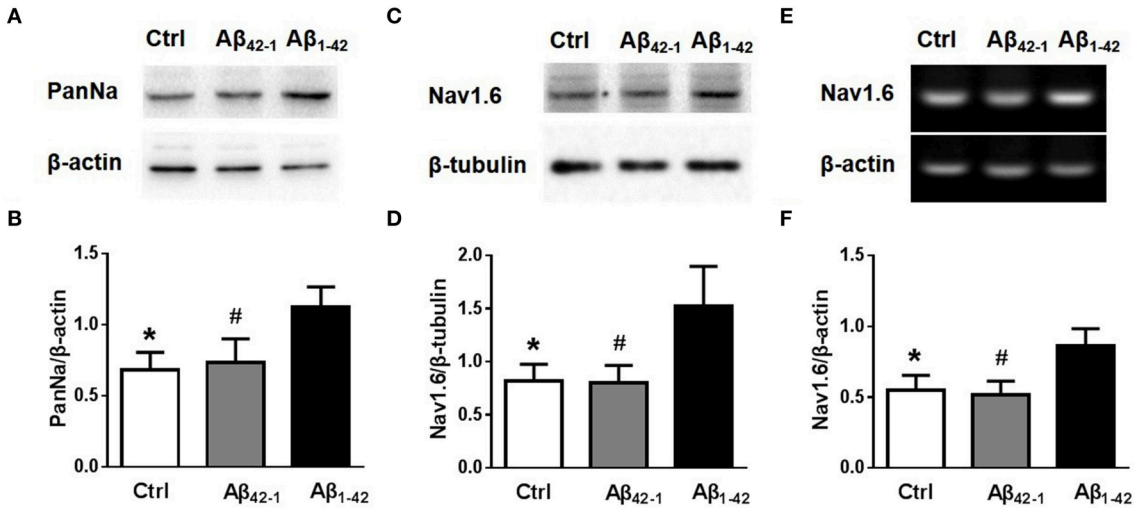

FIGURE 4 | $A\beta_{1-42}$ increases the expression of sodium channels. (A) Equal amounts of collected protein samples obtained from 12 DIV neurons after different treatments were analyzed by western blot to detect the protein expression level of sodium channel ($n = 3$ per group). **(B)** Quantification of protein levels of sodium channel. β-actin was used as an internal control ($n = 3$ per group). **(C)** Western blots of 12 DIV neurons after different treatments to detect the expression of Nav1.6 protein ($n = 3$ per group). **(D)** Quantification of protein levels of Nav1.6. β-tubulin was used as an internal control ($n = 3$ per group). **(E)** RT-PCR of Nav1.6 mRNA levels of 12 DIV neurons after different treatments ($n = 3$ per group). **(F)** Quantification of mRNA levels of Nav1.6. β-actin was used as an internal control ($n = 3$ per group). Mean ± SEM was displayed. *Presents $p < 0.05$ vs. $A\beta_{1-42}$ group; #presents $p < 0.05$ vs. $A\beta_{1-42}$ group.

[$n = 3$; $p_{\text{Ctrl vs. A}\beta1-42} = 0.0372$; $p_{\text{A}\beta42-1 \text{ vs. A}\beta1-42} = 0.0354$]. To further identify the increased expression of Nav1.6 after $A\beta_{1-42}$ treatment, mRNA expression levels were detected too. Paralleled with western blot analysis, Nav1.6 mRNA obtained from cultured neurons treated with $A\beta_{1-42}$ showed significantly increased than control groups [**Figures 4E,F**, $n = 3$; $p_{\text{Ctrl vs. A}\beta1-42} = 0.0257$; $p_{\text{A}\beta42-1 \text{ vs. A}\beta1-42} = 0.017$].

Morphological methods were also used to test the expression of Nav1.6. The localization of Nav1.6 in neurons was revealed by immunohistochemistry (**Figure 5A**). Consistent with the result from western blotting, most neurons incubated with $A\beta_{1-42}$ showed significantly deepening stain [**Figures 5A,C**; $n = 3$; $p_{\text{Ctrl vs. A}\beta1-42} = 0.0162$]. After obtaining these results, we wondered if the expression of Nav1.6 in APP/PS1 mouse brains would have changed. Coronal brain sections from 9 month old APP/PS1 mice and WT littermates were stained for MAP2, a maker of mature neurons, and Nav1.6 (**Figure 5B**; $n = 3$). We found, that APP/PS1 mice exhibited more Nav1.6 immunoreactivity than WT mice in cortex regions (**Figure 5D**; $n = 3$; $p = 0.0286$).

Taken together, our findings and previous studies suggest that $A\beta_{1-42}$ increased the excitability of cultured cortical neurons, and this effect was mediated by overexpression of Nav, with the Nav1.6 subtype perhaps accounting for much of this increase.

DISCUSSION

In addition to cognitive deficits, AD patients have an increased incidence of epileptic seizures. This incidence is even higher in patients with early-onset AD who overexpress human APP, the proteolysis of which generates Aβ (Palop and Mucke, 2009). Hyperexcitability is also detected in the brains of various AD transgenic mice (Tamagnini et al., 2015), including the APP/PS1 mice used here. Such aberrant increases in network excitability and compensatory inhibitory mechanisms in the hippocampus may contribute to the cognitive deficits in AD (Palop et al., 2007; Sanchez et al., 2012; Verret et al., 2012). However, the mechanisms underlying the hyperexcitability detected in AD brains are not fully understood.

In the present study, we investigated the excitability in cultured pyramidal neurons of APP/PS1 mice using patch clamp techniques. We found that the excitability of 12 DIV cultured pyramidal neurons from APP/PS1 mice was significantly greater than in WT littermates. Additionally, AP firing frequencies increased and $V_{threshold}$ decreased in neurons from APP/PS1 mice compared to WT mice. These results confirm previous studies that showed neuronal hyperexcitability in APP/PS1 mice by patch clamp methods. In recent years, many studies demonstrated that Aβ is associated with increased excitability of neurons *in vitro* and in animal models, leading to hypersynchronous network activity and higher risk for seizures (Minkeviciene et al., 2009; Busche et al., 2012; Born et al., 2014; Davis et al., 2014). In our current work, we observed the altered excitability of pyramidal neurons after treatment with Aβ. The higher firing frequency and lower threshold indicated an increased intrinsic excitability of pyramidal neurons.

Aβ-induced aberrant excitatory activity might occur through many different mechanisms. Previous studies have shown that Aβ can downregulate A-type K^+ currents, thereby increasing excitability of hippocampal pyramidal neurons (Good and Murphy, 1996; Chen, 2005). Elevated Aβ also causes GABAergic

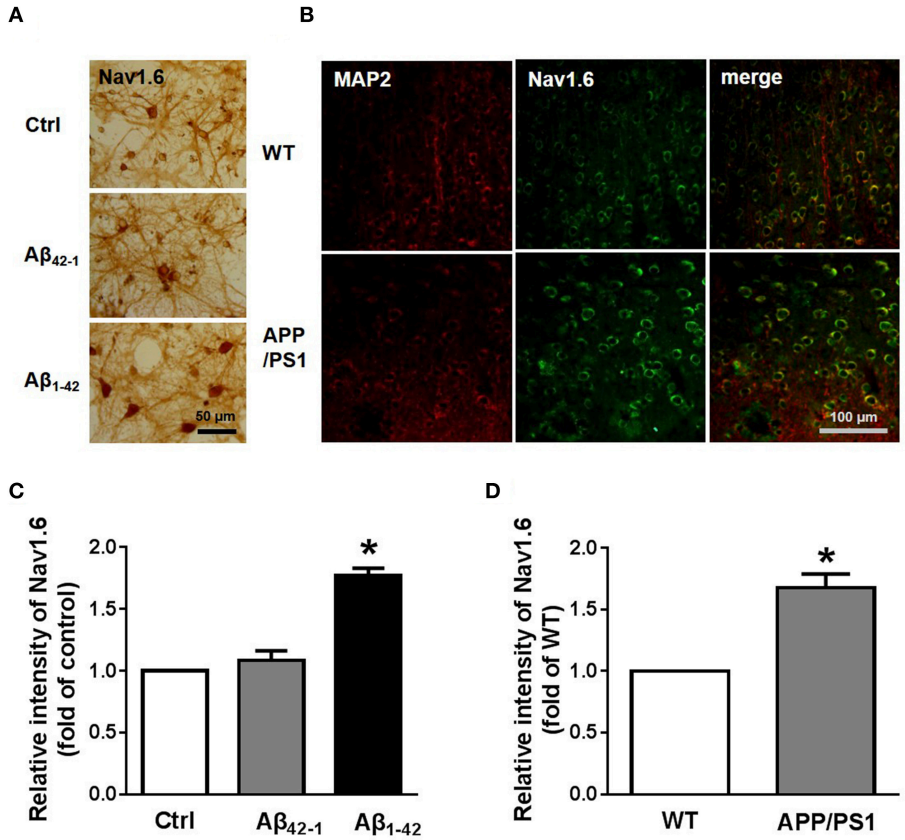

FIGURE 5 | Elevated expression of Nav1.6 in cultured neurons and APP/PS1 mice. (A) Immunohistochemistry images of 12 DIV primary cortical culture neurons after 24 h $A\beta_{42-1}$ or $A\beta_{1-42}$ treatments stained with anti-Nav1.6 antibody. Scale bar = 50 μm ($n = 3$ per group). **(B)** Immunostaining images of temporal lobe cortex sections obtained from 9 months APP/PS1 mice and its littermates co-stained with anti-Nav1.6 antibody and anti-MAP2 antibody ($n = 3$ per group). **(C)** Quantification of Nav1.6 immunohistochemistry images by using intensity analysis ($n = 3$ per group). **(D)** Quantification of fluorescence intensity of Nav1.6 ($n = 3$ per group). Mean ± SEM was displayed. *Presents $p < 0.05$ vs. control group.

dysfunction and attenuates excitatory synaptic transmission by decreasing the number of surface AMPA and NMDA receptors (Kamenetz et al., 2003; Hsieh et al., 2006; Shankar et al., 2007), as well as disrupting the development of aberrant synchrony in neural networks, damaging cognitive functions. Subsequent studies proved that neuronal activity regulates Aβ production (Kamenetz et al., 2003; Cirrito et al., 2005). Blocking neuronal electrical activity with TTX, a sodium channel blocker, decreased the cleavage of APP by β-secretase (Kamenetz et al., 2003). Recent studies found that blocking the network hyperactivity with the anti-epileptic drug lamotrigine, a voltage-dependent sodium channel inhibitor, reversed synaptic disorder and cognitive dysfunction in APP transgenic mice (Bakker et al., 2012; Sanchez et al., 2012; Zhang et al., 2014). These results indicate that Aβ, or other AD-related factors, plays a significant role in regulating neuronal activity at specific types of neurons as well as in wider neuronal networks, and Aβ and sodium channels have a certain relationship.

Voltage-dependent sodium currents play a critical role in action potential depolarization and firing frequency in many types of neurons (Kim et al., 2005; Baroni et al., 2013). We therefore hypothesized that the increased excitability induced by $A\beta_{1-42}$ could be due, at least in part, to an upregulation of the Nav current. As we expected, the peaks of the voltage-dependent sodium current were significantly increased after $A\beta_{1-42}$ treatment. The increased voltage-dependent sodium current could decrease action potential threshold and increase the probability of action potential generation in response to synaptic excitation, thus increasing the excitability of the neuron. However, Aβ upregulated sodium currents without significantly altering the voltage-dependence of activation and inactivation. We found that Aβ increased the expression of Nav and Nav1.6 in cultured neurons, indicating that the number of Nav channels could be altered by $A\beta_{1-42}$. These results provide a possible mechanism for the increased excitability of pyramidal neurons previously observed after Aβ treatment. Confirming the causal relationship between the aberrant excitatory activity induced by Aβ and cognitive decline in AD patients would be an important insight into the pathogenesis of AD and provide new therapeutic avenues.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: XW, SL. Performed the experiments: XW, XZ, and CJ. Analyzed the data: XW, XZ, ZX, and QM. Contributed reagents/materials/analysis tools: TZ, NL. Wrote the paper: XW, XZ.

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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.2016.00094

Supplementary Figure S1 | Biochemical characterization of $A\beta_{1-42}$. Eighty nanogram aged $A\beta_{1-42}$ and $A\beta_{1-42}$ monomer samples were analyzed by SDS-PAGE and detected with anti-6E10 antibody. After 48 h aggregated $A\beta_{1-42}$ with different molecular weight forms were displayed. aged presents $A\beta_{1-42}$ with aggregation treatment; mono presents $A\beta_{1-42}$ monomer.

Supplementary Figure S2 | Cell viability measurement of neuron treated with $A\beta_{1-42}$. Cell viability was determined by MTT assay in primary cultured neurons treated with 5 μM $A\beta_{42-1}$ or 5 μM $A\beta_{1-42}$ for 24 h. $A\beta_{1-42}$ induced slightly loss of cell viability in neurons. ($n = 3$, means 3 independent experiments). Mean ± SEM was displayed. MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide. *Presents $p < 0.05$ vs. control group.

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Proteomic and epigenomic markers of sepsis-induced delirium (SID)

Adonis Sfera[1,2]*, *Amy I. Price*[3], *Roberto Gradini*[4,5], *Michael Cummings*[2] and *Carolina Osorio*[1]

[1] Department of Psychiatry, Loma Linda University, Loma Linda, CA, USA, [2] Psychiatry, Patton State Hospital, Patton, CA, USA, [3] Evidence Based Health Care, University of Oxford, Oxford, UK, [4] Department of Pathology, Sapienza University, Rome, Italy, [5] IRCCS Neuromed, Pozzili, Italy

In elderly population sepsis is one of the leading causes of intensive care unit (ICU) admissions in the United States. Sepsis-induced delirium (SID) is the most frequent cause of delirium in ICU (Martin et al., 2010). Together delirium and SID represent under-recognized public health problems which place an increasing financial burden on the US health care system, currently estimated at 143–152 billion dollars per year (Leslie et al., 2008). The interest in SID was recently reignited as it was demonstrated that, contrary to prior beliefs, cognitive deficits induced by this condition may be irreversible and lead to dementia (Pandharipande et al., 2013; Brummel et al., 2014). Conversely, it is construed that diagnosing SID early or mitigating its full blown manifestations may preempt geriatric cognitive disorders. Biological markers specific for sepsis and SID would facilitate the development of potential therapies, monitor the disease process and at the same time enable elderly individuals to make better informed decisions regarding surgeries which may pose the risk of complications, including sepsis and delirium. This article proposes a battery of peripheral blood markers to be used for diagnostic and prognostic purposes in sepsis and SID. Though each individual marker may not be specific enough, we believe that together as a battery they may achieve the necessary accuracy to answer two important questions: who may be vulnerable to the development of sepsis, and who may develop SID and irreversible cognitive deficits following sepsis?

Keywords: Aquaporin-4 (AQP-4), astrocytes, cell cycle, lymphocyte proliferation test (LPT), T helper 17 cells, exosomes, microRNA

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***Correspondence:**
Adonis Sfera
dr.sfera@gmail.com

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INTRODUCTION

At present there are no biological markers to indicate vulnerability to sepsis or to the CNS dysfunction following it. Electroencephalography (EEG) and various instruments such as Confusion Assessment Method (CAM), Delirium Rating Scale (DRS) or Delirium Symptoms Interview (DSI) are currently used for diagnosing delirium, however they are not specific for SID and difficult to perform in sedated or intubated ICU patients (Zampieri et al., 2011). Recent studies have shown that delirium in general and SID in particular are unrecognized by clinicians in 32–66% of cases and because of their resemblance to psychiatric conditions, patients with delirium are frequently admitted to psychiatric wards, thus delaying much needed interventions (Leslie et al., 2008; Reeves et al., 2010). In order to avoid this problem the Society for Academic Emergency Medicine Geriatrics Task Force designated delirium screening a key quality indicator in elderly care (Terrell et al., 2009).

In our prior work in SID and delirium, we emphasized a potential epigenomic screening tool, the microRNA-6775 (miR-6775), which can be obtained from peripheral blood exosomes. This miR seems to block the transcription of CHRNA 7 gene which codes for alpha 7 nicotinic cholinergic receptors, while at the same time augments the expression of RNF-128, the Gene Related to Anergia in Lymphocytes (GRAIL), a gene involved in sepsis associated immune suppression (SAIS). Since SAIS represents a major cause of death in sepsis patients, miR-6775 may be a sepsis-stage marker in addition to a being a SID vulnerability screening tool (Sfera et al., 2014). We have hypothesized that delirium predisposing factors including low grade inflammation and the paucity of alpha 7 nicotinic acetylcholine receptors (nAChRs) may be the result of dysfunctional miR-6775 which silences the gene coding for these receptors. If this hypothesis is correct, miR-6775 may emerge as a marker of vulnerability to both sepsis and SID. In addition to epigenomic markers we drew attention to an astrocytic proteomic marker, the aquaporin-4 (AQP-4) protein which was found to be up-regulated in delirium (Sfera and Osorio, 2015).

In the present article, instead of focusing on individual biomarkers, we present a proteomic battery consisting of molecules released by the innate immune system, the initiator of septic responses. These markers can be obtained from the peripheral blood exosomes, a platform which carries to the periphery the molecular fingerprints of cells located in various tissues including the brain.

FROM THE IMMUNOLOGICAL TO THE NEUROLOGICAL SYNAPSE: PATHOPHYSIOLOGY OF SEPSIS AND SID

Critical care physicians have known for a long time that 70–80% of patients diagnosed with severe sepsis had been admitted to the hospital for reasons other than infections (Munford and Suffredini, 2010). Indeed, infection is necessary for a septic response, but not sufficient, as most infections remain localized and the inflammation does not spread throughout the body tissues. Moreover, sepsis-like global inflammatory responses are known to occur in burns, trauma or pancreatitis in the absence of infection (Balk, 2014).

Sepsis is thought to be ignited by a failure of the innate immune system to turn "off" after initiating an initial inflammatory response to intruding pathogens. The innate immunity "on" and "off" switch is located at the immunological synapse, the nanoscale junction between the T lymphocytes and the antigen presenting cells (APCs). This junction is populated by pattern recognition receptors (PRR) out of which the toll-like receptors (TLRs) are the best known. Activation of TLRs by pathogen toxins triggers "cytokine storms," release of non-specific pro-inflammatory molecules. Switching "off" the innate immune system is epigenomically controlled by microRNAs action on TLRs (Bayarsaihan, 2011; Stearns-Kurosawa et al., 2011). Turning the innate immunity "off" must occur when the more specific adaptive immune system comes "on line." Sepsis may be triggered by defective microRNAs which may be incapable of deactivating the TLRs and turn the innate immunity "off." In this scenario, both the adaptive and the innate immunity continue to work in parallel, eventually leading to immune exhaustion or SAIS.

MicroRNAs or miRs are short, 18–22 nucleotides in length, non-coding RNAs which have the ability to silence gene expression by inhibiting or degrading their messenger RNAs (mRNA). MicroRNAs were shown to modulate multiple biological processes, but those of interest for sepsis and SID include activation/deactivation of the innate immune system, cytoskeletal rearrangements in the innate immune cells (which enable cellular proliferation), and modulation of astrocytic volume (by changes in membrane permeability).

The CNS-immune interaction was demonstrated to be mediated by transmigration of peripheral lymphocytes and microphages through the blood-brain-barrier (BBB) and via the recently described lymphatic vessels in the dura mater (Louveau et al., 2015). The discovery of CNS lymphatics suddenly changed the perspective on the peripheral-central immune cooperation into a holistic one in which astrocytes, microglia and peripheral macrophages work in an innate immune continuum (Sternberg, 2006; Louveau et al., 2015). For example, the peripheral dendritic cells (DC) immigrate into the CNS and function as antigen presenting cells (APCs), while the activated microglia emigrate from the brain to the periphery, transporting inflammation (Sfera et al., 2014; Sfera and Osorio, 2015). These findings provide an explanation for the previously observed impairment in peripheral immunity following CNS pathology such as strokes, neurosurgical interventions, Alzheimer's disease (AD) and schizophrenia (Hochmeister et al., 2008; D'Agostino et al., 2012).

In the CNS, astrocytes and microglia are full members of the innate immune system which respond to peripheral infections by adopting a reactive stance (reactive gliosis). For example, reactive astrocytes undergo hypertrophy (edema) probably in an attempt to erect an additional physical barrier and restrict the spread of infection (Majewska and Szczepanik, 2006). Astrocytic swelling due to aquaporin-4 (AQP-4) up-regulation was documented in SID and may explain why most known delirium biomarkers are released by astrocytes (Papadopoulos and Verkman, 2013; Sfera et al., 2014; Thrane et al., 2014).

It is reasonable to assume that astrocytic swelling (astroedema) along with hypocholinergia (due to silenced CHRNA 7 gene) activate the TLRs, engendering low grade inflammation which is considered a delirium vulnerability marker in elderly (Court et al., 2001; Mitsis et al., 2009). For example, in post-operative delirium activation of TLR-4 (expressed by microglia and astrocytes) was demonstrated (Jalleh et al., 2012; Sofroniew, 2015). MicroRNA-130a is known to reduce astroedema by silencing the transcription of AQP-4 proteins (Sepramaniam et al., 2012). Moreover, miR-132 was demonstrated to restore CNS cholinergic signaling (Shaked et al., 2009). These epigenomic markers may represent a CNS compensatory response to astroedema and damaged cholinergic signaling.

PROTEOMIC BIOMARKERS OF SID

Aquaporin-4 (AQP-4) proteins in astrocytic end-feet constitute specific markers for astroedema described in SID. Exosomes carrying AQP-4 proteins from astrocytes to the peripheral blood may be utilized as biomarkers and therapeutic targets especially in SAIS (Papadopoulos and Verkman, 2013; Thrane et al., 2014; Sfera and Osorio, 2015).

Galectin-9 (Gal-9) and its receptor, the T cell immunoglobulin mucin-3 (Tim-3) expressed on T lymphocytes was shown to inhibit Th-17 proliferation both *in vitro* and *in vivo* (Oomizu et al., 2012). Gal-9 was demonstrated to play a major role in cell cycle regulation as it was shown to inhibit the cell cycle arrest in the G1 phase, promoting cellular proliferation. A dysfunctional Gal-9 may explain the aberrant neuronal cell cycle re-entry documented in AD as well as the exaggerated proliferation of macrophages and T-cells in the early phase of sepsis (Currais et al., 2009). In the CNS, Gal-9 is secreted by astrocytes and it was demonstrated to affect the astrocyte-microglia dialog (Steelman and Li, 2014). Interestingly, microRNA-155 (which modulates TLRs at the immunologic synapse) acts on the Tim-3 receptors, competing with Gal-9 (Cheng et al., 2015). Therefore, a significant drop in miR-155 level and/or an increase in Gal-9 level may suggest immune restoration in sepsis.

CRYAB (alphaB-crystallin) is a small heat-shock protein which was demonstrated to reduce T cell proliferation (including the CD-69 response). Animal studies show that Cryab-/- mice present with higher Th-17 cell count and more intense neuroinflammation, compared to wild-type counterparts (Arac et al., 2011). In the brain Cryab is secreted by astrocytes as an anti-inflammatory molecule; its presence in peripheral blood exosomes may indicate functional astrocytes, reflecting epigenetic integrity. CRYAB is involved in the process of microRNA maturation; its absence may result in dysfunctional microRNAs, including miR-6775, miR-155, miR-132, and miR-130 family. A drop in the level of this marker in peripheral blood exosomes may reflect the body-wide inflammation of sepsis with the imminent potential for SID onset (Ousman et al., 2007).

Ubiquitin-modifying protein A 20 has potent anti-inflammatory and anti-proliferative properties. For example it can decrease T cell differentiation into the neurotoxic Th-17 phenotype (Kool et al., 2011). Animal studies demonstrate that A 20 deficiency causes spontaneous neuroinflammation with reactive gliosis. In addition, it was demonstrated that that the A 20 protein deactivates the innate immunity at the level of the TLRs in the immunological synapse (Boone et al., 2004). Absence of TLR deactivation was demonstrated to result in exaggerated inflammatory responses, the "cytokine storms" characteristic of early sepsis. Micro RNA-155 epigenetically modulates the "on" and "off" activation of TLRs, suggesting involvement in sepsis and SID (O'Neill et al., 2011; **Table 1**). A drop in A 20 level along with an increase in miR-155 levels are markers of poor prognosis in sepsis and SID.

Vimentin is a major constituent of the cellular cytoskeleton which was found to orchestrate the Th-17 and possibly cancer cells migration into the CNS (Nieminen et al., 2006). Indeed, in sepsis an elevated Th-17 count was reported as vimentin equips T lymphocytes with both the motility and the amoeboid shape necessary for transmigration across the blood-brain-barrier (Nieminen et al., 2006). By rearranging the cytoskeleton vimentin facilitates proliferation of CD-69 lymphocyte in response to mitogens; this response can be measured by the lymphocyte proliferation test (LPT) (Nieminen et al., 2006; Ward et al., 2008). LPT is currently being used as a peripheral blood marker for AD and traumatic brain injury (TBI), based on the observation that lymphocytes derived from patients with AD present with a proliferation defect, a lower CD-69 count after mitogenic stimulation, compared to lymphocytes derived from normal individuals (Zhang et al., 2013). In fact the transition between elevated and depressed CD-69 response, may mark the precise point in time when a localized infection transitions to sepsis and SID. This transition may be captured by the stimulation index (SI) which represents the ratio of CD-69 expression level/endogenous CD-69 level. The SI may mirror the onset and progression of immune suppression and if obtained daily in ICU patients may detect sepsis onset. At the epigenetic level the CD-69 vimentin-induced response is modulated by miR-155 and miR-130 family, again rendering these miRs epigenetic markers for SID.

TABLE 1 | Potential proteomic markers for sepsis and SID.

Protein	Action	Effect
AQP-4	-Augments astrocytic water permeability -Promotes astroedema	Detrimental for SID
Galectin-9	-Inhibits Th-17 proliferation	Beneficial for sepsis/SID
CRYAB	-Decreases CD-69 lymphocyte proliferation -Promotes microRNA maturation	Beneficial in sepsis/SID
A 20 protein	-Deactivates TLRs -Turns innate immunity "off"	Beneficial in sepsis/SID
Vimentin	-Enables cytoskeleton for motility and proliferation	Detrimental in sepsis/SID

EPIGENOMIC BIOMARKERS OF SID

Approximately 60% of known miRs are expressed in the brain, rendering them almost ideal peripheral biomarkers for many CNS disorders (Song and Lee, 2015). Exosome-isolated miRs provide a higher diagnostic precision than miRs obtained from plasma (Stenvang et al., 2012).

MicroRNA-6775: silences the transcription of CHRNA-7 gene which codes for alpha 7 nicotinic acetylcholine receptors (nAChRs). When silenced CHRNA-7 gene augments the proliferation of Th-17 lymphocytes. Another action of microRNA-6775 involves activation of RNA-128, the gene related to anergia in lymphocytes (GRAIL), predisposing to SAIS (Sfera et al., 2014).

MicroRNA-130 family: nicknamed astromiRs, this miR family are modulators of cellular motility (Tu et al., 2014). Members of this family include:

MicroRNA–130b: promotes metastatic cancer aggressiveness by augmenting vimentin-induced cytoskeletal changes. MicroRNA-130b is believed to be an oncomiR as it augments

TABLE 2 | Potential epigenomic markers of sepsis and SID.

MicroRNA	Action	Effect
mir-6775	-Silences CHRNA-7 gene -Augments RNF-128	Detrimental for sepsis/SID Promotes low grade inflammation and SAIS
miR-130 a	-Silences transcription of AQP-4 gene -Decreases cell motility and proliferation (lowers CD-69 and Th-17 lymphocytes)	Beneficial sepsis/SID Lowers astroedema and Th-17 transmigration
miR-130b	-Increases cell motility and proliferation	
miR-155	-Increases CD-69 response -Activated TLRs by binding to galectin-9 receptor -Turns innate immunity "on"	Detrimental for sepsis/SID
miR-132	-Acetylcholinesterase inhibition -Restores cholinergic transmission	Beneficial for sepsis/SID

malignant aggression by enhancing cellular ability to migrate. We however characterize miR-130b as a motilimiR since metastasizing is occurs secondary to increased cellular motility. By the same mechanism miR-130b may promote Th-17 transmigration into the CNS (**Table 1**). For this reason miR-130b can be considered both a therapeutic target and a biomarker of SID (Leavy, 2010).

MicroRNA-130a (miR-130a): may also be a therapeutic target in addition to being a SID biomarker This miR, silences the transcription of AQP-4 proteins in astrocytic end-feet, reducing astroedema and neuroinflammation (Sepramaniam et al., 2012). In addition, miR-130a inhibits cellular locomotion and proliferation and is being credited with inhibition of metastatic spread. Since its action opposes miR-130b, we consider it an antimiR to 130a (Murugaiyan et al., 2011).

MicroRNA-155 (miR-155): In sepsis, a dysregulated miR-155 was shown to unleash inflammation via activation of TLRs preventing deactivation of the innate immune system upon activation of adaptive immunity. Others have shown that silencing miR-155 may be beneficial in experimental autoimmune encephalomyelitis (Murugaiyan et al., 2011), AD (Song and Lee, 2015), and other neuropsychiatric disorders (Hu et al., 2013). We believe that miR-155 is a plausible therapeutic target in SID in addition of being of diagnostic and prognostic benefit.

MicroRNA-132: acts as a natural cholinesterase inhibitor (also called a cholinomiR) as it enhances cholinergic signaling by inhibiting its degrading enzymes (Shaked et al., 2009; Nadorp and Soreq, 2014) (**Table 2**). Deficient acetylcholine is one of the best documented hypotheses of delirium. Daily levels of miR-132 obtained from peripheral blood exosomes in ICU patients with SID may reflect the prognostic trend.

TINY BUBBLES: AN EXOSOMAL TRANSPORTATION PLATFORM

Extracellular vesicles are tiny membrane structures which are divided by their size, into ectosomes, larger vesicles (over 100 nm), and exosomes, smaller vesicles (50–100 nm). They are released by a wide variety of cells including immune, CNS and cancer cells. Their cargo consists of proteins, DNA, microRNAs, and mRNAs which carry the signatures of their cells of origin throughout the body fluids. After release from their source, miRs circulate with the peripheral blood either bound to lipoproteins or incorporated in exosomes which offer protection from dilution and the ambient ribonuclease (Wagner et al., 2013). Therefore, assays of exosome-packaged miRs derived from the peripheral blood are more reliable compared to assays of non-exosomal miRs (Cheng et al., 2014). Exosome content can be analyzed with the help of simple commercial exosome isolation kits or by more complex ultracentrifugation (Corrado et al., 2013).

At the immunological synapse, exosomes carry pathogen-derived molecules between the T cell and the APC, committing T cells to particular phenotypes (Schorey et al., 2015). In sepsis it was demonstrated that naïve T cells differentiate into neurotoxic Th-17 lymphocytes (Martinez et al., 2008). We believe that this metamorphosis is enabled by the disinhibition of RNF-128 gene by dysfunctional miR-6775 which, probably acts on vimentin to enable transformation of naïve T cells into the highly mobile and aggressive Th-17 phenotype.

In the CNS, exosomes were demonstrated to be involved in neurotransmitter signaling at neurological synapses (Chivet et al., 2014; Glebov et al., 2015). For example, exosomal miRs in the prefrontal cortex synapses were recently demonstrated in schizophrenia and bipolar disorder (Banigan et al., 2013). In SID exosome-analysis may identify stage-of-disease markers and treatment targets. For example, astrocytes were shown to release exosomes containing miR-155 and miR-130 family which along with AQP-4 proteins which constitute SID biomarkers. Indeed the National Institute of Health recently awarded a grant for studying blood exosomes containing astrocytic proteins as diagnostic markers for CNS disorders (Crocker and Vella, 2015). In AD neural proteins such as amyloid beta, total tau and P-tau were recently isolated from peripheral blood exosomes (Fiandaca et al., 2015).

Exosomes were demonstrated capable of carry aquaporin proteins to the body periphery. For example, urinary exosomes containing aquaporin-1 (AQP-1) proteins are currently being utilized in nephrology as novel biomarkers of renal ischemia and reperfusion (Sonoda et al., 2009). In SID, the up-regulated AQP-4 proteins originating in astrocytic end-feet may be detectable in peripheral blood exosomes.

CONCLUSIONS

Sepsis and SID represent health care problems which translate into a major economic burden likely to increase along with the demographics of aging population. Biological markers with adequate sensitivity and specificity for SID could decrease not only the length of hospital stay, lead to new treatments, but also preempt the epidemic of irreversible cognitive deficit and dementia in elderly. With the same token the ability to predict vulnerability to delirium and subsequent dementia would facilitate the informed consent process of elderly individuals deciding for elective surgeries.

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Dehydration and Cognition in Geriatrics: A Hydromolecular Hypothesis

Adonis Sfera[1,2]*, *Michael Cummings*[2]* *and Carolina Osorio*[1]

[1] Department of Psychiatry, Loma Linda University, Loma Linda, USA, [2] Patton State Hospital, Patton, USA

Dehydration is one of the ten most frequent diagnoses responsible for the hospital admission of elderly in the United States. It is associated with increased mortality, morbidity and an estimated cost of 1.14 billion per year (Xiao et al., 2004; Schlanger et al., 2010; Pretorius et al., 2013; Frangeskou et al., 2015). Older individuals are predisposed to dehydration encephalopathy as a result of decreased total body water (TBW) and diminished sensation of thirst. We hypothesize that thirst blunting in older individuals is the result of a defective microRNA-6842-3p failing to silence the expression of the vesicular GABA transporters (VGAT) and alpha 7 cholinergic nicotinic receptors in the subfornical organ (SFO) of the hypothalamus. We hypothesize further that resultant dehydration facilitates protein misfolding and aggregation, predisposing to neurocognitive disorders. We completed a search of predicted microRNA targets, utilizing the public domain tool miRDB and found that microRNA-6842-3p modulates the SLC6A1 and CHRNA7 genes both of which were previously hypothesized to inhibit the thirst sensation by their action on SFO. The primary aim of this article is to answer two questions: Can prevention and correction of dehydration in elderly lower age-related cognitive deterioration? Can exosomal miR-6842 in the peripheral blood predict dehydration encephalopathy in elderly?

Keywords: dehydration, aquaporins, extracellular space, protein folding, protein conformational dynamics

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Megha Agrawal,
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Ravi C. Kalathur,
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Enno Klussmann,
Max Delbrück Center for Molecular Medicine, Germany

***Correspondence:**
Adonis Sfera
dr.sfera@gmail.com;
Michael Cummings
Michael.Cummings@DSH.ca.gov

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HYDRATION AND COGNITION

Dehydration is one of the most common medical problems in seniors diagnosed in 6.7% of hospitalized patients over the age of 65 (Warren et al., 1994). It leads to poor outcomes and increased health care expenditures. Novel studies reveal that if not prevented or treated promptly, dehydration results in longer intensive care unit (ICU) stay, higher hospital readmission rates and placement in long term facilities (Xiao et al., 2004; Frangeskou et al., 2015). On the other hand, preventing dehydration not only reduces healthcare expenditures, but also improves outcomes and the elderly patients' quality of life.

Dehydration is a contributing factor for delirium, a neurobehavioral syndrome recently demonstrated to be a strong risk factor for dementia (Inouye, 1998; Davis et al., 2012). It is therefore crucial to recognize and diagnose dehydration quickly, however at the present time there are no specific biological markers for this condition. Clinical signs, plasma osmolality and urine markers have poor specificity in elderly (George and Rockwood, 2004). For this reason potential epigenetic markers such as microRNA-6842-3p obtained from peripheral blood exosomes may contribute not only to early diagnosis, but also to prevention of dehydration.

Water is an essential body nutrient and its homeostasis is crucial for life. Early in the evolution, marine animals were surrounded by water, but survival on dry land required built-in, "portable" water (Warren et al., 1994). In humans, the muscle tissue is a genuine fluid reservoir, carrying over 80% of TBW (George and Rockwood, 2004).

The brain, in spite of being a highly lipophilic organ consists of 80% water (Tait et al., 2008). Most of the CNS intracellular water is stored in astrocytes. These cells are characterized by high aquaporin (AQP) expression which makes them four times more permeable to water than other brain cells, therefore true "brain cisterns" for times of water scarcity (Thrane et al., 2014). With the same token, because of their high AQP content, astrocytes are prone to pathological water retention and swelling. Novel studies demonstrate that astrocytes respond to peripheral dehydration by up-regulation of AQP-4 proteins on their end-feet processes probably in order to preserve water. For example, preclinical studies demonstrate that a hyperosmotic milieu induces AQP expression in astrocytes (Yang et al., 2013).

Overexpression of AQP-4 channels and augmented water intake transforms these cells into genuine "sponges" resulting in extracellular dehydration, extracellular space (ECS) hypovolemia. If severe enough this condition may turn into a medical emergency, dehydration encephalopathy or delirium.

The process of aging seems to undo the evolutionary advantage of "portable water" as elderly individuals are known to lose their fluid reservoirs by age-related decrease in both muscle mass and astrocyte density. For example, dehydration was demonstrated to accelerate the progression of AD which is also known to be associated with loss of astrocytes (Ogawa et al., 2011; Reyes-Haro et al., 2015; Rodríguez-Arellano et al., 2016).

It is well known that aging is associated with reduced acetylcholine (ACh) in the brain, but it is perhaps less emphasized that aging contributes to down-regulation of alpha7 nicotinic acetylcholine receptors (alpha7nAChR) (Utsugisawa et al., 1999; Akhmedov et al., 2013), rendering the CNS less responsive to ACh. This is significant for the sensation of thirst which is physiologically activated by ACh. Lower cholinergic activation predisposes to inflammation which is also involved in cognitive impairment. We discussed inflammation in the aging brain elsewhere and this subject will not be brought here (Sfera and Osorio, 2014). The alpha7nAChR are encoded by CHRNA7 gene which is subject to microRNA epigenetic regulation, including miR-6842.

Concerning the relationship between dehydration and impaired cognition nutrition studies demonstrate that a loss of only 1–2% of TBW may result in impaired cognitive performance; in elderly this percentage was shown to be even lower (Han and Wilber, 2013; Riebl and Davy, 2013). Furthermore, the link between hydration and cognition can be demonstrated by the neurocognitive disorders associated with up-regulation of AQP-4 expression primarily on asyrocytic end-feet (**Table 1**).

Novel studies demonstrate that both dehydration and aging were associated with AQP-4 up-regulation, therefore it should not come as a surprise that aging and water loss go hand in hand (Trinh-Trang-Tan et al., 2003). Interestingly, several amyloid-binding, neuroprotective compounds were demonstrated to down-regulate AQP-4 expression, further demonstrating the role of water in amyloid pathology (**Table 2**).

In addition, neuroimaging studies in dehydrated elderly, show decrease in gray and white matter volume (Streitbürger et al., 2012). However, it is important to keep in mind that most brain volumetric studies rely on diffusion tensor imaging (DTI) which detects water anisotropy and is therefore highly dependent on the brain fluid dynamics (Meng et al., 2004; Nakamura et al., 2014).

WATER AND PROTEIN MISFOLDING DISORDERS

Misfolded protein aggregates were shown to be involved in many human diseases, including neurocognitive disorders and diabetes type 2, but in spite of the increasing prevalence of these conditions, the reason proteins misfold is not completely understood.

Water has been known to play a major role in protein conformational dynamics (Lemieux, 1996; Phillips, 2002; Zhao et al., 2013). In order to become biologically active newly transcribed proteins must fold along specific axes like paper in the ancient Japanese art of origami (Collet, 2011; Chong

TABLE 1 | Disorders associated with cognitive deficit and AQP-4 up-regulation.

AQP-4/Cognitive deficit disorders	References
Cerebral amyloid angiopathy	Foglio and Fabrizio, 2010; Moftakhar et al., 2010
Alzheimer's disease	Nagelhus and Ottersen, 2013; Lan et al., 2015
Parkinson's disease	Subburaman and Vanisree, 2011; Zhang et al., 2016
Multiple sclerosis	Tanaka et al., 2007
Neuromyelitis optica	Saji et al., 2013; Zhang et al., 2015
Traumatic brain injury	Hu et al., 2005
Cerebral ischemia	Zador et al., 2009
Epilepsy	Binder et al., 2012; Alvestad et al., 2013
HIV encephalitis	St. Hillaire et al., 2005
Progressive multifocal leukoencephalopathy	Aoki-Yoshino et al., 2005; Florence et al., 2012

TABLE 2 | Neuroprotective compounds associated with AQP-4 down-regulation.

AQP-4 down-regulation	References
Rapamycin	Guo et al., 2014
Erythropoietin	Gunnarson et al., 2009; McCook et al., 2012
Curcumin	Laird et al., 2010; Wang et al., 2015
Purines	Morelli et al., 2010; Lee et al., 2013
Progesteron	He et al., 2014
Melatonin	Dehghan et al., 2013; Lin et al., 2013; Bhattacharyaa et al., 2014

and Ham, 2015). Recently it was demonstrated that water plays a crucial role in this process as it forms hydrogen bonds with the amino acid chains, facilitating their collapse into three dimensional molecular structures. In the presence of water, folding occurs almost instantly (140 ns), resulting in biologically active molecules available for chemical reactions at the opportune time (Sen and Voorheis, 2014; Vajda and Perczel, 2014). In the absence of hydration the folding process is significantly slower and the biomolecules may miss the timing of their reactions. This results in molecular overcrowding which predisposes to misfolding (Gregersen et al., 2006; Stoppini et al., 2009). Indeed, it was hypothesized by others that biomolecular crowding relative to the fluid volume is inductive of misfolding and aggregation (Tokuriki et al., 2004; Yerbury et al., 2005).

Novel studies in protein conformational dynamics demonstrate that both protein misfolding, their repair and removal can take place in the intra and the extracellular compartment. The chance of protein misfolding is higher in the extracellular space (ECS) which is a rougher environment exposing these biomolecules to a higher degree of shear and tear (Ker and Chen, 1998; Genereux and Wiseman, 2015). For this reason, we focus our study on the ECS where hypovolemia may facilitate protein misfolding and aggregation.

It was hypothesized that adequate water circulation via aquaporin (AQP) channels is essential for clearing beta amyloid and for preventing its build-up characteristic for Alzheimer's disease (AD) (**Figure 1**). The glymphatic system paradigm suggests that insufficient amyloid clearance and its subsequent aggregation is the result of impaired water movement (Xie et al., 2013). This model, however pays less attention as to why proteins misfold in the first place.

The hydromolecular hypothesis is therefore complementary to the glymphatic model, but also differs from it by elevating water from an inert medium to an active participant in cognition (via protein folding) (Levy and Onuchic, 2006). This hypothesis raises another interesting question: do proteins participate in information processing directly?

Novel studies in neuroscience demonstrate that proteins participate in cognition by their ability to access logic gates, the elementary building blocks of digital circuits (Qi et al., 2013). These molecules are endowed with abilities to adaptively change their shapes in Transformers-like fashion, assembling and disassembling in response to electronic signals or electromagnetic fields (Kidd et al., 2009; Ausländer et al., 2012). For example, proteins were shown to assemble in the neuronal post-synaptic membrane into heteroreceptor complexes which may engender memory "bar codes" (Fuxe et al., 2007; Chen et al., 2012). Calcium-calmodulin-dependent kinase III, a component of neuronal microtubules, was hypothesized to store long term memory by reorganizing its spatial structure in response to synaptic activity (Smythies, 2015). Interestingly, water plays a

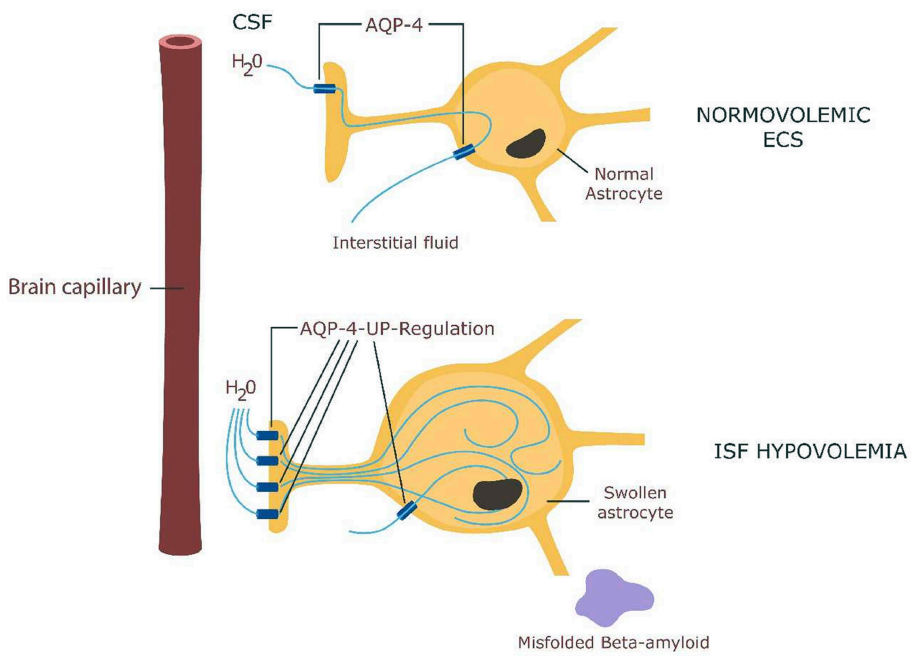

FIGURE 1 | Astrocyte swelling as a result of AQP-4 channels up-regulation with interstitial fluid hypovolemia and beta-amyloid misfolding.

major role in this model. Several studies revealed that dendritic spine biomolecules may play a crucial role in associative memory as they endow the neural circuits with Boolean logic (Craddock et al., 2012; De Ronde et al., 2012; Qi et al., 2013). Furthermore, proteins are endowed with Lego-like abilities to interlink, engendering large intra and extracellular biomolecular networks with hypothesized roles in cognition (Chen et al., 2012; Mancuso et al., 2014). In light of this data we believe that alteration of the normal protein conformation may impair cognition directly, rather than indirectly by damaging synapses and neurons which is the traditional view.

EPIGENOMIC REGULATION OF THE SUBFORNICAL ORGAN (SFO)

Elderly individuals are prone to dehydration as a result of blunted thirst sensation and loss of TBW as discussed above (Cowen et al., 2013; Hooper et al., 2014). Recent preclinical data reveal that the subfornical organ (SFO) of the hypothalamus functions as a "thirst center" in the mammalian brain, regulating the basic instinct of water intake (Oka et al., 2015). Since the SFO lacks a blood-brain-barrier (BBB) it may be well positioned to detect peripheral dehydration and respond to it by increasing the sensation of thirst lowering water output. The SFO contains sensitive osmoreceptors which convert peripheral changes in osmolality into an excitatory neuronal signal, triggering both the sensation of thirst and the release of arginine vasopressin (AVP) by the posterior pituitary (Azizi et al., 2008).

It was recently demonstrated that the SFO contains both excitatory and inhibitory neurons which can be activated by the ECS water volume and osmolality (Oka et al., 2015). ECS hypovolemia activates the SFO excitatory neurons (which express ETV-1 transcription factor), triggering thirst. ECS normovolemia, on the other hand activates the SFO inhibitory neurons (which express the vesicular GABA transporter (VGAT)], inhibiting the sensation of thirst.

These genetically distinct neuronal groups may explain both dehydration and psychotic polydipsia. For example, excessive activation of excitatory, or failure to activate inhibitory SFO neurons may result in psychotic polydipsia. The opposite may be true in dehydration.

Several prior studies revealed that the sensation of thirst may also be activated by the stimulation of SFO neuronal cholinergic receptors. The SFO neurons express both nicotinic and muscarinic cholinergic receptors, while the SFO astrocytes express only alpha 7- nAChRs (Honda et al., 2003; Tanaka, 2003; Ono et al., 2008). Age-related paucity of these receptors interferes with ACh activation of the thirst sensation. The glial water channels consist of AQP-9 expressed by astrocytes and AQP-4 expressed by tanycytes (**Figure 2**).

In addition to decreasing the expression of alpha 7 nAChRs, the aging process was documented to augment the expression of AQP channels on astrocytic end-feet as part of an age-related senescence-associated secretory phenotype (SASP). SASP is characterized by low grade inflammation, increased accumulation of misfolded protein aggregates and astrocyte swelling induced by AQP up-regulation (Picciotto and Zoli, 2002; Salminen et al., 2011; Akhmedov et al., 2013).

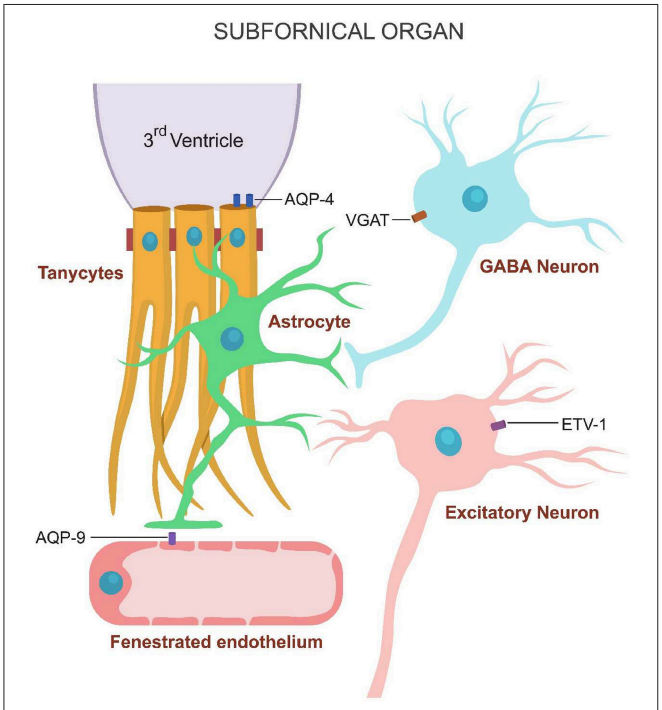

FIGURE 2 | Water channels expressed on the SFO cells. Inhibitory GABAergic neurons express VGAT, astrocytes AQP-9 and tanycytes AQP-4.

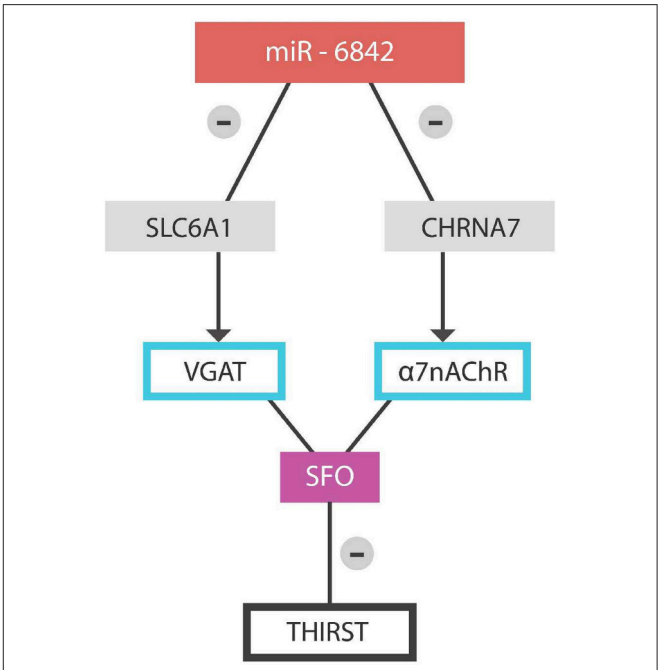

FIGURE 3 | Physiologically, microRNA-6842 silences SLC6A1 and CHRNA7 genes, activating the sensation of thirst.

Peripheral dehydration was demonstrated to alter the expression of several SFO- related genes (Hindmarch et al., 2008). One of these genes is SLC6A1 which expresses VGAT on the cellular membranes of the SFO inhibitory neurons.

Method: we conducted a search of miRDB, a public online database for microRNA target prediction and functional annotations. The targets in miRDB are predicted by the bioinformatics tool, MirTarget. MirTarget was developed by analyzing thousands of miRNA-target interactions from high-throughput sequencing experiments. We searched the human database for the genes of interest SLC 32A1 and CHRNA 7, coding for VGAT and alpha 7 nicotinic cholinergic receptors respectively. We conducted a separate search for each of the two genes by utilizing the gene symbol SLC 32A1 and CHRNA 7. The results revealed that 131 microRNAs modulate the SLC 32A1 gene and 57 microRNAs the CHRNA 7 gene. Analyzing this data, miR by miR we found one common microRNA modulating both genes, the miR-6842 (**Figure 3**).

A dysfunctional miR-6842 may fail to silence the SLC6A1 gene, preventing inhibition of the SFO GABAergic neurons with resultant thirst blocking. The same is achieved via failure to inhibit the CHRNA-7 gene, thus preventing ACh-induced thirst.

CONCLUSIONS

The hydromolecular hypothesis endeavors to explain the relationship between dehydration and decreased cognition in elderly as resulting from protein misfolding and aggregation in the context of low interstitial fluid volume (ECS hypovolemia). Defective proteins may affect cognition either directly via impaired information processing in the brain biomolecular networks, or indirectly via neuronal and synaptic damage, or both.

MicroRNA-6842 may constitute a biological marker with predictive value for dehydration encephalopathy in elderly as it regulates two genes involved in the sensation of thirst.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Corpora Amylacea of Brain Tissue from Neurodegenerative Diseases Are Stained with Specific Antifungal Antibodies

Diana Pisa[1], *Ruth Alonso*[1], *Alberto Rábano*[2] *and Luis Carrasco*[1]*

[1] Centro de Biología Molecular "Severo Ochoa," Universidad Autónoma de Madrid, Madrid, Spain, [2] Department of Neuropathology and Tissue Bank, Unidad de Investigación Proyecto Alzheimer, Fundación Centro de Investigación de Enfermedades Neurologicas, Instituto de Salud Carlos III, Madrid, Spain

The origin and potential function of corpora amylacea (CA) remains largely unknown. Low numbers of CA are detected in the aging brain of normal individuals but they are abundant in the central nervous system of patients with neurodegenerative diseases. In the present study, we show that CA from patients diagnosed with Alzheimer's disease (AD) contain fungal proteins as detected by immunohistochemistry analyses. Accordingly, CA were labeled with different anti-fungal antibodies at the external surface, whereas the central portion composed of calcium salts contain less proteins. Detection of fungal proteins was achieved using a number of antibodies raised against different fungal species, which indicated cross-reactivity between the fungal proteins present in CA and the antibodies employed. Importantly, these antibodies do not immunoreact with cellular proteins. Additionally, CNS samples from patients diagnosed with amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD) also contained CA that were immunoreactive with a range of antifungal antibodies. However, CA were less abundant in ALS or PD patients as compared to CNS samples from AD. By contrast, CA from brain tissue of control subjects were almost devoid of fungal immunoreactivity. These observations are consistent with the concept that CA associate with fungal infections and may contribute to the elucidation of the origin of CA.

Keywords: corpora amylacea, neurodegenerative disease, fungal infection, Alzheimer's disease, amyotrophic lateral sclerosis

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Megha Agrawal,
University of Arkansas at Little Rock, USA

Reviewed by:
Isidro Ferrer,
University of Barcelona, Spain
Ian Paul Johnson,
University of Adelaide, Australia

***Correspondence:**
Luis Carrasco
lcarrasco@cbm.csic.es

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INTRODUCTION

Corpora amylacea (CA) are glycoproteinaceous inclusions that accumulate in the brain during the course of normal aging and to a greater extent in some neurodegenerative diseases, particularly Alzheimer's disease (AD) (Mrak et al., 1997; Keller, 2006; Song et al., 2014). Abundant CA are found in a subset of patients with temporal epilepsy, where extensive deposits of CA replace the pyramidal layers of the cornu ammonis (Nishio et al., 2001; Kovacs and Risser, 2014). In addition to the central nervous system (CNS), CA are found in other organs and tissues, such as normal prostate glands, prostate cancer and several other malignant tissues (Christian et al., 2005; Morales et al., 2005; Hechtman et al., 2013a,b; Badea et al., 2015). CA are amorphous rounded, laminated bodies approximately 10-50 μm in diameter.

The composition of CA has been analyzed in some detail. They mostly contain polyglucans (over 85% are hexoses) with a minor component (4%) of proteins (Robitaille et al., 1980; Nishimura et al., 2000; Sfanos et al., 2009). The rounded core is formed by different calcium salts, principally calcium phosphate and calcium oxalate depending on the bodies analyzed (Magura and Spector, 1979; Nakamura et al., 1995; Kodaka et al., 2008). A wide range of proteins are found in CA and a number of them have been characterized using specific antibodies (Singhrao et al., 1994). For example, ubiquitin, heat-shock proteins, Bcl-2, and c-Jun (Martin et al., 1991; Cisse et al., 1993; Botez and Rami, 2001) in addition to tau and several blood proteins such as thrombospondin-1 and some complement components may be detected in CA (Singhrao et al., 1993, 1995; Meng et al., 2009; Day et al., 2015). A detailed characterization of prostate CA by proteomic analyses has confirmed that lactoferrin is the most abundant protein, together with myeloperoxidase, S100 calcium-binding proteins A8 and A9, which form human calprotectin, and α-defensins, which form part of neutrophil granules (Sfanos et al., 2009). A number of S100 proteins including calprotectin, an inflammatory protein, are also present in CA from normal human brains (Hoyaux et al., 2000). Indeed, immunohistochemistry analysis suggests that the source of calprotectin in CA is prostate-infiltrating neutrophils, leading to the concept that chronic inflammation results in prostate cancer (De Marzo et al., 2007; Sfanos et al., 2009, 2014). By contrast, the suggestion that CA are built up of breakdrown products from neurons and oligodendroglial cells has also been proposed (Singhrao et al., 1994). Along this line, proteomic analyses of brain CA from multiple sclerosis patients detected the presence of cytoskeleton proteins and glycolysis enzymes (Selmaj et al., 2008). A number of microorganisms are suggested as the potential source of the chronic inflammation that triggers the formation of CA. Among these, several bacteria such as *Chlamydia trachomatis*, *Escherichia coli,* and *Pseudomonas* spp., protozoa such as *Trichomonas vaginalis* and viruses known to contribute to different types of cancer, including human papillomavirus, have been considered (Sfanos et al., 2009, 2014). Furthermore, a correlation between fungal infection and prostatic cancer has been reported (Sutcliffe et al., 2014). Prostatic CA are thought to give rise to prostatic calculi, and electron microscopy examination suggests the presence of microbial infection (Dessombz et al., 2012). Therefore, the traditional notion that CA result from precipitated proteins of glandular secretions is being replaced by the concept that they represent a response to a microbial infection.

We have recently reported the presence of fungal proteins in CNS from AD patients (Alonso et al., 2014a; Pisa et al., 2015a,b), and also in patients diagnosed with amyotrophic lateral sclerosis (ALS) (Alonso et al., 2015). Fungal infections elicit a neutrophil response, leading to the production of defensins and other molecules that participate in the innate immune response (Cunha et al., 2014; Lionakis, 2014). Neutrophils play a pivotal role against fungal infections (Lionakis, 2014). Undoubtedly, lactoferrin is considered a marker of inflammation and infiltration. Transferrin and lactoferrin are iron-binding proteins which function to maintain low levels of ferric ions in blood, mucus and tissues (Johnson and Wessling-Resnick, 2012). In this manner, some microbial infections are controlled since an increase in free iron leads to microbial growth (Samaranayake et al., 1997; Bullen et al., 2006; Mehra et al., 2012). Additionally, both myeloperoxidase and calprotectin are involved in the control of fungal infections (Murthy et al., 1993; Metzler et al., 2011). Finally, defensins are a family of small cationic peptides that can perturb the plasma membrane of *C. albicans*, leading to increased membrane permeability (Schroeder et al., 2011). As indicated above, polyglucans are the most abundant macromolecule in CA and interestingly these polysaccharides are also quite abundant in the fungal cell wall (Free, 2013). In the current study, we assessed the presence of fungal proteins in CA from different CNS regions obtained from control individuals and patients with several neurodegenerative diseases. Our findings provide strong evidence that fungal proteins are localized in the periphery of CA from patients diagnosed with neurodegenerative diseases.

MATERIALS AND METHODS

Description of Control Subjects and Patients

We analyzed samples from patients diagnosed with AD, ALS, and Parkinson's disease (PD) in addition to control individuals without neurological disease. The age and gender of the subjects investigated in this study are listed in Supplementary Table 1. All samples were supplied by a brain bank (Banco de Tejidos CIEN) and were analyzed anonymously. The Ethics Committee of the Universidad Autónoma de Madrid approved the study. The transfer of samples was carried out according to national regulations concerning research on human biological samples. In all cases, written informed consent was obtained. For patients with dementia, informed consent for brain donation was given on a postmortem basis by a next-of-kin following the procedure established by the external ethical committee of the brain bank. Accordingly, a next-of-kin of the patient gave credit through an informed consent document to the fact that the patient had never opposed to be a brain donor during his/her life. An ethics committee external to the bank approved all ethico-legal documents, including written informed consent.

Brain samples were processed according to a common postmortem protocol followed by Banco de Tejidos CIEN. Briefly, rapid neuropathological autopsy was performed upon call by the donor's proxies (mean postmortem interval, 4.5 h). Immediately after extraction, the right half of the brain was sliced and frozen, while the left half was fixed by immersion in phosphate-buffered 4% formaldehyde for at least 3 weeks. A full neuropathological study was performed in the left half brain after fixation. Neuropathological diagnosis and staging of all disease entities was performed according to consensus criteria. Various neuropathological variables related to AD, vascular, Lewy and TDP (TAR DNA-binding protein) pathologies in addition to the presence of hippocampal sclerosis were recorded for full classification of cases.

Antifungal Antibodies

Candida famata, *C. albicans*, *C. glabrata,* and *Syncephalastrun racemosum* were grown in YEP (yeast extract peptone) medium (1% yeast extract, 2% Bacto peptone) as described (Pisa et al., 2008). Fungal cells were centrifuged and washed in phosphate-buffered saline (PBS). *Phoma betae* was purchased from Allergon AB (Engelholm, Sweden). Fungal cells were autoclaved and lyophilized. Rabbit antisera against *C. famata*, *C. albicans*, *C. glabrata*, *P. betae*, and *S. racemosum* were obtained by inoculation of 1 or 2 mg of dried fungi in 0.5 ml PBS, previously mixed with an equal volume of Freund's adjuvant. Rabbits were inoculated up to three times every 3 weeks and the antibody titer and specificity of the sera were tested by immunohistochemistry and immunoblotting using fungal proteins. The protocols employed were approved by the ethics committee of Centro de Biologia Molecular "Severo Ochoa" (identification number: ES280790000180). The optimal dilution for immunofluorescence staining for each antibody was assayed using both isolated *Candida* spp.

The specificity of the antifungal antibodies obtained was tested by immunofluorescence against different *Candida* spp. The cross-reactivity of each antibody against the different fungal species can differ, for instance anti-*C. glabrata* antibody immunoreacted with *C. glabrata*, *C. albicans*, *C. tropicalis*, *C. parapsilosis,* and *C. Krusei*, whereas anti-*C. albicans* does not recognize *C. Parapsilosis* and *C. Krusei*. By contrast, anti-*S. racemosum* only immunoreacts with *C. Krusei*. Besides, none of the antifungal antibodies obtained immunoreacted with cultured human cells or human brain sections from healthy subjects (Pacheco et al., 2007; Pisa et al., 2015a,b).

Immunohistochemistry Analysis

CNS tissue was embedded in paraffin following standard techniques and cut into 5-μm sections using a microtome (Microm HM355s, Walldorf, Germany). For immunohistochemical analysis, paraffin was removed and sections were rehydrated and boiled for 2 min in 10 mM citrate buffer and then incubated for 10 min with 50 mM ammonium chloride. Subsequently, tissue sections were incubated for 10 min with PBS/Triton X-100 (0.1%) followed by 20 min with 2% bovine serum albumin in PBS. Sections were incubated overnight at 4°C with a mouse monoclonal antibody raised against human α-tubulin (Sigma), human phospho-PHF-tau, clone AT100 (Thermo Scientific), or human neurofilament protein, clone 2F11 (Dako), all at 1:50 dilution, or a rabbit polyclonal antibody raised against proteins obtained from *C. glabrata* at 1:500 or *C. famata, C. albicans, P. betae,* and *S. racemosum* at 1:100 dilution. Thereafter, sections were washed with PBS and further incubated for 1 h at 37°C with donkey anti-mouse IgG secondary antibody conjugated to Alexa 555 (Invitrogen) at 1:500 for α-tubulin, tau and neurofilament, and donkey anti-rabbit IgG secondary antibody conjugated to Alexa 488 (Invitrogen) at 1:500 dilution for antifungal antibodies. To visualize nuclei, sections were then stained with DAPI (4,6-diamino-2-fenilindol) (Merck) and treated with autofluorescence eliminator reagent (Merck). The use of this reagent is important to avoid autofluorescence, since there is lipofuschin in the aging brain. All images were collected and analyzed with a LSM710 confocal laser scanning microscope combined with the upright microscope stand AxioImager.M2 (Zeiss), running Zeiss ZEN 2010 software. The spectral system employed was Quasar + 2 PMTs. Images were deconvoluted using Huygens software (4.2.2 p0) and visualized with Fiji/ImageJ (NIH, Bethesda, MD) software.

RESULTS

Fungal Proteins Are Present in CA from AD Patients

A variety of cellular proteins constitute part of CA (Sfanos et al., 2009). To assess the presence of fungal proteins in CA, we carried out immunohistochemistry analysis using a specific rabbit polyclonal antibody raised against *C. glabrata*, which does not cross-react with human proteins (Pisa et al., 2015a,b). Initially, we tested this antibody on tissue sections from different CNS regions, including lateral frontal cortex (LFC), cerebellar córtex (CEC) and entorhinal cortex/hippocampus (ERH) from one AD patient. Double immunofluorescence staining was performed using a second antibody that detects tau protein. CA were more abundant in ERH regions than in LFC or CEC (**Figure 1**). CA from all three CNS regions (LFC, CEC, and ERH) stained positive with the anti-fungal antibody (**Figure 1**). The external laminar structures and the envelope surrounding the central core of CA were clearly immunoreactive for the *C. glabrata* antibody (green), revealing the presence of fungal proteins in this region (**Figure 1**). In some instances, the entire envelope was positive, while in other sections only a part of the external envelope was immunoreactive. By contrast, the anti-tau antibody (red) stained only some of the CA, particularly from CEC sections (**Figure 1**). This result is consistent with previous findings describing tau protein in CA (Singhrao et al., 1993; Day et al., 2015). Of note, not all CA contained tau protein inasmuch as it was undetectable in some CA sections analyzed by confocal microscopy. Thus, although tau can be detected in some CA, it is not an abundant protein.

To further assess the presence of fungal proteins in CA, tissue sections were immunolabeled with additional antibodies raised against *C. famata*, *C. albicans. S. racemosum,* and *P. betae* (green) together with a monoclonal antibody against neurofilaments (red). As shown in **Figure 2**, CA inclusions from the different CNS regions from AD patient 1 immunoreacted with all four antifungal antibodies, further supporting the existence of fungal proteins in CA. None of the antifungal antibodies recognize cellular proteins from neural cells in CNS sections (Pisa et al., 2015a,b). The localization of the immunopositive structures using the additional antibodies was similar to that observed with the *C. glabrata* antibody, strengthening the notion that fungal proteins are present in CA from different CNS regions. By contrast, neurofilament staining was more irregular, with strong immunoreactivity in some CA, and weaker or no staining in other CA inclusions. This was evident in CEC sections double-labeled with the anti-*C. albicans* antibody and in LFC and CEC sections labeled with the anti-*S. racemosum* antibody; in

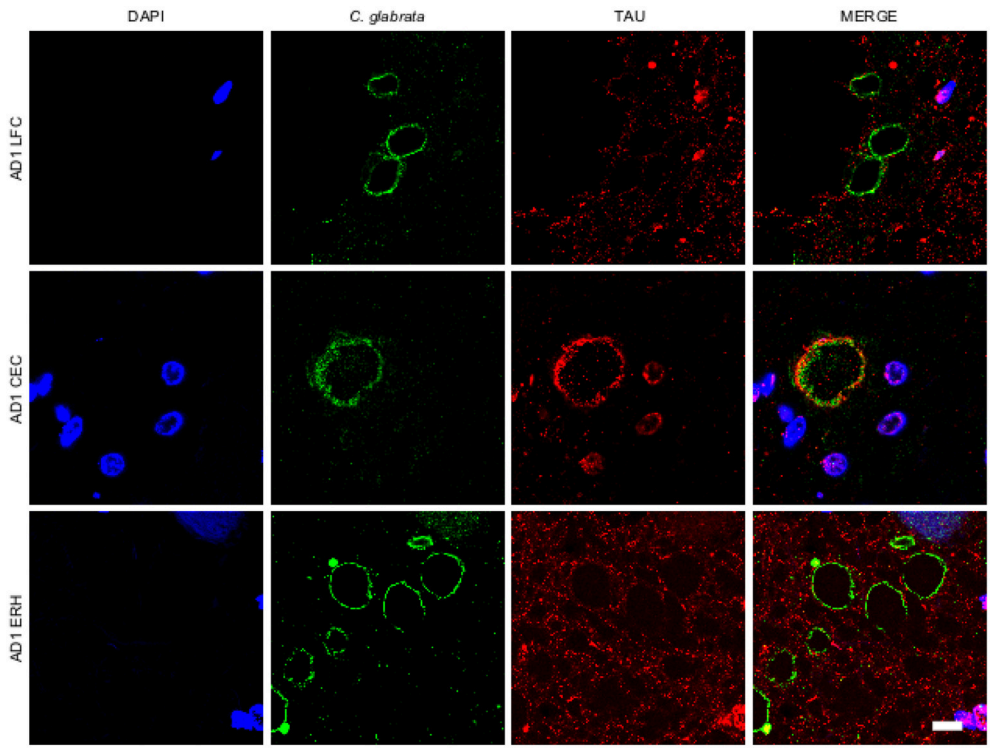

FIGURE 1 | Corpora amylacea are detected in different regions of the CNS. Tissue sections (5 μm) from different CNS regions of patient AD1 were tested as follows: lateral frontal cortex (LFC), cerebellar cortex (CEC) and entorhinal cortex/hippocampus (ERH). Immunohistochemistry analysis was carried out by double immunofluorescence staining employing a rabbit polyclonal anti-*C. glabrata* antibody (green) and a mouse monoclonal anti-tau antibody (red). Sections were mounted and observed by confocal microscopy after incubation with the corresponding secondary antibodies, as described in Materials and Methods. Overlapping red and green pixels appear as orange/yellow. DAPI appears in blue. Scale bar: 10 μm.

both cases, the neurofilament signal was more intense than the fungal signal, as manifested by the punctate yellow staining in merged images in some of the sections. The fact that neurofilaments are detected in some CA but not in others underscores the concept that the protein composition of CNS CA is nonhomogeneous. This lack of homogeneity can be revealed only by immunohistochemistry and not by proteomic analyses of purified CA.

We also analyzed ERH sections from other AD patients (AD2-AD11; Supplementary Table 1). Immunohistochemmistry was performed using anti-*C. albicans* and anti-*P. betae* antibodies (green) and anti-human α-tubulin antibodies were used to mark microtuble structures (red). Of note, the anti-tubulin antibody immunoreacts not only with human cells but also with several eukaryotic species, including fungal cells. Consequently, in the instances where both green and red signals co-localized, it may be because of the presence of fungal tubulin. Fungal proteins were detected in ERH CA inclusions from all 10 additional AD patients (**Figure 3**). As the antifungal antibodies are polyclonal, they can cross-react with a number of fungal proteins. The positive immunoreactivity with one of these antibodies does not demonstrate that the fungal species present is the same as that employed to raise the antibody. However, since each antibody immunoreacts with different fungal antigens, differences in the immunostaining provides a clue to indicate that the fungal species differ. Accordingly, this technique cannot establish the precise fungal species present in each sample and DNA sequencing would be required (Alonso et al., 2014a,b; Pisa et al., 2015b). The majority of the CA inclusions from different patients immunoreacted with both anti-*C. albicans* and anti-*P. betae* antibodies, although staining for *P. betae* was more robust in patients AD3, AD8, AD10, and AD11 than in the other patients (**Figure 3**). This finding suggests that the fungal species present in each patient differ. Additionally these results support the use of a panel of anti-fungal antibodies to comprehensively determine the presence or absence of fungal proteins in CA. An important conclusion from this analysis is that the location of fungal proteins in CA inclusions rules out the possibility that fungal infection was due to postmortem contamination since the formation of CA occurs over long time periods (months or even years).

Analysis of CA from ALS and PD Patients

Our recent finding of fungal infection in CNS from ALS patients (Alonso et al., 2015), prompted us to test for fungi in CA inclusions in ALS samples. We examined tissue sections from motor cortex (MC), medulla (MD) and different levels of the spinal cord (SC1, SC2, and SC3) of an ALS patient (ALS1)

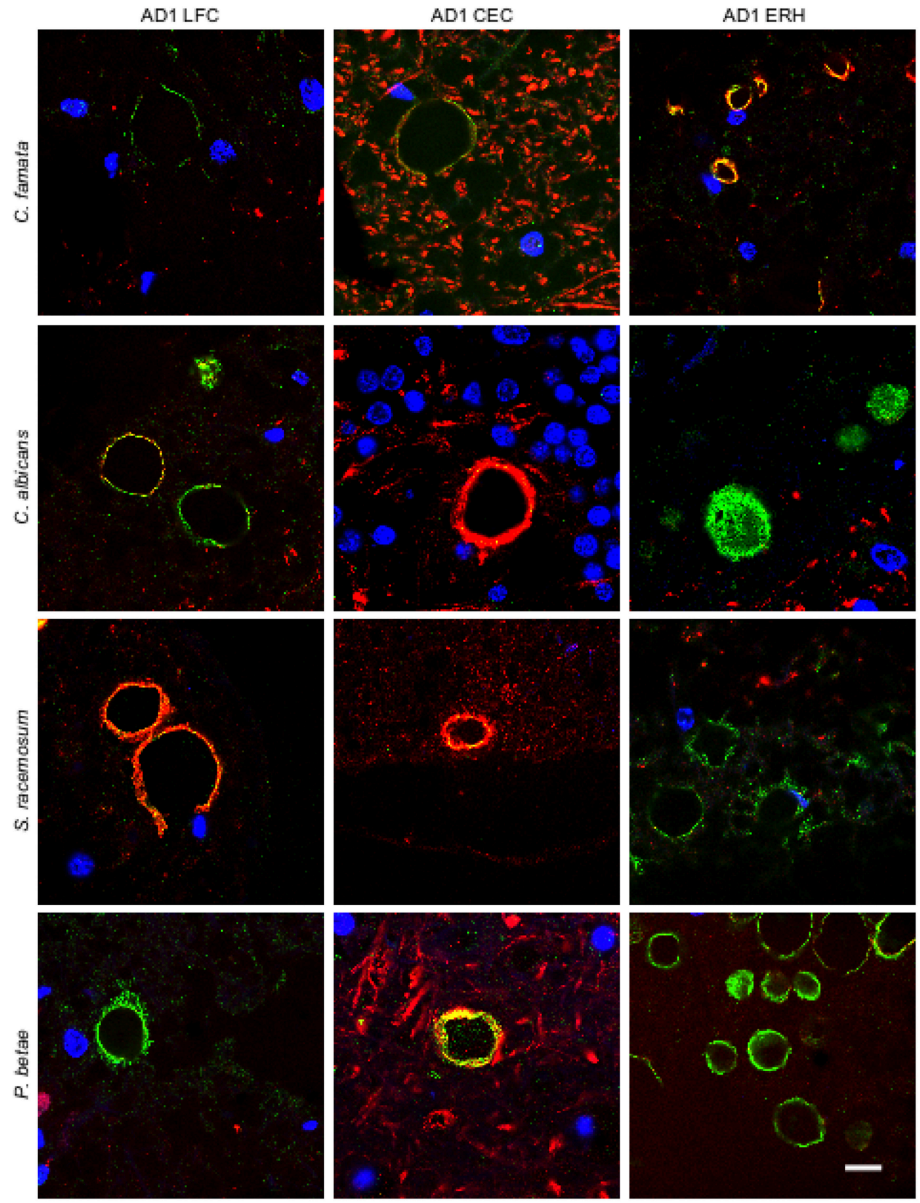

FIGURE 2 | Immunoreactivity of corpora amylacea against different antifungal antibodies. Tissue sections analyzed from patient AD1 are indicated in the figure. Immunohistochemistry analysis was carried out using a monoclonal antibody to human neurofilaments (red) and rabbit polyclonal antibodies raised against the following fungi: *C. famata, C. albicans. S. racemosum,* and *P. betae* (green). Sections were mounted and examined by confocal microscopy after incubation with the corresponding secondary antibodies. Orange/yellow corresponds to red and green pixels. DAPI (blue) and scale bar: 10 μm.

using anti-*C. albicans* and anti-*P. betae* antibodies. Double immunolabeling of the CNS from patient ALS1 with α-tubulin (red) revealed fungal proteins (green) at the periphery of CA inclusions in different regions (**Figure 4**). Of note, CA inclusions were also detected in different regions of the CNS of this patient, including the spinal cord. The numbers of CA inclusions observed in these samples were, however, lower than those found in AD patients. We also tested tissue sections from different CNS regions of five additional ALS patients (ALS2-ALS6) using the same antibodies. CA inclusions were detected in all ALS patients examined and all were positive for fungal protein. For clarity, only one field with each antibody and only one CNS region for each ALS patient is shown (**Figure 4**). In general, fungal proteins (green) were detected at the periphery of CA inclusions, but in some instances fungal proteins were observed throughout CA bodies, including the central portion. Additionally, anti-α-tubulin (red), reactivity was in the main detected in association with material present in CA, but in a few instances α-tubulin was detected in the surrounding areas. The observations in ALS tissue indicate that there is a similarity between ALS CA and AD CA

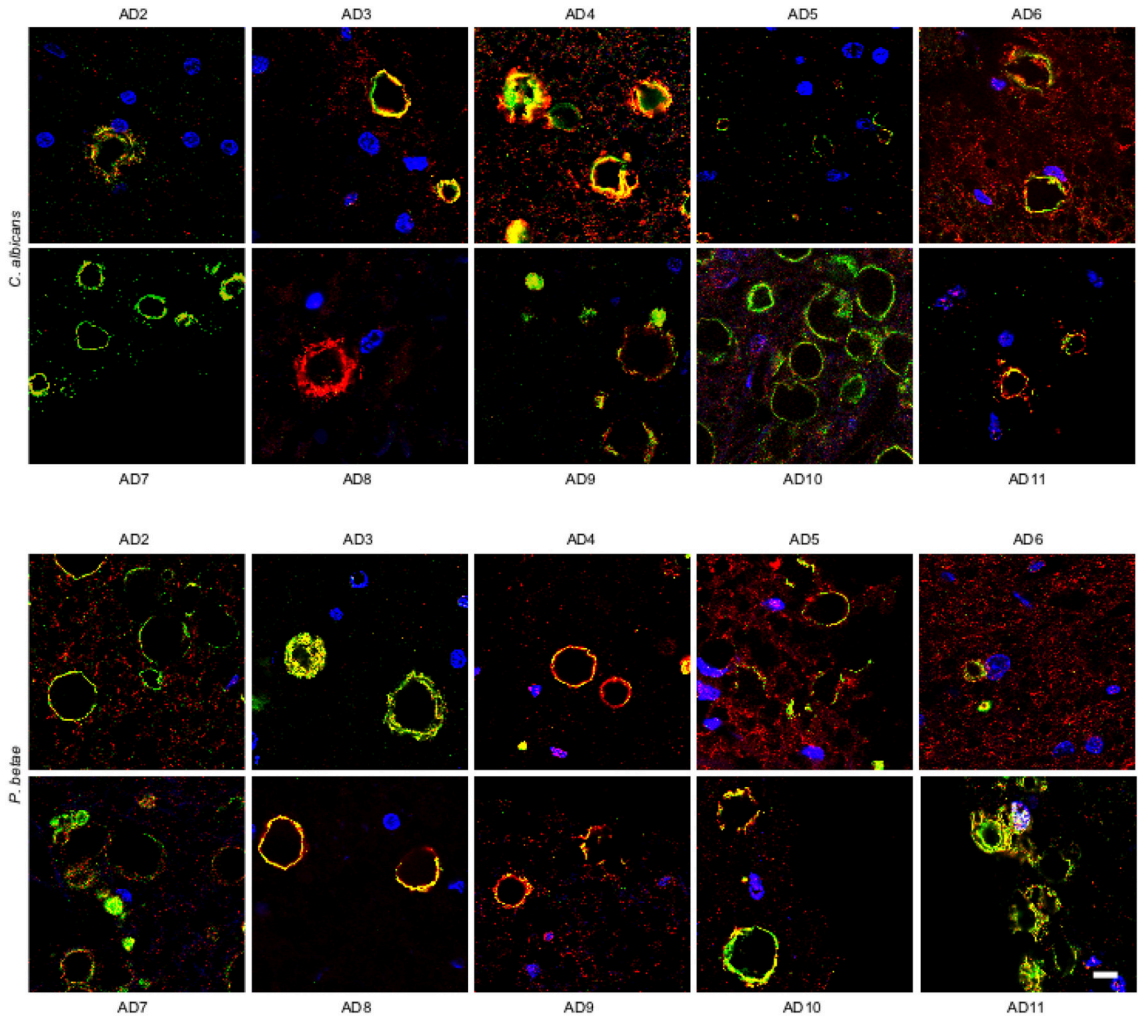

FIGURE 3 | Detection of fungal proteins in corpora amylacea from different AD patients. ERH sections from 10 AD patients (AD2-AD11) were incubated with rabbit polyclonal antibodies against *C. albicans* and *P. betae* (green) and a monoclonal antibody against α-tubulin (red). Sections were mounted and examined by confocal microscopy after incubation with the corresponding secondary antibodies. DAPI and scale bar as in **Figure 1**.

(**Figures 1**, **2**), and also further support the concept of CA protein heterogeneity.

We also tested for fungal proteins in CA from brain samples of one PD patient. As before, several CNS regions were analyzed using antifungal antibodies (green) and anti-α-tubulin antibodies (red) (**Figure 5**). The CNS regions examined in this PD patient included pons (PN), mesencephalon (MSP), hypothalamus (HT), callosal body (CB), and caudate and lenticular nuclei (CLN). CA were also detected in this PD patient and were found in all CNS regions analyzed. Furthermore, immunoreactive fungal proteins were prominent in CA bodies and the distribution of the immunolabels was similar to those observed in ALS and AD patients. Similarly to ALS patients, the number of CA inclusions in the different regions examined in the PD patient was much less than in AD patients. Analysis of CNS samples from five additional PD patients using anti-*C. albicans* and anti-*P. betae* antibodies is shown in **Figure 5**. Once again, CA inclusions were detected in all PD patients analyzed and immunoreactivity to fungal proteins in these CA was revealed with the two antifungal antibodies employed. The location of fungal proteins (green) and the distribution of human α-tubulin were again similar to the pattern observed with CA from ALS patients.

Study of CA from Control Individuals

It is thought that the formation of CA inclusions are related to the aging process, even in healthy subjects (Song et al., 2014). We examined ERH sections from the CNS of five control subjects using the two antifungal antibodies indicated above. In general, CA inclusions were clearly much less abundant than those observed in CNS tissue from patients diagnosed with neurodegenerative diseases. Nevertheless, the CA inclusions in control subjects exhibited a modest immunoreactivity against the two antifungal antibodies employed. For example, CA from control subjects C2 and C3 immunoreacted with the

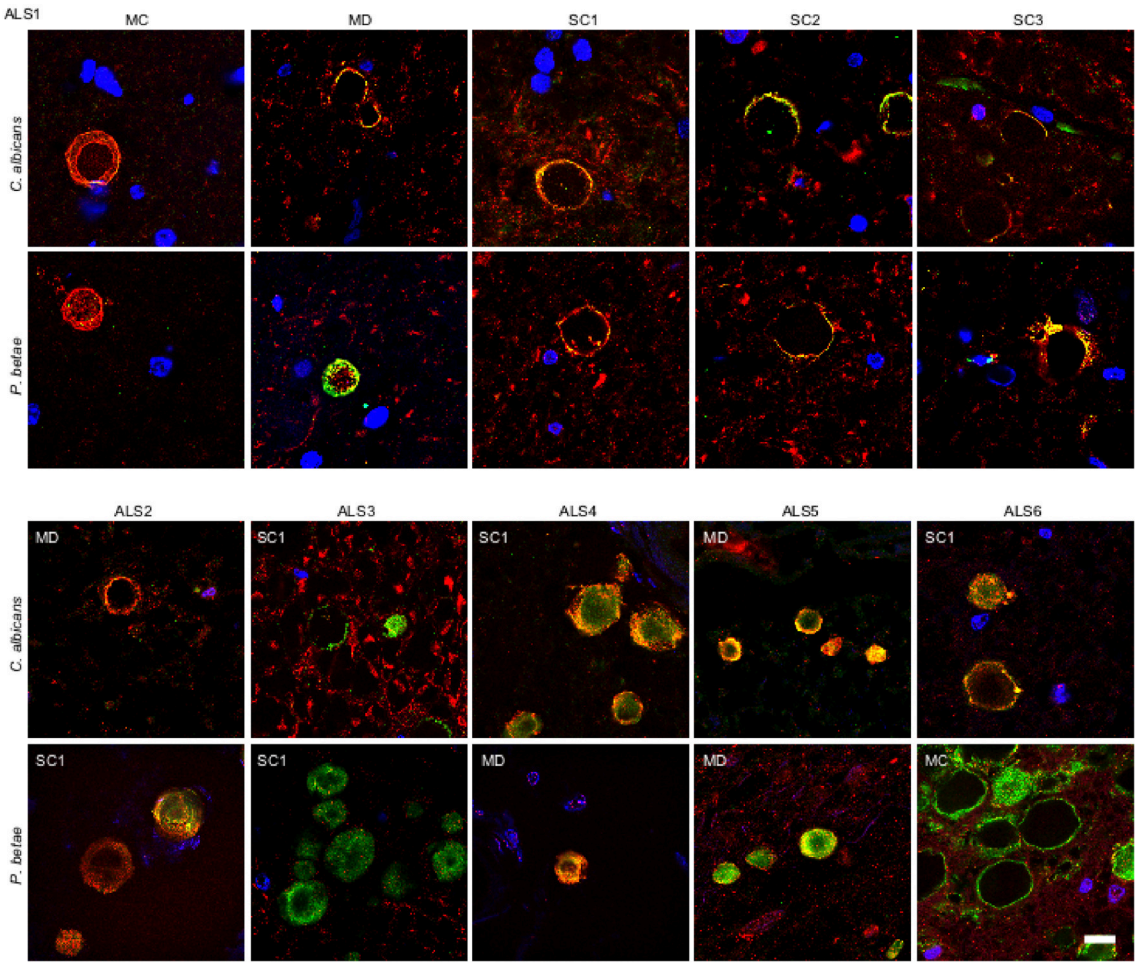

FIGURE 4 | Fungal proteins in corpora amylacea from ALS patients. Tissue sections of patient ALS1 were obtained from the following regions: primary motor cortex (MC), medulla (MD) and different levels of the spinal cord (SC1, SC2, and SC3) (upper panels). Different regions of the CNS from five additional ALS patients (ALS2–ALS6) were also analyzed (lower panels). Sections were incubated with rabbit polyclonal antibodies against *C. albicans* and *P. betae* (green) and a monoclonal antibody against α-tubulin (red). Sections were mounted and examined by confocal microscopy after incubation with the corresponding secondary antibodies. DAPI and scale bar as in **Figure 1**.

anti-*C. albicans* antibody, while C4 and, to a lesser extent, C3 immunoreacted with the anti-*P. betae* antibody (**Figure 6**). Similar to CA from neurodegenerative patients, the external perimeter of CA exhibited punctate immunoreactivity when detected; however, the labeling intensity was lower than that observed in AD patients. These findings indicate that in general the amount of fungal proteins in CA from control individuals is much lower than from equivalent neurodegenerative disease patients and in some cases no immunoreactivity is detected. The absence of fungal proteins in some CA might be determined by the particular section examined. Nevertheless, the amount of fungal proteins in control CA inclusions is very low. Alternatively, it is theoretically possible that the fungal proteins, if present, cannot be detected with the antibodies employed in this study. An estimation of the number and positiveness of CA in different patients and control subjects is shown in **Figure 7**. Certainly, the vast majority of CA from the different patients analyzed is stained with antifungal antibodies, whereas only a very minor portion of CA can be considered as positive in control subjects. On the other hand, in general this quantitation reveals higher numbers of CA in AD patients, particularly from ERC areas where CA are more abundant both in patients and controls. However, one limitation of this quantitation is that these numbers can vary depending on the specific tissue section analyzed. As previously noted by other researchers, the amount of CA is higher close to blood vessels (Nishio et al., 2001).

Collectively, these findings reveal the presence of CA inclusions in several regions of the CNS from patients with neurodegenerative diseases. CA are more abundant in the ERH of AD patients than in other regions but there are higher quantities of CA from ALS and PD samples than in controls. Importantly, fungal proteins are detected in CA from all patients with neurodegenerative diseases tested by means of specific antibodies.

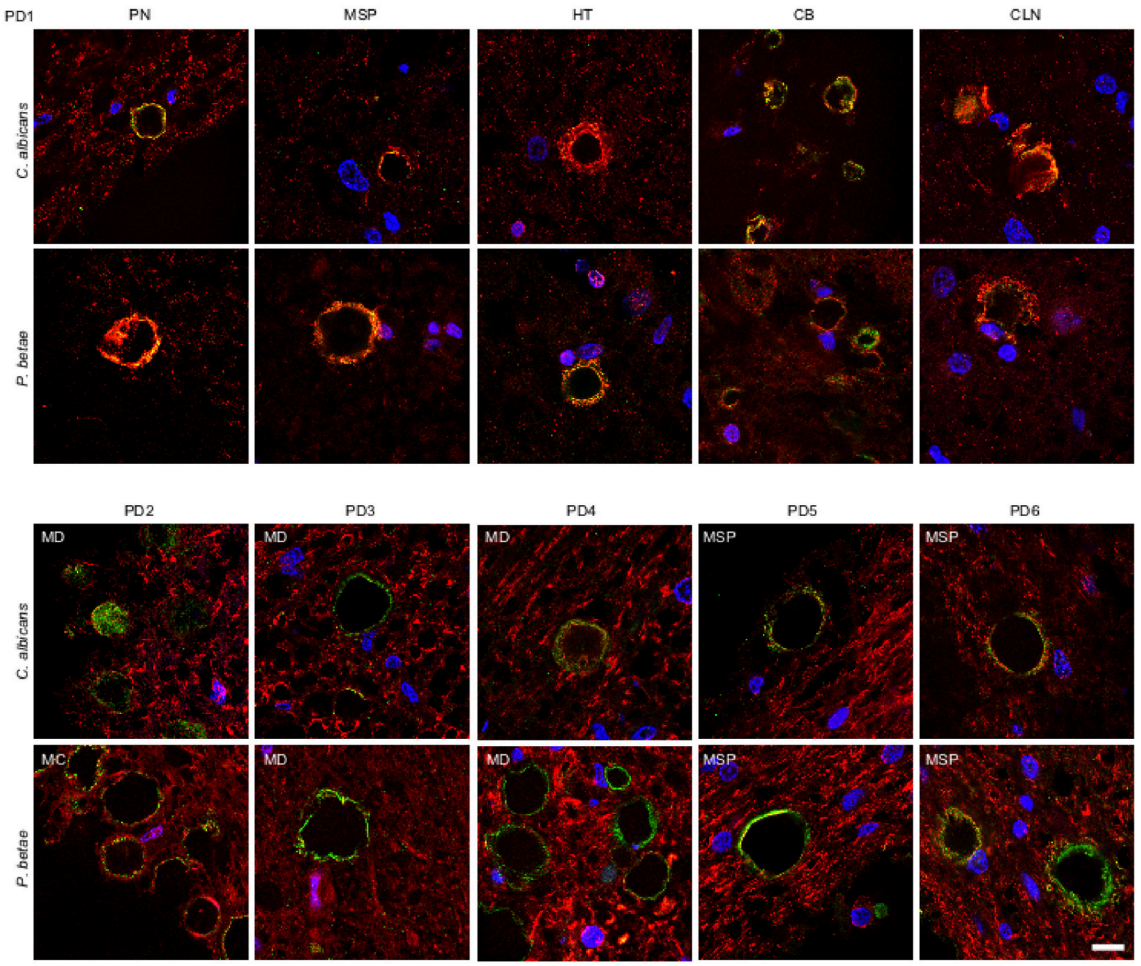

FIGURE 5 | Detection of fungal proteins in corpora amylacea from PD patients. Tissue sections of patient PD1 were obtained from the following regions: pons (PN), mesencephalon (MSP), hypothalamus (HT), callosal body (CB), and caudate and lenticular nuclei (CLN) (upper panels). Different regions of the CNS from five additional PD patients (PD2-PD6) were also analyzed (lower panels). Sections were incubated with rabbit polyclonal antibodies against *C. albicans* and *P. betae* (green) and a monoclonal antibody against α-tubulin (red). Sections were mounted and examined by confocal microscopy after incubation with the corresponding secondary antibodies. Overlapping red and green pixels appear as orange/yellow. DAPI and scale bar as in **Figure 1**.

DISCUSSION

Although great progress has been made in our understanding of the protein composition of CA inclusions (Sfanos et al., 2014), a number of components remain unidentified. Furthermore, the precise origin and potential function of CA inclusions is obscure and subject to speculation (Mrak et al., 1997; Keller, 2006; Song et al., 2014). The proposal that CA originate through neurodegeneration is based on findings showing that several proteins from neural cells can be detected in CA (Singhrao et al., 1993; Selmaj et al., 2008). Along this line, CA are thought to be formed by cellular debris and/or breakdown products from brain cells since cleaved tau protein is found in CA (Day et al., 2015). Another suggestion is that CA arise from a conglomeration of proteins that interact after neuron degeneration and from extravasated blood proteins after breakdown of the hematoencephalic barrier (Meng et al., 2009). Following this idea, CA could be envisaged as aggregated proteins and polyglucans, which together with calcium salts accumulate extracellularly. Thus, some proteins may form insoluble aggregates that are integrated as the amyloid component of CA (Kimura et al., 1998; Vogl et al., 2012). The fact that glial CA inclusions contain heat-shock proteins, heme oxygenase-1 and ubiquitin suggests the existence of oxidative stress, perhaps mediated by advanced glycosylation of cellular proteins (Cisse et al., 1993; Lowe et al., 1993; Iwaki et al., 1996). The possibility that CA are formed from mitocondria inside cells and progressively increase in size leading to cell disruption has also been suggested (Schipper, 2004). Indeed, the expression of heme oxygenase-1 in cultured rat glial cells induces mitochondrial dysfunction and the formation of corpuscles reminiscent of CA (Sahlas et al., 2002; Schipper et al., 2006; Song et al., 2014).

Alternative proposals for the origin of CA center on the idea that microbial infection with concurrent inflammation is

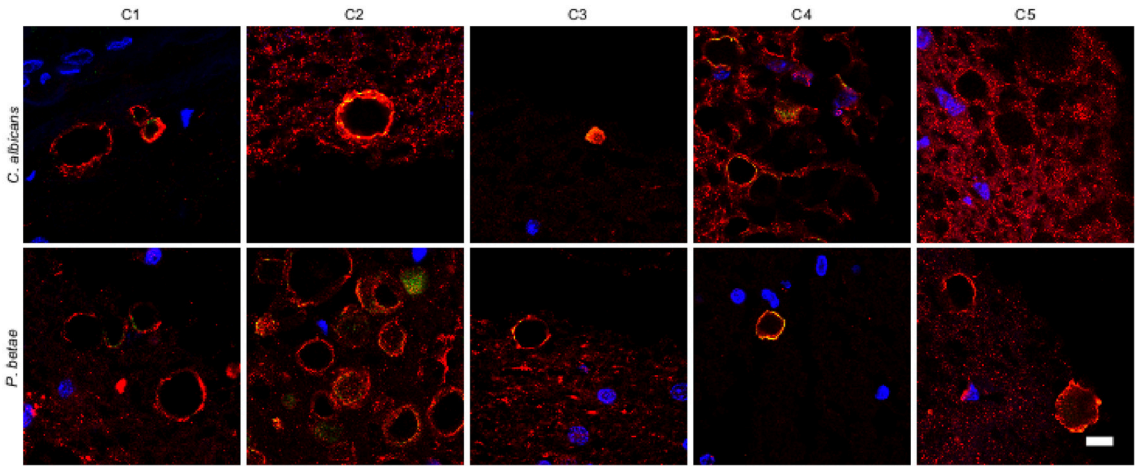

FIGURE 6 | Analysis of corpora amylacea present in the CNS of control individuals. Tissue sections from the ERH of five control individuals (C1–C5) were analyzed by immunohistochemistry. Sections were incubated with rabbit polyclonal antibodies against *C. albicans* and *P. betae* (green) and a monoclonal antibody against α-tubulin (red). Sections were mounted and examined by confocal microscopy after incubation with the corresponding secondary antibodies. Overlapping red and green pixels appear as orange/yellow. Note the near absence of activity against the fungal antibodies. DAPI and scale bar as in **Figure 1**.

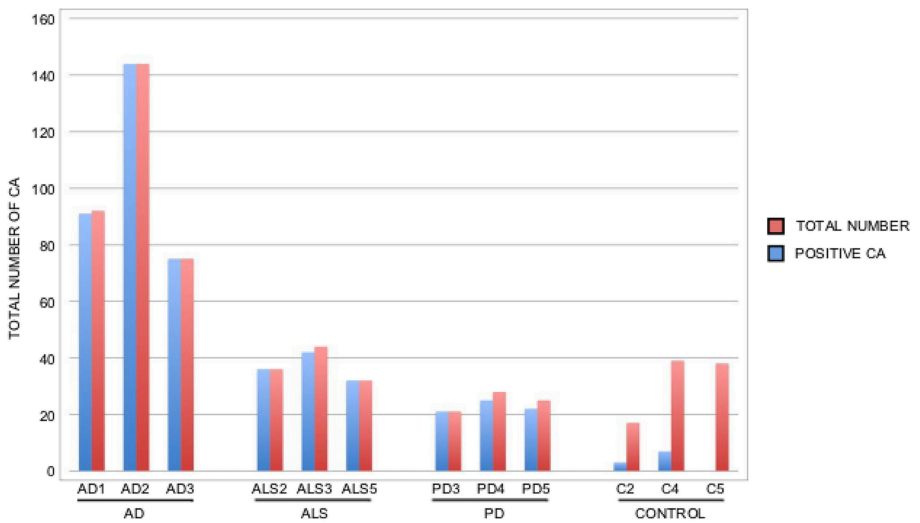

FIGURE 7 | Quantitation of the number and positiveness of CA from different patients and control subjects. Brain sections from the patients indicated in the Figure were examined by confocal microscopy and the total number of CA in three different fields was estimated, as well as their staining with anti-*P. betae* antibodies. Wide fields were analyzed using 40x magnification. The sections examined were ERC from AD patients and control subjects, MD from ALS patients, PD3 and PD5 and MC from PD4. Red bar: total number of CA in the three fields; blue bar: positive CA with antifungal antibody.

an intrinsic component of CA formation (Heinonen et al., 1992; Sfanos et al., 2014). In this vein, the present study demonstrates the existence of fungal proteins in CA from the CNS, but predominantly in patients diagnosed with neurodegenerative diseases since the yield in control individuals is very low. These observations suggest that fungal infection is not necessarily involved in the formation of CA, however, microbial infections and particularly mycoses may enhance and/or trigger the build-up of CA inclusions. Alternatively, if fungal proteins do not participate in CA formation *per se*, it is possible that in tissues infected with fungi, some fungal proteins are conglomerated together with cellular proteins in these inclusions. As CA formation occurs progressively, and the location of these proteins is specific to the CA envelope, the possibility that fungal infection is caused by postmortem contamination is unlikely.

The possibility that CA play a role in the pathology of AD and other diseases has been raised in the literature (Mrak et al., 1997; Keller, 2006; Schipper et al., 2006; Sfanos et al., 2014). If correct, it is conceivable that fungal proteins are also implicated in the severity of symptoms in some of these diseases, mediated by the formation of larger amounts of CA. The possibility that

CA, at least in some cases, are formed by mycoses is supported by several lines of evidence. First, CA contain several salts, including calcium phosphate and calcium oxalate, and these salts have also been detected as deposits in fungal infections (Tanaka et al., 1993; Nakagawa et al., 1999; Modem et al., 2006; Rassaei et al., 2009). Second, proteins from neutrophil granules appear in CA and it is well established that fungal infections elicit a neutrophil response (Murthy et al., 1993; Metzler et al., 2011; Cunha et al., 2014). Third, polyglucans form a major component of CA inclusions and this macromolecule is also an integral constituent of the fungal cell wall and is secreted to the external medium (Chaffin et al., 1998; Free, 2013). The finding of keratan sulfate and high mannose glycoconjugates in CA inclusions was initially interpreted as the result of an accumulation of glycoconjugates normally present in the brain tissue matrix through aging (Liu et al., 1987). Nonetheless, it must also be considered that high mannose glycoconjugates are also produced by fungal cells (Chaffin et al., 1998). Therefore, the composition of CA does not discard the possibility that they are related to fungal infection; rather, it is quite feasible that they can originate from mycoses. The findings reported in the present study lend strong support to this possibility. Interestingly, calprotectin exhibits potent anti-*Candida* activity (Sohnle et al., 1996; Okutomi et al., 1998; De Marzo et al., 2007), and this is also the case for defensin (Schroeder et al., 2011), an additional component of CA. Given that amyloid β peptide, which is very abundant in senile plaques, has strong potency against *C. albicans* (Soscia et al., 2010), it is conceivable that an antifungal response occurs in brains from patients diagnosed with some neurodegenerative diseases. Future work aimed at purifying and characterizing the range of polysaccharides present in CA may help to determine whether CA arise as a result of fungal infection.

AUTHOR CONTRIBUTIONS

DP and RA carried out the experiments. AR managed the human brains and provided the tissue sections. LC designed the experiments and wrote the manuscript. All authors discussed the results obtained and participated in the correction of the 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.2016.00086

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